



Stem Cells and Innate Immunity in Aquatic Invertebrates: Bridging Two Seemingly Disparate Disciplines for New Discoveries in Biology

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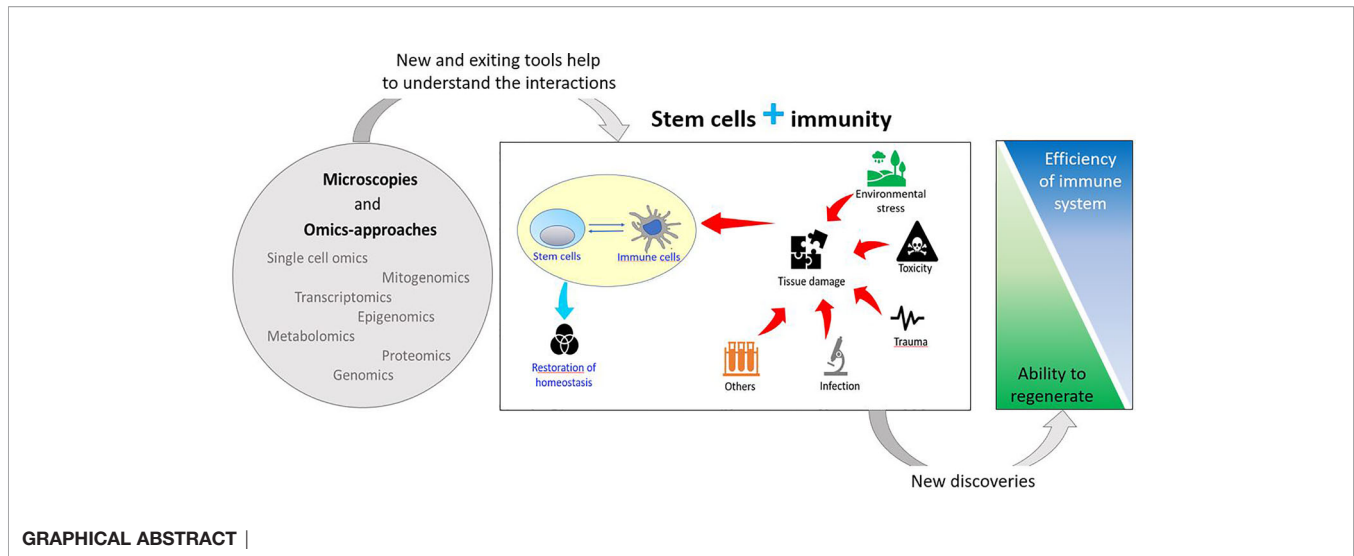
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The scopes related to the interplay between stem cells and the immune system are broad and range from the basic understanding of organism's physiology and ecology to translational studies, further contributing to (eco)toxicology, biotechnology, and medicine as well as regulatory and ethical aspects. Stem cells originate immune cells through hematopoiesis, and the interplay between the two cell types is required in processes like regeneration. In addition, stem and immune cell anomalies directly affect the organism's functions, its ability to cope with environmental changes and, indirectly, its role in ecosystem services. However, stem cells and immune cells continue to be considered parts of two branches of biological research with few interconnections between them. This review aims to bridge these two seemingly disparate disciplines towards much more integrative and transformative approaches with examples deriving mainly from aquatic invertebrates. We discuss the current understanding of cross-disciplinary collaborative and emerging issues, raising novel hypotheses and comments. We also discuss the problems and perspectives of the two disciplines and how to integrate their conceptual frameworks to address basic equations in biology in a new, innovative way.

Keywords: stem cells, immune cells, tissue regeneration, aquatic invertebrates, system biology, omics technology



INTRODUCTION

Stem cells are multipotent, self-renewing entities that generate one or more differentiated daughter cell types (1, 2). Conversely, immune cells are differentiated cells devoted to immunosurveillance and able to detect and respond to non-self stimuli. Stem and immune cells are thus commonly discussed as separate entities and considered to belong to disparate branches of biological disciplines with limited or even no interconnections. Yet, following Morin's (3) view for any complex system as "composed of interweaving interactions characterized by complementary, antagonistic, concurrent relationships so that no one exists and can be understood in isolation from the whole", it becomes natural to consider these two complex cellular networks, as not fully understood in isolation from each other. Stem and immune cells are essential for animal survival and fitness. Indeed, the two systems contribute to homeostasis by perceiving physiological changes in the systemic environment and, consequently, they generate differentiated cells or mount an immune response, respectively (4–6).

There are multiple examples in mammals, showing intermingled immune and stem cell functionalities (see below), whereas immunotoxicology and pharmaceutical research are additional scientific fields interfacing immunologists and stem cell biologists. As an example, human and murine colony-forming units-granulocyte/macrophage assays have been validated for assessing xenobiotic-induced myelotoxicity, and *in vitro* bone marrow stem cell assays are now commercially available and routinely used in pharmaceutical screening. The assessment of myelotoxicity provides a broad measure of the potential impacts of chemicals on the growth and development

of immune cells, where all immune-related cells develop from pluripotent, hematopoietic stem cells residing in the bone marrow. This is not the situation in aquatic invertebrates, where stem cell-based bioassays have not yet been adequately explored as ecotoxicological tools (7).

Aquatic invertebrates represent the great majority of animal biodiversity and are an extremely promising group of organisms for stem cells and immune research. They can benefit from a more straightforward body organization, the abundance of adult stem cells spread throughout the body, and the lack of a complex immune network by lacking an adaptive immunity (8). Therefore, they represent ideal organisms to study the interplay between stem and immune cells.

Hematopoiesis is the driver for generating circulating immune cells able to face non-self and, in vertebrates, it has been the subject of intensive research by immunologists and stem cell biologists (9, 10). Conversely, in the majority of aquatic invertebrates, stem cell origin has not yet been adequately explored, while the immune response has been further confirmed as a relevant endpoint in ecological risk assessment of both legacy and emerging contaminants (8, 11).

Various stimulation experiments were carried out, and various techniques were used in aquatic invertebrates to study the hematopoietic tissue (HPT) in relation to the (circulating) immune cells (i.e., hemocytes, coelomocytes). The variations in haemocyte number are, most likely, mainly regulated by release from the HPT, perhaps complemented by storage and release of hemocytes in other sites (12). The majority of the cells in the HPT are able to proliferate, and proliferation can be increased significantly after the injection of saline or bacterial toxin like lipopolysaccharide (LPS) (13). There is a dynamic change in the HPT in response to LPS-induced blood loss, similar to that observed in response to an infection by bacteria (which contain LPS) or fungi (which contain laminarin) (14).

Although in many cases, the hematopoietic sites are still unknown, the mesothelium lining the coelomic spaces or the vasculature plays a vital role in the formation of the

Abbreviations: ASC, Adult stem cell; DIC, Differential interference contrast; ENF, En1-lineage-naive fibroblast; ENP, En1-lineage-past fibroblast; EP, Engineered nanoparticle; HPT, Hematopoietic tissue; HSC, Hematopoietic stem cell; ISH, *In situ* hybridization; LPS, Lipopolysaccharide; MC, Morula cell; ROS, reactive oxygen species; SCT, Single cell transcriptomics; SEM, Scanning electron microscopy.

coelomocytes/hemocytes in spiralian coelomates as a source of hematopoietic stem cells (HSCs) that colonize specific lymphoid organs or lymph glands (15–17). Among invertebrate deuterostomes, in addition to the coelomic epithelium, the pharyngeal region acquires a role in hematopoiesis (18, 19).

In the present review, we will focus on the relationships between stem cells and the immune system in aquatic invertebrates, compared to vertebrates. We will highlight some selected examples stemming from the established collaboration within the MARISTEM COST Action (8). The introduction of modern techniques in biological research allows us to discuss new tools and frontiers in biological research aimed to study the links between stem and immune cells.

LINKS BETWEEN STEM CELLS AND IMMUNITY

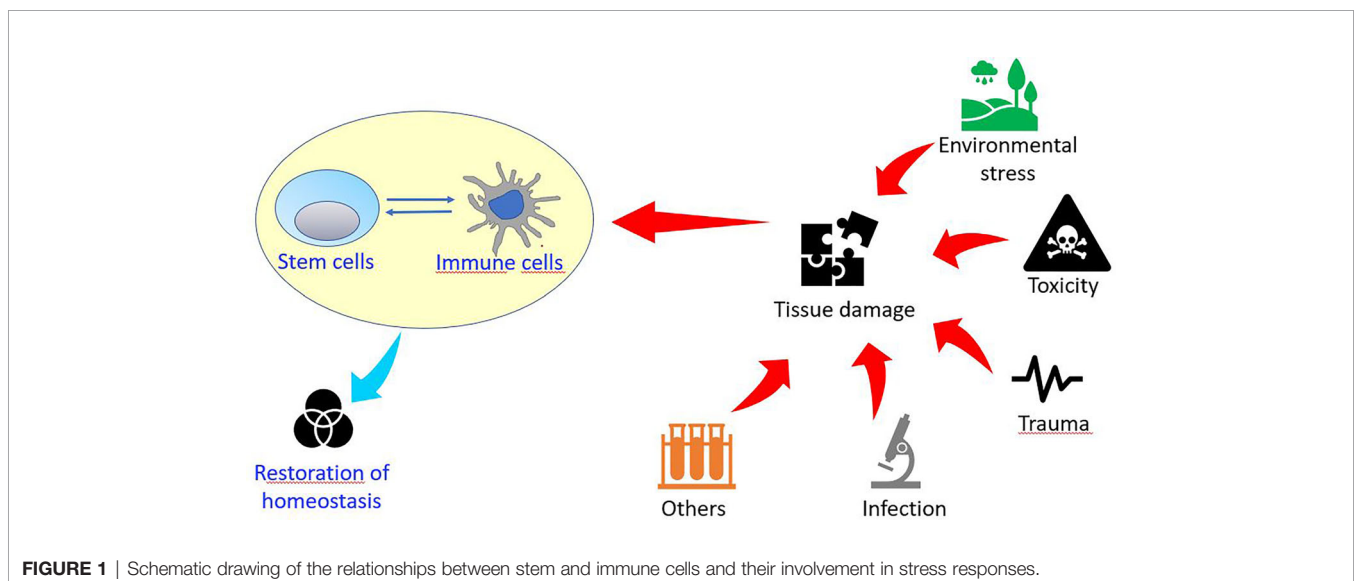
The interplay between immune cells and stem cells is reciprocal (20), and the relationships between stem cells and immune responses have been particularly well studied in mammals. For example, the damage of the hair follicle bulge, the niche where skin stem cells reside, induces these cells to release stress signals that stimulate immune cells. Recruited immune cells, in turn, stimulate stem cell proliferation to restore niche integrity, thus depicting a scenario in which the two counterparts are finely intermingled in a reciprocal tuning (21). Mammalian mesenchymal stem cells can modulate immune responses by acting as antigen-presenting cells and immune suppressors (22, 23). In addition, HSCs discriminate non-self and respond to the infection through increased proliferation and differentiation of immune cells. The latter, during their differentiation, consequently to the recognition of non-self, acquire epigenetic modifications in immune genes that are transmitted to their descendants (24). Conversely, immune cells can regulate stem cells activity and differentiation. For instance, macrophages, far from being just professional

phagocytes, can govern HSCs differentiation to blood cells and modulate stem cells differentiation in the mammary gland, epidermis and the intestinal crypts (25).

In addition, the immune system plays an important role in tissue/organ regeneration processes as immune cells contribute to the modulation of stem cell activation to facilitate regeneration. Regenerative organisms generally present limited pro-inflammatory responses, absence of scar formation and poor immune responses, suggesting an inverse correlation between regeneration capability and sophisticated adaptive immune system (20, 26, 27) (**Figure 1**).

Immune cells affect mammalian regeneration and tissue repair both positively and negatively (28). Different specific subtypes of macrophages (M) and T cells play opposite roles during wound healing, influencing the choice between regeneration and fibrotic repair. In accordance, regenerative capacity is impaired if anti-inflammatory processes initiate too early or if inflammation persists too long (20, 29). During scar healing, M (IFN-gamma) inflammatory macrophages activate T helper cells that release inflammatory cytokines inhibiting stem cells, while M (IL-4) pro-fibrotic macrophages induce extracellular matrix deposition. On the contrary, during scarless healing (M(IL-10)), anti-inflammatory/anti-fibrotic macrophages activate subtypes of regulatory T cells that secrete anti-inflammatory cytokines, while the tissue-resident subtype $\gamma\delta\tau$ cell may activate stem/progenitor cells by releasing growth factors (27).

Direct evidence of the interaction between the immune system and tissue regeneration has also been provided in the axolotl, where it has been demonstrated that macrophages, a crucial component of the innate immune system, infiltrate at the wound site and are essential for limb regeneration and their reduction causes regeneration failure and considerable fibrosis. Indeed, macrophage depletion induced deregulation of dedifferentiation factors, causing the disruption of the early regenerative blastema, formed by the accumulation of dedifferentiated multipotent cells (30). Moreover, myeloid cells



are implicated in salamander lens regeneration (31) and the reduction of a specific subset of macrophages also influenced blastema formation and fin tail regeneration in zebrafish *Danio rerio* (32).

The cross-talk between immune cells, neural stem and progenitor cells and their progeny seems to determine both the efficacy of endogenous regenerative responses and the mechanism of action (33). Neurons do not efficiently regenerate in mammals and several studies suggest that the inflammatory response to injury impedes neurogenesis (34–36). In the zebrafish brain, which has the capacity to regenerate and replace neurogenic activity, recent work showed that inflammation is necessary and sufficient to initiate neurogenesis *via* progenitor cell activation. A similar phenomenon is also known in crustacean neurogenesis (37). Immune-mediated regeneration has also been discovered in skeletal muscle, a well-studied model for adult mammalian regeneration that employs activation of satellite cells, the resident progenitors of the muscle (38).

Unlike vertebrate immune systems, armored with the innate and the adaptive forms of immunity, invertebrates, which include more than two millions species and belong to more than 30 phyla, use only the ancient form of germline encoded innate immunity (39). Innate immunity in aquatic invertebrates, as in the vertebrates, relies on recognition elements (their nature not yet disclosed in most taxa), tightly associated with a wide range of effector mechanisms. While many prominent properties of innate immunity systems are shared by all multicellular organisms (40–46), the evolutionary origin remains poorly understood, as well as the roles of stem cells in immunological pathways of aquatic invertebrates (47, 48). Here, we present

information on the relationships between stem and immune cells deriving from research on selected taxa of aquatic invertebrates.

Planarians

Planarians are highly regenerative organisms for which the potential role of the innate immune system during tissue regeneration is still largely unknown (20, 49). Their amazing regenerative capability resides in a heterogeneous population of adult stem cells, the neoblasts, which contains the sigma-neoblasts, a subpopulation of pluripotent stem cells (50). Following injury, neoblast proliferates and accumulates below the wound producing the blastema, where the lost structures differentiate (51, 52) (Figure 2).

Two peaks of apoptosis are required for proper tissue regeneration and reticular cells, a macrophage-like cell type, are recruited to clear the debris at the site of injury (53). Recently, the presence of subsets of cathepsin-positive cells was found. They have a dendritic-like shape and phagocytic activity and their specification is determined by the transcription factor foxF-1 (54).

Data support a connection between immune cell activity, stem cells, and regenerative mechanisms. Indeed, upon injury, planarians macrophages produce maresins, mediators of inflammation resolution, and when their synthesis is blocked by the lipoxygenase inhibitor baicalein, the tissue regeneration rate is reduced (55). The infection with bacteria (*Pseudomonas* sp.) has been linked to the inhibition of tissue regeneration through the activation of TAK1/MKK/p38 innate immunity signaling (56). Among the genes likely involved in innate immunity, some were found specifically activated following wounding in the presence of LPS (57). It has been demonstrated that planarians are resistant to

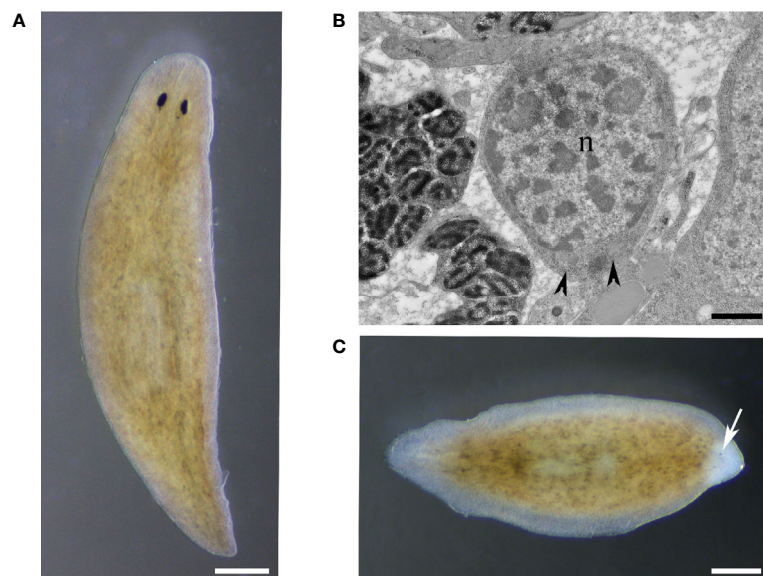


FIGURE 2 | (A) Intact planaria (*Dugesia japonica*, asexual G1 clone). **(B)** Electron micrograph of a neoblast, the planarian stem cell, with scanty undifferentiated cytoplasm containing free ribosomes and mitochondria. n, nucleus; arrowhead, mitochondria. **(C)** A tail fragment is regenerating the head, 3 days after amputation. Arrow indicates the unpigmented blastema containing the new differentiating eye cups. Scale bar: 100 μ m in **(A, C)**, and 800 nm in **(B)**.

infection by a broad range of pathogenic bacteria and the key resistant gene, *MORN2*, which is conserved in humans, has been identified (58), thus making planarians a useful model for the identification of innate resistance mechanisms (59). Given the availability of RNAi, transcriptomic and proteomic approaches in planarians, as well as the possibility of single-cell gene profiling by flow cytometry analysis and single cell-transplantation (60, 61), these invertebrates represent a model for understanding the mechanisms integrating immune responses to stem cells and tissue regeneration. As an example, identifying fundamental genes involved in reticular cell differentiation and their silencing by RNAi could select organisms with inhibited immune system which could serve to study regenerative performance.

Bivalve Mollusks

Bivalves, the second largest group in Mollusca, are represented by several species, all of ecological importance in aquatic ecosystems, as well as of considerable economic value in estuarine and coastal areas (62). Their ability to face infection and their disease susceptibility have been deeply investigated in farmed species (i.e., as mussels, oysters, clams) and provided enormous progress in the characterization of their circulating cells, the hemocytes, including the immune cells and their cellular receptors and signal transduction pathways (63).

In the Mediterranean mussel *Mytilus galloprovincialis*, one of the most studied bivalve species, different subpopulations of hemocytes identified by flow cytometry have been functionally characterized with large and small granular hemocytes, representing phagocytes that can produce high levels of reactive oxygen species and nitric oxide upon activation (64). In contrast, small hyaline cells are not phagocytic and perform other defense mechanisms; they also include hemoblast-like cells that are suggested as possible stem cells in bivalves. The hemocyte half-life was determined to be between 22 and 28 days (65) in various bivalve species, thus suggesting the need for a continuous hemocyte replacement from stem/precursor cells. In the blue mussel *Mytilus edulis*, basal proliferation can account for up to 20% of the circulating cells indicating an essential process of hemocyte homeostasis (66). Although the identification of stem cells in mollusks is of physiological interest when considering the long-life span (years) and the extreme longevity of certain species (67), current knowledge on pluripotent cells in general, their location and their differentiation pathways, with special reference to immune cells, is still limited. Therefore, the origin of hemocytes remains an unanswered question in mollusks, including bivalves (16). In the Manila clam, *Tapes philippinarum*, newly formed hemocytes with stem cell-like features (according to cytochemical methods) can derive from the proliferation of undifferentiated circulating cells called hemoblasts (68). To date, the identification of a population of cells in the sub-epithelial connective tissue and vessels, resulting in positive for both SOX2, a stemness marker, and superoxide dismutase, a hemocyte marker, suggests that these cells are hemocyte progenitor cells and is still the only report on adult somatic progenitor cells in bivalves. It represents an essential first step in elucidating the hematopoietic process (65). The expression of

SOX2 is significantly induced by LPS and Poly I:C, two well known activators of immune cells, in the freshwater species *Anodonta woodiana* (69), indicating an influence of immune cells on stem cells.

Overall, it is likely that adult bivalves do not produce hemocyte precursors or mature hemocytes from distinct hematopoietic organs, as it occurs in other molluscan taxa like gastropods (16). Multiple or ubiquitous sites of hematopoiesis may exist, comprising a system in which stem cells present in both tissues and circulation receive decisive signals from neighboring cells. The experimental challenge with bacteria, leading to changes in hemocyte subpopulations, was useful in identifying the expression of both proliferation and differentiation markers in circulating hemocytes (70–72).

Available information on bivalve hematopoiesis in larval stages has been recently reviewed (73). Based on multiple functional and molecular evidence for the presence of immune cells in the early developmental stages of various bivalve species, the author hypothesized that the development of the immune system is inextricably linked with the formation of the digestive system from the earlier trochophora, a key stage when the formation of the first ciliated digestive organs (such as the mouth, esophagus, and digestive mass) occurs. In this view, at the early stages of development, when pathogens can enter the body *via* feeding, immune cells would play a double role as digestive cells and immune cells. Developmentally, hemocytes are closely related to the epithelial cells lining the vascular system (endothelia) and the body cavity (mesothelia) and, in the absence of a circulatory system and HPT in early larvae, immune cells may be produced by precursor cells of the digestive system or associated mesothelial cells. Only later, when the circulatory system develops, hemocytes may be produced by tissues lining vessels and sub-epithelial cells (73).

Crustaceans

Crustaceans represent a morphologically and physiologically diverse group of organisms, comprising between 40,000 and 60,000 species (74). They belong to an evolutionary very successful group of aquatic invertebrates characterized by life-long growth, able to enlarge their organs as adults and regenerate lost appendages.

Research on immune response in crustaceans has been largely driven by the need to understand diseases in aquaculture and to develop knowledge of environmental effects within a fishery context (75). Investigation of stem and progenitor cells in crustaceans has been primarily focused on the research of replacement of damaged and aged cells in the tissues to ensure homeostasis, immune responses and regeneration of lost appendages (76). This knowledge on how stem cells and immune cells communicate, sense damage and co-operate to help tissues to cope with stress is available only for some species of crustaceans.

Crustaceans have an open circulatory system, with a highly developed cardiovascular system. Like other invertebrates, their hemocytes have essential roles in immunity, performing recognition, cell–cell communication, lysis of foreign cells, melanisation, encapsulation, phagocytosis, and cytotoxicity.

They are also involved in other physiological processes, such as the repair of the cuticle (77) or damaged skeletal muscles (78) and neurogenesis (79), where orchestrated action of immunity and stem cells is needed to maintain or restore homeostasis.

The number of circulating hemocytes can vary significantly and depends on the physiological state (12). Crustaceans have three main types of hemocytes in their hemolymph, i.e., hyaline cells, semigranular cells, and granular cells (Figure 3), as well as immature progenitor cells. Indeed, crustacean hemocytes are produced and start their differentiation within the HPT, but the final differentiation into functional hemocytes occurs once the hemocytes are released into the circulation (80). Circulating hemocytes do not divide and aged cells must be continuously replaced by the release of new cells from hematopoietic organs (81). The variations in hemocyte number are, most likely, mainly regulated by the release of cells from the HPT, and perhaps complemented by storage/release of hemocytes in/from other sites. Although research on the role of hemocytes in crustaceans is rapidly progressing, the knowledge on HPT in different species is still limited.

In decapods, HPT comprises lobules with hemocyte precursors at different stages of differentiation (82). Each lobule is surrounded by connective tissue and contains stem cells, prohemocytes, and mature hemocytes, which are then released into the hemocoel (83). There are large variations in lobule morphology among species (13). Stem cells exclusively occupy the apical parts of the hematopoietic lobules and are closely attached to the extracellular envelope of the lobules. In the shrimp *Sicyonia ingentis*, the endothelial cells, capsular cells, and stromal cells, together with the extracellular matrix envelope hematopoietic lobules, and provide a niche-like microenvironment for the stem cells (82).

The HPT is not the only tissue harboring stem cells. The E-cells of the hepatopancreas, the neurogenic stem cells of the brain, the satellite cells of the heart and skeletal musculature are

examples of adult stem cells (ASCs). Some of these cells are more or less continuously active, like the neurogenic and hematopoietic stem cells (84, 85), while others are periodically activated like the myogenic satellite cells (86) or the E-cells (87).

A cross-talk between immune cells and stem cells was identified by Benton et al. (88), who demonstrated that hemocytes are a source of adult-born neurons in crayfish and showed that the immune system is a key contributor to adult neurogenesis. Neurogenesis is an ongoing process in the brains of adult organisms. Noonin et al. (14) described the anterior proliferation organ in *Pacifastacus leniusculus*, located at the anterior part of the HPT, near the brain, as an organ regulating stem cell activity, demonstrating a physical link between the HPT and the brain. Benton et al. (88) also reported that manipulation of circulating hemocyte levels results in highly predictable changes in the number of neurogenic niche cells. These data show that the neurogenic niche is dynamically regulated by the immune system.

Crustaceans can partly regenerate their hepatopancreas, hearts and ovaries: here, similar ultrastructural features were observed in regenerating tissues of early adults and old specimens and included active stem cells (89). The immune cells have only recently emerged as key components and prominent effectors of stem cell behaviors to help stem cells in maintaining tissue integrity and driving regeneration (25). Common functions of the immune response in regeneration include scavenging cellular debris and activating progenitor cells as well as production of cytokines, and complement factors (90). In the hepatopancreas, hovering between cell proliferation and cell death (mainly apoptosis) plays an important role in the maintenance of tissue homeostasis. Hemocyte infiltration, in the form of an inflammatory-like reaction, was also observed in the hepatopancreas of *Palaemonetes argentinus* under stress conditions leading to increased desquamation of necrotic epithelium (91).

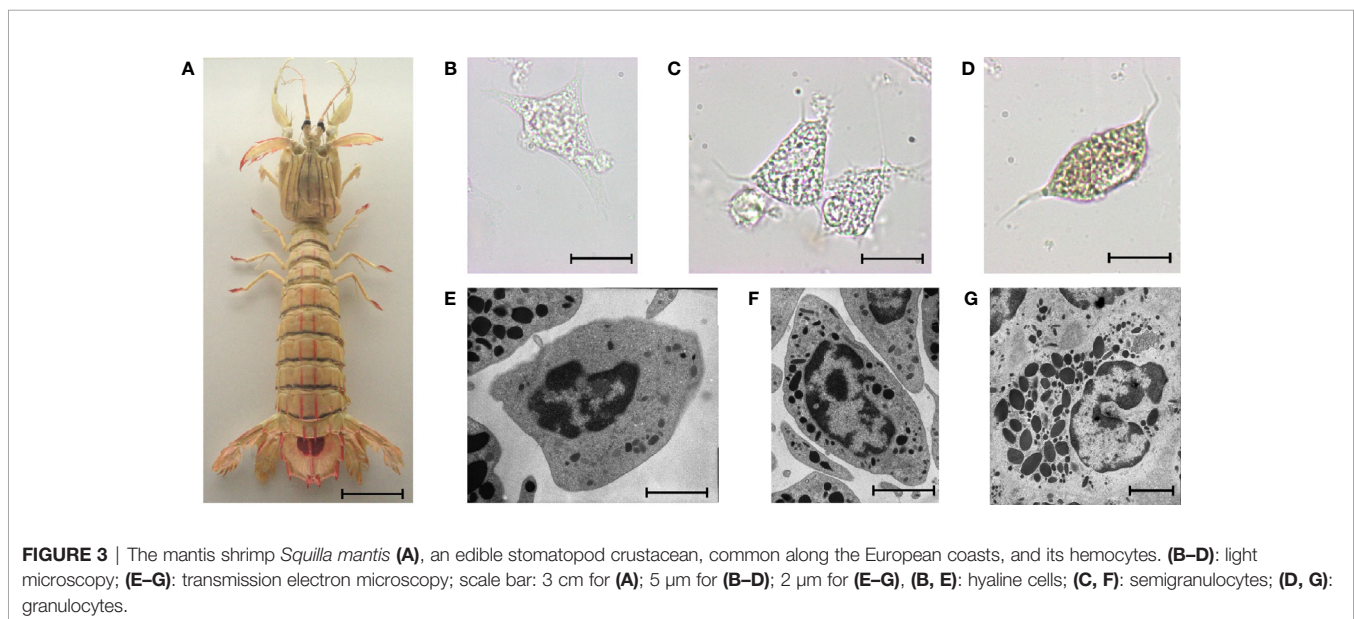


FIGURE 3 | The mantis shrimp *Squilla mantis* (A), an edible stomatopod crustacean, common along the European coasts, and its hemocytes. (B–D): light microscopy; (E–G): transmission electron microscopy; scale bar: 3 cm for (A); 5 μm for (B–D); 2 μm for (E–G). (B, E): hyaline cells; (C, F): semigranulocytes; (D, G): granulocytes.

Echinoderms

Echinoderms are very successful and ancient marine invertebrates, which appeared more than 500 years ago, before the Cambrian explosion (92). Many of them (e.g., sea urchins, sea cucumbers) play a central role in the safeguarding of certain marine ecosystem integrity and are constantly exposed to environmental and anthropogenic pressure, including predation, climate changes, pathogens, and pollutants (e.g., chemicals, nanomaterials, plastics) (93). This favors phenotypic plasticity both in the embryonic and adult life-cycle stages, thus conferring a notable ability to generate multiple phenotypic shapes and responses towards an always-changing world (94).

Echinoderm immune cells are the so-called coelomocytes, a heterogeneous population of cells freely circulating in all coelomic spaces, including the perivisceral coelomic cavities, the open water-vascular system, the perihemal system, but also in the main tissues and organs (95–97). A general consensus on echinoderm coelomocyte types is lacking, possibly due to the actual diversity of morphotypes among the different classes (93) but mostly to the diversity of techniques and protocols used to analyze them (98). For this reason, from two to up to twelve cytotypes are reported in the literature (97, 99–105). The most common types are phagocytes, red and white amoebocytes, and vibratile cells (93, 97). Gorshkov et al. (101) described mature and immature coelomocytes, the latter possibly representing a progenitor population of the former. Echinoderm immune cells carry out many functions, such as clot formation, phagocytosis, encapsulation, clearance, oxygen transport, inflammation and cell recruitment at the wound and regenerative sites (93). They are a big source of bioactive molecules secreted into the coelomic fluid to maintain homeostasis and intercellular crosstalk (106). Under homeostatic conditions, these cells work together to maintain physiological balance within the organism while, under perturbation, the stimulus may induce an energetically expensive reprogramming of the immune cells, resulting in either a reduced reactivity (tolerogenic immunological response) or an increased response (potentiation) (107, 108).

The exact origin of coelomocytes is still a matter of debate. The coelomic epithelium is the favored HPT, as proposed by the pioneering work of Bossche and Jangoux (109) and further supported by more recent studies (105, 110, 111). Other structures/organs have also been proposed such as Tiedmann's body, the axial organ and more recently the pharynx (19, 112, 113). It must be stressed, however, that all these "alternative" structures are always more or less directly linked to coelomic spaces and, therefore, to coelomic epithelium. A recent study by Sharlaimova et al. (105, 114) provided ultrastructural evidence of the release of different cell types from the perivisceral coelomic epithelium, thus demonstrating a hematopoietic role for this tissue. According to their model, the free-swimming population of coelomocytes is physiologically replenished by the coelomic epithelium through both apical detachment of differentiated cells and small scarcely differentiated cells. The latter could be regarded as stem/progenitor cells of at least a subpopulation of coelomocytes (114). The presence, although rare, of these undifferentiated cells within the coelomic lining would be

consistent with the proliferative activity often reported in this tissue in both physiological and regenerating conditions (19, 110, 115).

Some echinoderms possess extraordinary regenerative abilities (116, 117) in which immune cells play a fundamental role. Sea urchin diseases, consequent to lesions associated with body injuries or abrasions, induce loss of spines and other appendages and a bald patch, sometimes associated with a green mark probably due to a cellular infiltration into the affected area, which starts wound repair and drives tissue regeneration and recovery. Regardless of the origin of the wound (disease, autoamputation, or traumatic insult), immune cells are recruited first to create a clot (117) then to clear debris and pathogens as well as secrete numerous signaling molecules inducing appropriate cell proliferation and differentiation program essential for successful regeneration (20). Besides providing new circulating coelomocytes, the coelomic epithelium can also provide progenitor cells for the reconstruction of the regenerating tissue as reported in crinoids (116). This can occur thanks to a dedifferentiation process followed by epithelial-mesenchymal transition, allowing migration of scarcely differentiated cells in the underlying developing connective tissue (117–120). After arm amputation, trauma-stressed sea stars (*Asterias rubens*) show an increase both in the total number of the circulating coelomocytes and in the levels of Hsp70 stress protein within the cells. Besides, a protein known to be involved in coelomocyte adhesion (toposome) is massively expressed in the coelomic epithelium of trauma-stressed arms of *A. rubens* (121) (**Figure 4**).

Ascidians

Ascidians are members of the subphylum Tunicata, the sister group of vertebrates (122). Their defense from environmental assaults relies mainly on the tunic and the circulating immune cells in the adults, and, likely, on the test cells in early embryonic stages (**Figure 5**).

The tunic is the external layer embedding the ascidian body (**Figures 5A–C**). It includes a gelatinous matrix rich in fibers (collagen, tunicin). It is always speckled with several types of free cells, some of which are permanently located within the tunic, while other cell types perambulate between the tunic and the body wall (123, 124). In some species, the tunic contains hemolymphatic vessels (125). Among the various types of cells found within the tunic, there are cells functioning in innate immunity and allorecognition (phagocytes), chemical defense (bladder cells), light protection (pigmentary cells), photosymbiosis (phycocytes), tunic contraction (myocytes); for some of the tunic cells (e.g., granulocytes, amoebocytes), the functions are unknown (123). The tunic is secreted by the epidermal cells and tunic cells derive from both the epidermis and the hemocytes that enter the tunic in response to infections (123, 125, 126).

Circulating immune cells of ascidians are represented by phagocytes and cytotoxic morula cells (MCs), the latter containing the enzyme phenoloxidase inside their granules (127) (**Figure 5D**). Both the immunocyte types assure the organism

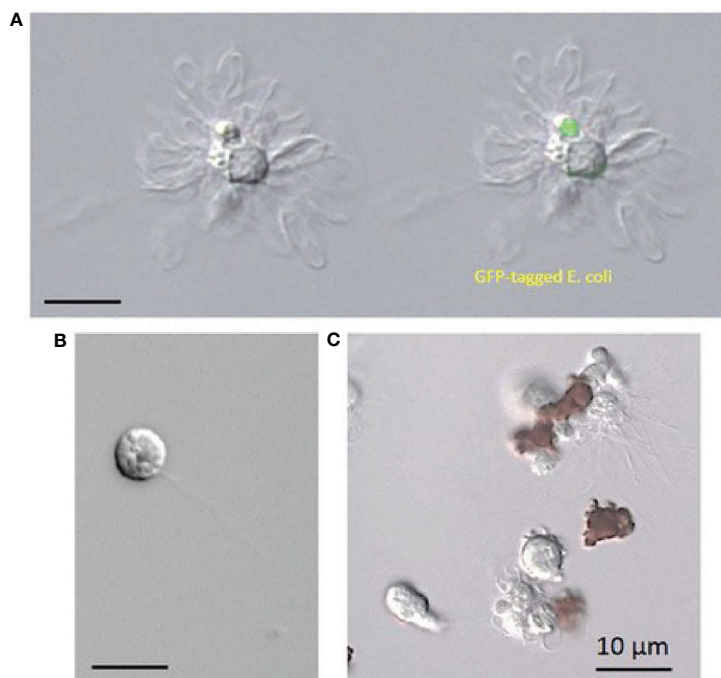


FIGURE 4 | Immune cells from *Paracentrotus lividus* sea urchin collected as a total cell population in an anticoagulant solution containing EGTA. **(A)** Phagocytes, **(B)** vibratile cell, **(C)** red and white amoebocytes.

defense against non-self and, in colonial species, trigger the inflammatory response consequent to allorecognition (128).

In *Botryllus schlosseri*, a model species for the study of immunobiology (**Figure 5A**), allorecognition and asexual reproduction, including whole-body regeneration (119, 129–131), colonies increase their size through periodical cycles of asexual reproduction requiring the involvement of stem cells. Unlike most species, where the body is long-lived and maintained by cellular replacement, *B. schlosseri* regenerates new colonial units (buds) on a biweekly basis from stem cells that remain for life, replacing the previous generation's zooids and, partly, the circulatory cells (129, 132–137). Circulating immune cells influence the process as they assure the cross-talk between blastogenic generations at the generation change (takeover) (**Figure 5D**): cytotoxic MCs increase the transcription of the complement factor 3 (C3) and stimulate phagocytosis, required for the removal of apoptotic cells in tissues of old zooids (138, 139). Phagocytes are also required for the completion of the takeover and the formation of new buds as the prevention of phagocytosis blocks the process (140).

By limited dilution transplantation of cells that express the high enzymatic activity of aldehyde dehydrogenase, a stemness marker, and from a set of serial engraftment assays, Laird et al. (141) revealed that multipotent stem cells are responsible for stable long-term chimerism and budding in *B. schlosseri* colonies. A major adult somatic stem cell niche was further identified in the anterior ventral side of the endostyle (18), the long glandular groove extending medially at the ventral face of the zooid

pharynx (**Figures 5E–G**). It is immersed in the hemolymphatic flow through the large subendostylar sinus and other sinuses (142). Cells that contribute to gonad formation were identified in cell islands along the endostyle (143). These stem cells and stem cell niches were identified by direct visualization of cells that exhibit fundamental and important aspects of mammalian stem cell biology, including self-renewal, homing into developing buds, expansion, differentiation, and multilineage potential (18, 141, 143). The involvement of circulating stem cells in the formation of new buds is also supported by the case of vascular budding or whole-body regeneration, where new buds can originate from the aggregation, inside the tunic vessels, of undifferentiated circulating cells even in the absence of adult zooids (144–152).

Ascidian eggs are enveloped by outer and inner follicle cells, the vitelline coat and the test cells (125). In *B. schlosseri*, all these cells share common surface antigens with hemocytes and tunic cells (153). Test cells constitute an ascidian-specific cell envelope encased in oocyte depressions beneath the other egg envelopes. During development, test cells adhere to the tunic surface and are discharged at hatching (154). Test cells are of maternal origin and are first detected during early oogenesis (155). They express markers that can be associated with immune defense but also with the stem lineages: (i) DDX1 (156) known to function as sensors of viral and bacterial RNAs and in inhibition of virus replication (157, 158); (ii) a IAP orthologue [IAP28 (159); **Figure 6**] that was correlated with cell proliferation control (160) and activation of caspases leading to apoptosis,

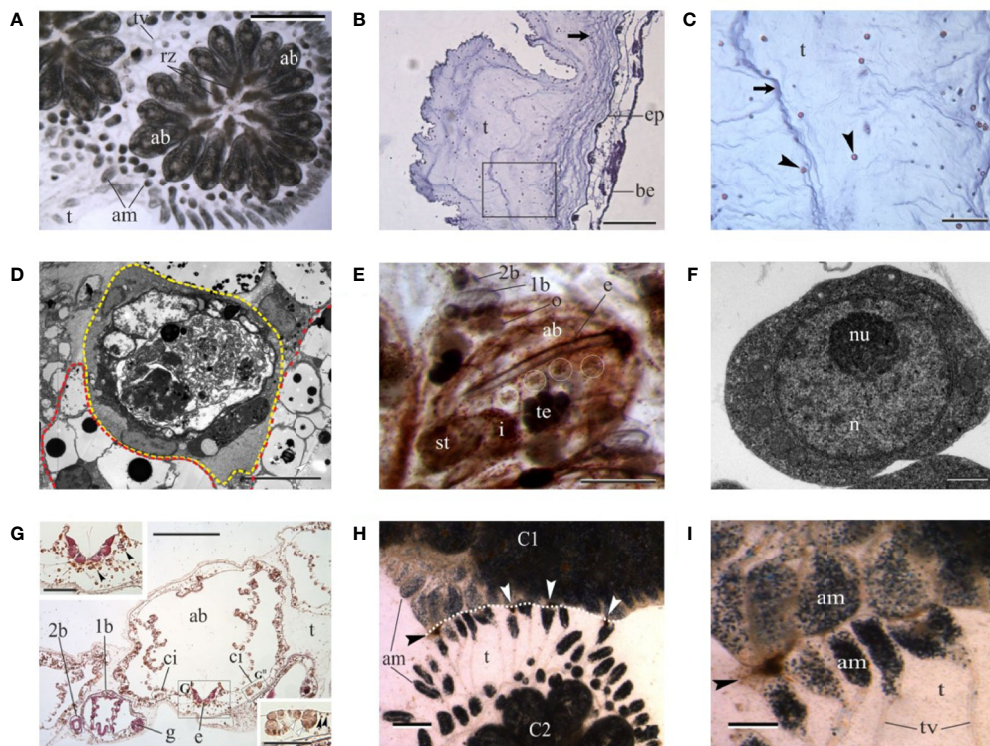


FIGURE 5 | (A) Colony of the tunicate ascidian *Botryllus schlosseri*. The colony has concluded the change of generation and adult blastozooids (ab) coexist together with regressing zooids (rz). am, blood ampullae in tunic; t, tunic; tv, tunic vessel. Dorsal view. Scale bar: 2.2 mm. **(B, C)** Histological sections of the tunic of the solitary ascidian *Ciona robusta*. Square area in **(B)** is enlarged in **(C)** to show tunic fibers (arrows) and cells (arrowheads). Hematoxylin–eosin. be, branchial epithelium; ep, epidermis. Scale bar in B: 200 μ m; in C: 50 μ m. **(D)** Transmission electron microscopy of immunocytes in *B. schlosseri*. Yellow dotted line: large phagocyte with phagosomes of different size. Red dotted line: morula cells characterized by large vacuoles with an electrondense core. Scale bar: 10 μ m. **(E)** Ventral view of an adult blastozooid (ab) of *B. schlosseri* with its primary bud (1b) bearing a secondary bud (2b). White circles label the cell islands in the left ventral body wall. Whole mount, ventral view; hematoxylin. o, oocyte in gonad rudiment in primary bud; i, intestine; st, stomach; te, testis. Scale bar: 500 μ m. **(F)** Transmission electron microscopy of a candidate stem cells in *B. schlosseri*. n, nucleus; nu, nucleolus. Scale bar: 1 μ m. **(G)** Transverse histological section of an adult blastozooid (ab) of *B. schlosseri* with its primary bud (1b). The latter bears a secondary bud (2b). The endostyle (e) niche and the cell island (ci) on the right are enlarged in upper left and bottom right insets, respectively. The contralateral cell island is contained by the black circle. Note in insets that small hemoblasts (black arrowheads) are recognizable in both the endostyle niche and in the cell island. In the latter, they are close to large phagocytes (white arrowheads). g, gonad rudimenti in primary bud; t, tunic. Hematoxylin–eosin. Scale bar in G: 200 μ m; in G¹–G¹¹: 50 μ m. **(H, I)** Regression between two incompatible colonies (C1 and C2) of *B. schlosseri*. Necrotic areas (arrowheads) are recognizable between the contacting tunics (white dotted line). am, blood ampullare; t, tunic; tv, tunic vessels. Scale bar in H: 1.2 mm; in I: 0.47 mm.

depending on its subcellular location; (iii) Vasa and DDX3 (PL10), RNA helicases highly associated with soma/germ stem cells and germ lineages [(156, 161); **Figure 6**]. PL10 is also a modulator of the NF- κ B pathway, known to be involved in responses to stress and viral/bacterial infections.

Allorecognition in Aquatic Invertebrates: Stem Cell-Related Immunity

The term allorecognition is generally defined as the capability of intraspecific recognition of non-self. Here, we will consider the animal immune-related phenomena of allograft rejection/acceptance and colony specificity that usually implies the triggering of an inflammatory reaction leading to the killing of the alien tissues.

Allorecognition is central to the understanding of innate immunity in vertebrates (39), and is one of the most highlighted features of aquatic invertebrates. It reflects the

facility for self and non-self discrimination between conspecifics, a phenomenon that evolved over 600 million years ago, in concert with the development of multicellularity (162, 163), and reveals, with the few available examples on the formal genetics of allorecognition in marine invertebrates, levels of polymorphism at allorecognition loci that exceed typical levels associated with other polymorphic loci (164). One such example is the colonial tunicate *B. schlosseri*, where populations from several sites, worldwide, revealed 80 to 300 allorecognition alleles at the fusibility locus/site (165–168). Indeed, while no homology has been established between marine invertebrates and vertebrates allorecognition genes, and not even among those of different invertebrate species (47, 169–171), a wide range of marine invertebrates, including sponges, cnidarians, bryozoans, and ascidians, reveal allorecognition competencies that represent high specificity and accuracy (44, 172, 173), also reflecting highly conserved effector mechanisms for innate immunity in the

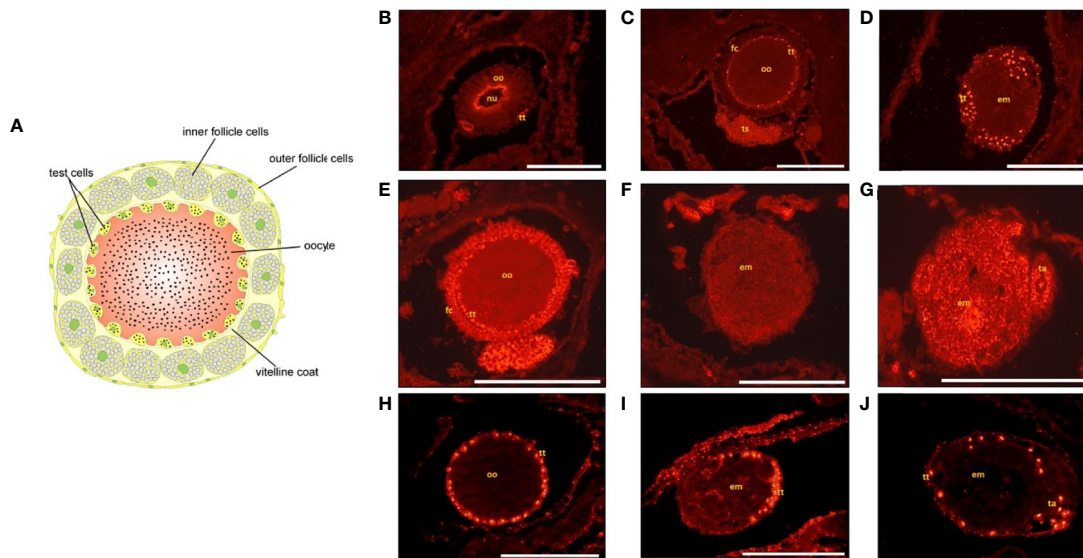


FIGURE 6 | (A) Scheme of *Ciona robusta* oocyte showing the envelopes surrounding it. Test cells are located under the vitelline coat. Right: immunohistochemical analysis of *Botryllus schlosseri* oocytes (**B, C, E, H**) and embryos (**D, F, G, I, J**) with Vasa (**B–D**), PI10 (**E–G**) and IAP antibodies (**H–J**). Bar=250 μ m.

animal kingdom (40, 42–44, 46). The discovery of specific and complex non-transitive hierarchies in marine invertebrates (174–176) is dictated by a variety of effector mechanisms that are tailored to recognize different conspecifics (177).

Allorecognition in aquatic invertebrates has been probably developed for purposes such as the regulation of gametic compatibility (164, 178), for the discriminatory machinery of within-organism conflicts (179), as a platform to purge nascent selfish cells (39), as a partisan tool following the completion of each major evolutionary transmission (180) and for protection against predatory cell lineages of the same species, part of the costs of fusion with non-self, where stem cell lineages play a major role (47, 181–184). It is manifested by specificity, immune priming, allogeneic maturation and natural chimerism (176, 185–188), many of them managed by stem cells. These allorecognition phenomena exhibit suites of effector mechanisms (specific and non-specific) that are based on extreme allotypic diversity, commonly emerged in wide groups of marine invertebrates, and are recurrently seen in nature in the wake of tissue contacts among conspecifics. The genetic blueprints of allorecognition in marine invertebrates were elucidated in just two model species, the hydrozoan *Hydractinia symbiolongicarpus* (189, 190) and the colonial ascidian *B. schlosseri* (171). Yet, transcriptomic profiling for other allorecognition responses also exists for other marine invertebrates, like sponges (191) and corals (44).

Ascidian Allorecognition as an Immune Phenomenon

In solitary ascidians, allorecognition is observed morphologically as fusion/rejection reactions of allografts (192, 193) or *in vitro* as “contact reaction” responses among co-cultured hemocytes (194–196). Both allorejection and contact reactions trigger inflammatory

responses with a decisive role of cytotoxic hemocytes, known as MCs (197) that underwent degranulation and induced cytotoxicity (99). In *Styela plicata*, during the allograft rejection, there is significant recruitment of MCs within the allograft bed and the neighbor tissues within days of transplantation, followed by a slower increase of circulating undifferentiated cells, commonly known as lymphocyte-like cells or hemoblasts, in the tunic surrounding the graft. This response reaches a peak around day 30 from the transplantation (99, 193). Since MC-induced inflammation leads to cell death (see below), the hemoblast increase suggests the activation of hematopoietic activity with the release in the circulation of new cells, in order to replace the effete cells. This idea is further corroborated by the observation that, in *Styela clava*, the injection in the tunic of allogeneic hemocytes induces the proliferation of hemoblasts with a peak at 5 days post-injection (198).

In colonial ascidians, allorecognition manifests itself as colony specificity occurring whenever colonies contact each other. This leads to either a fusion between genetically compatible colonies, with the formation of a chimeric colony (**Figure 7**), or a rejection between incompatible colonies, with the appearance of a region of dead tissue along the contact border. In *Didemnum vexillum* allogeneic partners may, initially, form chimeric entities. Subsequently, zooids of differing genotypes coordinatively retreat away from the fusion zones and within a few days, they detached leading to the formation of zooid-depauperate tunic zones (199). The genetic control of fusion/rejection was particularly studied in the colonial species of the genus *Botryllus*. As previously reported, fusibility is controlled by a highly polymorphic fusibility/histocompatibility locus and fusion always occurs when contacting colonies share at least one allele at this locus (200–202). Fused colonies share both the tunic and the circulatory system.



FIGURE 7 | *Botryllus* chimera. Two genetically compatible colonies fused into a single chimeric colony, sharing the tunic and the circulatory system, after their contact.

Without a common allele on the fusibility locus, an inflammatory (rejection) reaction follows the initial limited fusion of the tunics that allows the diffusion of soluble factors from one colony to the alien one. The outcomes of the rejection reaction are a series of melanic, necrotic spots along the contact border known as points of rejection (128, 203–206). At the cellular level, upon the recognition of the alien factor(s), MCs exit the colonial vasculature and enter the tunic at the contact points where they degranulate. The released materials, mainly the enzyme phenoloxidase and its polyphenolic substrata, induce a local cytotoxicity (128, 205–209). A “contact reaction”, similar to the one observed in solitary ascidians, leading to the release of fluorescent material from MCs and their ultimate death, together with that of neighboring cells, can also be observed when hemocytes from incompatible colonies are mixed *in vitro* (210). This implies that the dead cells must be replaced by new cells entering the circulation from hematopoietic sites. Indeed, experiments carried out in the Japanese species *Botryllus primigenus* indicate that X-ray irradiation can alter the intensity of the rejection reaction, likely affecting stem cells, commonly known as X-ray-sensitive cells (211). As a second step in allorecognition, the progressive resorption of one compatible partner frequently occurs (203, 212–214). The resorption of the zooids can be mimicked by the injection in the colonial vasculature of MCs from a fusible partner (215).

Ascidian Allorecognition and Stem Cell Lineage Competition

When colonies of *B. schlosseri* with opposite genotypes (AAbb and aaBB, respectively), relative to two Mendelian loci controlling zooid pigmentation, were separated after a short (few days) period of fusion, and crossed with the double recessive aabb, in addition to the expected offspring phenotypes (Ab and aB), a certain

number of colonies with the aB and Ab phenotypes were obtained from the AAbb x aabb and aaBB x aabb crosses, respectively. This is indicative of the persistence of germ stem cells from the previously fused colony competing with the host stem cells to produce gametes (216). As, during the cyclical (weekly) generation replacement (blastogenesis) of *Botryllus* colonies (129), stem cells leave their temporary niches and, through the circulation, move to new niches in the growing buds (18, 143), it was possible to record the production of heterochthonous offspring for 15 blastogenetic generations and, in few cases, the entire offspring was heterochthonous, indicative of the complete germline parasitism of the host gonad by the alien stem cells (216).

Somatic parasitism can also be demonstrated when compatible colonies sharing one allele at the Fu/Hc locus (e.g., with AC and AD genotypes) were fused for at least four days before their separation. When AC colonies previously fused with AD colonies and AD colonies previously fused with AC colonies were challenged with BC and BD colonies, respectively, it was possible to observe an alteration of fusibility as colonies alternated fusibility and rejection with BC and BD colonies. This alteration, in few cases, was observed up to 2 years from the initial fusion and is indicative of the persistence of a renewing population of cells from the alien colony responsible for the observed effect. This is supported by the observation that the electrophoretic patterns of the enzyme phosphoglucoisomerase and amplified fragment length polymorphism in the AC colonies previously fused with AD colonies combine the pattern of AC and AD colonies (217–219).

In addition, as reported before, both somatic and germ cell parasitism can be obtained through the injection, in the circulation of compatible colonies, of hemocytes selected based

on their high aldehyde dehydrogenase activity, a marker of stem cells in mammals (141).

Even in the case of colony resorption, the somatic and germline of the resorbed colony is not lost. Germ stem cells can invade the host gonads and give rise to germ cell parasitism with the production of heterochthonous offspring (203, 217, 219–221); somatic stem cells also persist in previously fused colonies, as demonstrated using microsatellites as molecular markers (220, 221). In addition, a chimera can express one or another genotype according to changes in seawater temperature (219), indicative of the capability of a colony to modulate its stem cell reservoir when the environment changes.

Although there are no direct data on the proliferation of circulating hemoblasts following allojection (but see (184) for hemocyte xenotransplantations) or alloresorption, in both cases a MC-driven inflammatory event is activated leading to the ultimate death of these cells upon their degranulation (215). This suggests that, similarly to allograft rejection and contact reaction in solitary ascidians, new hemocytes should enter the circulation as a product of hematopoiesis activation. The relationships between inflammation and hematopoiesis, still unclear, deserves further investigation in the next future.

Stem Cells-Immune Cells Cross-Talk in Stress and Toxicity

Immune and stem cells of invertebrate species have already been recognized as valuable models in ecotoxicology for both *in vitro* and *in vivo* studies. However, rarely the behavior of both cell type and their cross-talk in preserving and restoring homeostasis have been used as ecotoxicological endpoints (7, 11).

Recently, the potential application of aquatic invertebrate ASCs in ecotoxicology has been underlined in a very comprehensive review (7). The authors point out that, due to their lower genetic complexity, aquatic invertebrate ASCs represent a valuable and reliable tool for understanding fundamental biological processes, mode of action of pollutants and mechanisms of epigenetic toxicity. It was suggested that ASCs from selected invertebrate species having a key role in aquatic ecosystems could be harnessed in ecotoxicological testing.

However, up to now, the majority of ecotoxicological studies largely involved the use of immune cells instead of stem cells (11, 97, 222, 223). Few contributions investigating immune cells responses towards pollutants include aspects of hematopoietic tissue or circulating stem-cells/stem-like cells. Ambrosone et al. (224) investigated the effects of engineered nanoparticles (EPs) on regeneration success and proliferation of stem-cells of *Hydra vulgaris* using the bromodeoxyuridine immunodetection assay. Also, gene expression of the transcription factor Hymc1, involved in stem cell proliferation/differentiation, was monitored. Furthermore, the impact of EPs as well as of 4-methylumbelliferon) on hematopoietic organ and circulating hematopoietic stem cells of silkworm (*Bombyx mori*) larvae was investigated (225–227). The rate of hematopoiesis in hematopoietic organ *in vitro*, apoptosis of circulating hemocytes, and internalization of EPs by prohemocytes (227) referring to it as “hematopoiesis toxicity” were reported (226).

These kinds of studies benefit from the extensive knowledge of hemocyte proliferation and of the final steps of hemocyte maturation in the silkworm (228). Similarly, Oweson et al. (229) reported the effects of manganese on circulating immune cells of the common sea star *A. rubens*. Manganese induces the proliferation of the cells of the coelomic epithelium, a putative hematopoietic organ of echinoderms (see above), and increases the number of circulating coelomocytes. At the same time, coelomocytes showed stress response and their phagocytic capacity was negatively affected. Such findings can be considered as a building block in the understanding of the interplay between immune and stem cells in invertebrate species. The complex “defence” machinery used by them to face external stressors, including exposure to pollutants clearly identifies the involvement of both in the organism’s response and ability to recover and evolve. Taking into account the increasing environmental changes faced by aquatic invertebrate species in their natural environment and the variety of stressors to which they must adapt to survive, greater knowledge of the interplay between the immune system and stem cells would be useful for predicting new future scenarios and identify those species more prone to deal with it (Figure 8).

STEM AND IMMUNE CELLS OPEN QUERIES NEW TOOLS, AND FUTURE PERSPECTIVES

Research on aquatic invertebrate immunity and stem cells suffers from the scarce consideration of this group of animals as model organisms for biological research, with few notable exceptions represented by *Hydra* and planarians. Moreover, the lack of suitable *in vitro* models as well as of specific protocols for cell identification and cell culture limit their application also in ecotoxicity. Here below, we present some critical points that deserve consideration in the near future and can lead to rapid and significant advancements if tackled correctly.

Identification of Stem and Immune Cells

The paucity of available molecular markers to precisely identify stem and immune cells in aquatic invertebrates is still a limitation. Recently some progress has been made as for instance, in mussel hemocytes. Freshly isolated hemocytes from the mussel *M. galloprovincialis* have been shown to be highly responsive to mammalian stem cell growth factor in terms of both increased proliferation, and changes in immunoreactivity to the HSC marker CD117, or Kit-ligand receptor [(230); Canesi, personal communication] (Figure 9).

Up to now, light and electron microscopy have been the major methods for the identification of stem and immune cells of aquatic invertebrates. Light microscopy could be associated with immunohistochemistry or *in situ* hybridization to reveal the location of enzymatic activities or molecular markers. In addition, differential interference contrast and phase-contrast microscopy were also used for cell characterization (231–235).

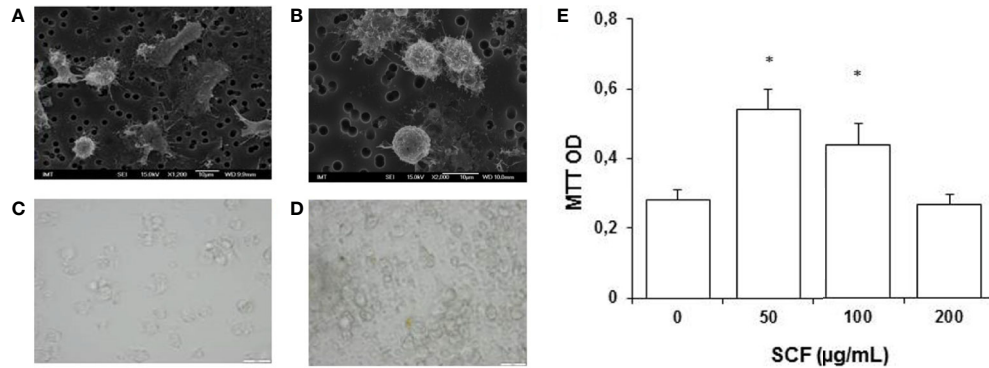


FIGURE 8 | *Mytilus galloprovincialis*. (A, B) Scanning electron microscopy (SEM) image of freshly isolated cells (courtesy of Dr. Manon Auguste). (C, D) Light microscopy of control hemocytes (C) and hemocytes exposed to human Stem Cell Growth Factor (SCF) (24 h, 50 µg/mL, in the presence of 5 µg/mL Con-A (D)). (E) Effects of SCF on hemocyte proliferation evaluated by the MTT assay. *P < 0.05 Mann-Whitney U test.

Scanning electron microscopy (SEM) was successfully applied to distinguish various hemocyte populations in aquatic invertebrates, as well as visualize the putative stem cells of some organisms (232, 236–239). Holland and Somorjai (240) used serial block-face scanning electron microscopy to distinguish the population of stem cells in invertebrate chordate.

Despite demanding sample preparation and time-consuming inspection of samples, transmission electron microscopy is the most widespread technique for the study of the ultrastructure of invertebrate circulating cell and putative stem cells (231, 232, 236, 241–250). However, new imaging technologies are emerging, such as optical coherence tomography, optical coherence phase

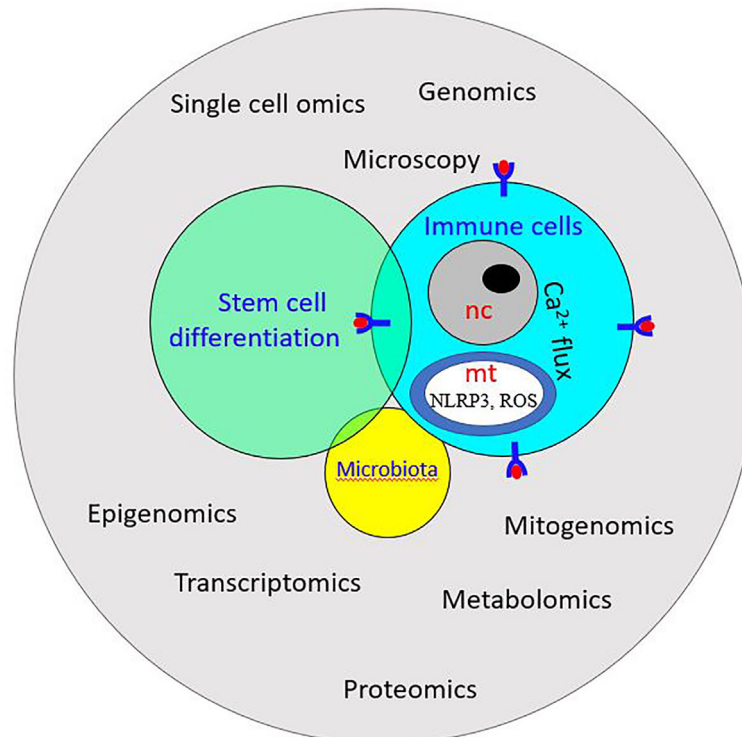


FIGURE 9 | Schematic illustration of system biology: it exploits -omics technology to study the interplay among stem cells, immune cells, and microbiota. ROS, Reactive oxygen species; NLRP3, NOD-like receptor P3; nc, nucleus; mt, mitochondrion.

microscope, microcirculation imaging, confocal reflectance microscopy, super-resolution microscopy, microcomputed tomography, FIB/SEM milling and subsurface imaging, radionuclide imaging that, if applied to aquatic invertebrates, can lead to a finer description of cell morphology and better identification and discrimination of stem and immune cells.

In crustaceans, the identification of different hemocyte lineages based on their morphology (251) was upgraded by studying the expression of some hematopoietic transcription factors (GATA, RUNX) and signal pathway molecules (JAK/STAT, Notch) (252). In addition, specific protein markers have been used for the identification of semigranulocytes (Kazal-type proteinase inhibitor and crustacean hematopoietic factor) and granulocytes (superoxide dismutase and mannan-binding lectin) (253, 254).

Homologues of the vertebrate stemness genes have been identified in many aquatic invertebrates studied so far (255–258) and were used as stem cell markers in various species (259–265). Furthermore, the application of flow cytometry, together with different markers of stem cells and hematopoietic cells, to circulating hemocytes/coelomocytes or to dissociated cells from early larval stages, can represent a powerful tool for investigating stem cells and innate immunity in adult and developing aquatic invertebrates. Recently, using flow cytometry sorting, whole-transcriptome sequencing, and diverse transplantation essays, Rosental et al. (266) identified hematopoietic stem cells and hematopoietic niches in the tunicate *B. schlosseri*. This study revealed that the subendostylar sinus is a hematopoietic stem cell niche while its molecular signature further suggests that the vertebrate hematopoietic bone marrow niche evolved from an organ resembling the *B. schlosseri* endostyle (266). The comparison of the HSC molecular signature during embryogenesis and blastogenesis, showed a similar early/late pattern of the HSC-associated gene enrichment, suggesting similar molecular dynamics of HSC development in embryogenesis and blastogenesis in *B. schlosseri* (137).

In Vitro Cell Cultures

Although many efforts have been spent over many years of research, no established cell line from aquatic invertebrates is available today. This represents a serious obstacle to the full elucidation of the stem cell and immune cell properties in this group of animals and to promote their application in various disciplines (267, 268). The main limitations in cell harvesting lie in the *in vitro* requirements which leads to the failure of most invertebrate primary cultures within 24–72 h post cell isolation (7, 267), a topic that has been recently tested from an experimental point of view (269). However, some progress has been made as reported below. Conkling et al. (270) were able to keep in culture dissociated sponge cells for more than one month observing a significant increase in their number, Munroe et al. (271) have developed a genetic algorithm as an optimization tool for the development of sponge cell culture media, and efforts have been invested in studies on the establishment of sponge transgenesis (272). Ventura et al. (273) succeeded in maintaining primary cell cultures from dissociated tentacle cells of the cnidarian *Anemonia viridis* for one month. In crustaceans, long-term primary cultures of crayfish HPT cells were possible

with the use of astakine, a new invertebrate cytokine directly involved in hematopoiesis, thus allowing the study of the molecular mechanism involved in the production of hemocytes (84, 252, 274). Transfection methods were also studied on the hematopoietic primary culture system from *Cherax quadricarinatus* (275) and efforts were invested in long-term ostracod epidermal cultures (276).

Recently, a method for the harvesting and short-/long-term maintenance of primary immune cells from sea urchin *Paracentrotus lividus* has been proposed (277). This method uses the coelomocyte culture medium, containing a high-affinity Ca^{2+} chelator, for short-term culture (≤ 48 h), and the artificial seawater as the master medium that maintains cell survival and *in vitro-ex vivo* physiological homeostasis over 2 weeks. Such method could unravel complex cellular phenomena and is pivotal in establishing the sea urchin invitrome (107, 108).

'-Omics'-Based Technologies

Microscopy and molecular analysis of aquatic invertebrate stem-cell behavior can be fostered by the -omics tools pioneered for studying regeneration and immune responses. The last two decades have seen the generation of draft genomes of various aquatic invertebrate species (163, 278–293). The increasing number of data has been accompanied by increasing quality of assemblies and annotations, for example in oysters (294) and mussels (295). Studies on the genome of selected species are contributing to elucidate the molecular mechanisms underlying the processes of proliferation and differentiation of immune cells from stem cells. Moreover, the application of transcriptomic approaches to early developmental stages is helping to unravel the molecular signature of undifferentiated cells, including the expression of stem cell markers, transcription factors, and epigenetic modifications. For instance, Mao et al. (296) reported transcriptomic evidence of the molecular basis underlying functional differentiation of hemocytes in the bivalve *Crassostrea* sp., whereas Söderhäll and Junkunlo (297) recently reported a complete proteomic analysis from purified cell types from the crayfish HPT, the anterior proliferation center. This last work allowed the detection of several cell type-specific proteins and new putative biomarkers within the crayfish hematopoietic lineage that can be used to increase the understanding of how the differentiation process is regulated, are described.

Besides the nuclear genome, the mitochondrial genome's omics approach can provide further information on the relationships between stem and immune cells. The release of DNA and/or reactive oxygen species by mitochondria represents a signal influencing immune cell transcription (298). Mitochondria can also regulate immunity through the alterations of metabolic pathways and are associated with NLRP3 inflammasome activation (299): mitochondria-induced transcriptional changes can lead to entirely different outcomes in immune cells (300). Mitochondria are also essential for stem cell plasticity (301) and it has been reported that mitochondria have a role in deciding stem cell fate by keeping the records of divisional histories (302, 303). Indeed, stem cell fate decisions are controlled by a complex balance between mitochondrial-nuclear interactions and Ca^{2+} flux that further modulates mitochondrial features during proliferation, metabolism, differentiation, and apoptosis (302, 304, 305).

Single-Cell Transcriptomics

Stem and immune cells live in a complex environment aimed at protecting tissue integrity and homeostasis upon changes in functional demands; they have various genetic backgrounds and are endowed with disparate receptors on their plasma membrane (306). All these features are under the control of their genome, whose expression varies upon cell type. Today, it is possible to associate each cell with its “single-cell transcriptomics” (SCT) profile. Current SCT studies offer unprecedented opportunities to trace the evolutionary origins of every cell within a tissue/organ and reveal their activation pathways (258) and encompass a broad spectrum of organisms and cell types (307–316). SCT approaches can be used to elucidate how stem cells differentiate into the set of cells that make the body of the larval/adult organisms (258, 317, 318). The application of this technique to stem and immune cells of aquatic invertebrates can provide researchers with valuable information on transcriptome trajectories, regulatory differentiation cascades, and common gene networks underlying cell differentiation/activation during development and upon nonself recognition and response.

Epigenetics Modifications

Cytosine methylation is one of the main covalent base modifications in eukaryotic genomes. It is involved in the epigenetic regulation of gene expression in a cell-type-specific manner, is reversible, and can remain stable through cell division. In mammals, epigenetic changes associated with stem cell self-renewal and differentiation (319, 320) as well as with the activation of immune cells (321) have been reported. Whereas invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* do not exhibit cytosine methylation and consequently do not have CpG rich and poor regions but rather a steady CpG frequency over the genome (322), some studies provided evidence of epigenetic modification in planarian stem cells (323) and in tissues of buds and regenerating zooids in colonial tunicates (324), but no investigation was addressed to aquatic invertebrate immune cells. Therefore, the study of histone methylation patterns can provide relevant information on its role in the maintenance of stem cell identity by chromatin remodeling and transcriptional control of pluripotency genes as well as in immune cell activation. In addition, the techniques of chromatin immunoprecipitation followed by sequencing (ChIP-Seq) for genome-wide profiling of DNA-binding proteins, histone modifications, or nucleosomes offer the possibility of a better understanding of the importance of chromatin modification in stem cell differentiation and immune cell activation. This can elucidate the gene regulatory networks characterizing the interplay between the transcriptome and the epigenome (325–327) in the cross-talks between stem and immune cells.

Stem Cells, Immune Cells, and Gut Microbiota

It is widely accepted that the gut community of microorganisms has beneficial effects on animals and host-microbiome

interactions contribute to the general organism homeostasis. Various data suggest the importance of gut microbiota in shaping an organism's immune system/response (328–331). The opposite is also true: the immune system influences the microbial community in the gut (332). A clear demonstration of the close relationships among gut microorganisms and immune cells in humans comes from the observation that patients with inflammatory bowel disease are characterized by restricted biodiversity and unbalanced bacterial composition in their intestines associated with immune dysregulation (333, 334). Gut microbiota is also important for the establishment of the innate immune memory or immune priming in both vertebrates and invertebrates (331, 335, 336). Stem cell activity is also influenced by the gut microbiota, although few researchers focused on this (337, 338). Studies on the gut microbiota in aquatic invertebrates are still at the very beginning (339–343). However, since the gut microbiota of invertebrates is simpler than that of vertebrates (341), the former can be used as a suitable model to study its intimate dialogue with stem and immune cells. Indeed, Arnold et al. (56) reported evidence that innate immune responses and the machinery required for regeneration (which includes neoblasts) are modulated by the shift in gut microbial composition in the planarian *Schmidtea mediterranea*. Liberti et al. (344) recently provided an exhaustive review on the interaction between the immune system and the gut microbiota in the solitary ascidian *Ciona robusta*, but no data are available on the modulation of the stem cell activity by the microorganisms hosted in the gut.

Systems Biology: Multi-Omics Approach

System biology is the integrative study of biological systems. It exploits the high volume of data from genomics, proteomics, metabolomics, and other omics- technologies and combines mathematical and computational models to characterize functional changes across different contexts (345, 346). Studies using a multi-omics approach integrating metagenomics, metatranscriptomics, metaproteomics and metabolomics data are already ongoing to clarify the molecular basis of some human diseases (347, 348). As far as aquatic invertebrates are concerned, multi-omics approaches, involving bioinformatics tools for the analysis of high throughput sequencing data, database designs for proteomics and metabolomics data, use of algorithms to detect transcriptional and translational changes is a new venture that will certainly help researchers in understanding their biological systems. A multi-omics approach was recently followed for studying the interaction between the host and the gut microbiota in the solitary ascidian *C. robusta* (344). Another aspect of system biology is the combination of omics-based data and morphological data. For instance, in the compound ascidian *B. schlosseri*, transcriptomic data were combined with multiple microscopy techniques to investigate the changes in molecular and morphological signatures of cells during embryogenesis and blastogenesis (314). This comprehensive approach will certainly help in identifying differentiation/activation pathways for stem and immune cells.

CONCLUSION

Our review presents the *state-of-the-art* of the current knowledge on the relationship between stem cells and the immune system in selected aquatic invertebrate phyla. We point out the need to bridge two seemingly disparate disciplines, stem cell biology and the immune system research. The need to integrate current knowledge, methodological tools and concepts to provide a more holistic understanding of how organisms maintain and restore homeostasis using stem cells and immune system is underlined. Here we propose aquatic invertebrates as suitable models to deeply investigate the interplay between stem and immune cells, starting by addressing the basic equations in biology and further applying the acquired knowledge in other fields as ecotoxicology, biotechnology, and medicine. A parallel to vertebrates is presented to demonstrate how these two fields of research can be interwound.

The integrated analysis of existing information needs to be coupled with the generation of new data and the new tools available will allow cross-species extrapolations. The biological read-across can only be achieved by understanding similarities and differences in biological pathways through the involvement of a variety of species.

AUTHOR CONTRIBUTIONS

LB - substantial contributions to the conception, and drafting the work, revising it critically for important intellectual content. AK - substantial contributions to the conception and drafting the

work, revising it critically for important intellectual content. AS - conception and drafting the work. LR - conception and drafting part of the work. LM - conception and drafting part of the work. BuR - conception and drafting, AR - conception and drafting. AV - provide approval for publication of the content. BeR - provide approval for publication of the content. LC - conception and drafting. CA - conception of the work. AP - conception of the work. BT - provide approval for publication of the content. AJ - provide approval for publication of the content. AD - provide approval for publication of the content. SN - provide approval for publication of the content. MS - conception and drafting part of the work. IC - provide approval for publication of the content. DD - substantial contributions to the conception, and drafting the work, revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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