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Evolutionary Perspective on the Hematopoietic System through a Colonial Chordate: Allogeneic Immunity and Hematopoiesis

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Abstract

Evolution and selection have shaped diverse immune systems throughout phylogeny, the vast majority of which remain unexplored. *Botryllus schlosseri* is a colonial tunicate, a sister group to vertebrates, that develops as a chordate, then metamorphoses to an asexually reproductive invertebrate that every week makes the same body plan from budded stem cells. Genetically distinct *B. schlosseri* colonies can fuse to form a chimera, or reject each other based on allogeneic recognition. In chimeras, circulating germline and somatic stem cells participate in development; stem cells compete in all individuals in the fused colonies, with rejection preventing germline parasitism.

Here we review the isolation and characterization of *B. schlosseri* hematopoietic stem cells (HSC) and their niches, and the role of the immune effector cells in allorecognition.

Keywords

Comparative Immunology; Allorecognition; Tunicates; HSC; Cytotoxic Cells; Evolution of the Immune System; Hematopoietic System; Innate Immunity

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Introduction

Pathogenic microbes and internal pathogenic cells most likely evolved simultaneously with multicellular life forms they could parasitize. The rapid evolution of pathogens was mirrored by the development of innate and anticipatory adaptive immune systems. Throughout evolution the immune system has evolved a heterogeneous and highly diverse repertoire of cells and mechanisms that identify and target pathogens as well as determine the tolerance to harmless antigens like self and symbionts.

While we know a great deal about immunity in a small set of model organisms, the wide array of genes and cells that constitute diverse immune systems in nature are still unknown. The study of immune systems of non-classical model organisms have revealed significant discoveries including the discovery in multicellular organisms phenomena such as phagocytosis (starfish,1), toll-like receptors (flies, 2), VLR novel adaptive immune system (lamprey, 3), somatic immune receptor diversification at a single cell level (urchin,4), resembling somatically recombined Ig and TCR molecules in lymphocytes of most vertebrates, and in single celled organisms restriction endonucleases and the CRISPR–CAS immune system (prokaryotes, 5, 6). Tunicates, like *Botryllus schlosseri*, the closest living invertebrate relatives of vertebrates, are situated at a critical evolutionary juncture: their larval stage shares developmental and structural characteristics with all chordates, including a notochord, neural tube, segmented musculature and gill slits (7–8). Larvae settle and metamorphose into sessile individuals (Figure 1a), which lose most of their chordate cells by programmed cell death and cell removal. Tunicates reproduce either sexually (solitary species), or sexually and asexually (colonial species like *B. schlosseri*). These two reproductive modes give rise to nearly identical adult body plans, including digestive and respiratory systems (branchial sac), endostyle, a simple tube-like heart, siphons, a neural complex with larval specific and adult invertebrate specific neural elements, ovary and testis (9; Figure 1b). In *B. Schlosseri*, the larva develops after fertilization as a chordate, then metamorphoses to an asexually reproductive invertebrate that every week makes the same body plan from budded germline and somatic stem cells. Colonies grow via such cycles of stem cell mediated asexual reproduction, where buds develop and produce genetically identical individuals (zooids; 10). Every week the developed buds in a colony replace the older generation following a synchronized programmed cell death [and macrophage removal] of the parent zooids (11; Figure 1c). A defined vasculature with circulating blood cells including cells with lymphocyte-like and macrophage-like morphology connects all zooids and buds in the colony and extends outward with ampullae into a tunic that covers the entire organism (12–13; Figure 1).

When two colonies touch, they either fuse vasculature and share blood, or undergo a genetically determined immune rejection and permanently separate. (14–15; Figure 1e–f). This self-nonself recognition process is controlled by Botryllus Histocompatability Factor (BHF), a single polymorphic histocompatibility gene (16). For fusion to occur at least one shared *BHF* allele is required. Thus, *B. schlosseri* undergoes a natural allorecognition process with features of experimentally produced parabionts, in which successful ‘transplantation’ occurs only with genetically compatible colonies.

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Upon fusion, circulating somatic and germline stem cells from both colonies compete for dominance of the somatic and germline organs and contribute to the formation of new buds (10, 17–19). Highly successful germline stem cells can outcompete less successful stem cells in the gonads of their chimeric partner, resulting in heritable dominance of a single germline origin of gonads in the chimera (10, 17–19). In a significant fraction of these chimeras, the budding cycle in all of the individuals of one of the chimeric partners fails to develop or persist (20). It appears that this developmental arrest is an immune cell based rejection of bud cells that operate within a *BHF* histocompatible pair that involves inflammatory and cytotoxic cells, comparable to mammalian chronic rejections (20). Since fusion and stem cell competition among germ line stem cells is restricted to individuals that share a BHF allele, we hypothesized that allogeneic rejection restricts genotype replacement to histocompatible kin, preventing germline predation from unrelated conspecific colonies.

A comparison of the *B. schlosseri* genome with those of several invertebrates, solitary tunicates and vertebrates, revealed enrichment for genes essential to the immune system and hematopoiesis that are predicted to have evolved in a common ancestor of *B. schlosseri* and vertebrates (21). These hematopoietic and immune behaviors and innovations of a colonial tunicate prompted us to characterize the molecular and cellular mechanisms underlying hematopoiesis and immunity in *B. schlosseri* (22).

Evolution of HSCs and the Hematopoietic Organ

HSCs are multipotent, self-renewing cells that generate all mature blood and immune cell populations in an animal's life (19). In mammals, the blood-forming organ is the bone marrow, where HSCs reside in specialized niches that support their self-renewal and maintenance of an undifferentiated state. Since the identification of HSCs by prospective isolation (23), several model systems have been studied to elucidate HSC biology (24–27). We found that the mammalian and tunicate candidate HSCs (cHSC) share a transcriptome signature, suggesting that they are homologous and originated from a common ancestor (Figure 2a; 22). Among the genes expressed in *B. schlosseri* isolated hematopoietic progenitor cell populations, we found high enrichment for gene sets predominantly expressed in human HSC, myeloid populations, and early but not mature lymphoid populations (22). Consistent with previous studies (21, 28–31) this analysis indicates that the evolution of T and B or VLRA and VLRB cell adaptive immunity progressed rapidly in the jawless vertebrates, with much of the genetic repertoire in place by the emergence of jawed vertebrates. However, homologs of human genes with specific expression in HSC and blood progenitor populations, including T and B progenitor cells, appear early in metazoan evolution (21–22, 32–33). It is of interest that the MHC and elements of vertebrate immune receptors appear to be absent in both *B. schlosseri* and lampreys, and in all other sequenced invertebrates to date; as *omnis DNA e DNA*, the origin of these elements and how they appeared in jawed vertebrates remains an unsolved mystery.

The hematopoietic stem cell niche is an interactive structural unit organized to facilitate cell-fate decisions in a proper spatiotemporal manner (24, 26, 34). In adult mammals under normal circumstances, the main site of haematopoiesis is the bone marrow (24, 26, 34). The *B. schlosseri* HSCs are localized in the endostyle; 327 genes that are significantly expressed

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in the endostyle are also expressed in human hematopoietic bone marrow (Figure 2b; 22, 35), suggesting a common origin for the hematopoietic bone marrow niche and the endostyle. The tunicate endostyle is a long glandular groove extending medially at the ventral face of the zooid branchial sac along its anterior posterior axis consisting of eight distinct anatomical zones and is immersed by blood flow through the large subendostylar sinus and other sinuses (36). The endostyle is connected to the *B. schlosseri* central nervous system; several zones within it produce mucous and potentially also produce digestive enzymes and hormones (36), indicating a diversity of functions in this organ. The endostyle expresses thyroid transcription factor 1 homologs and has an iodine-concentrating activity, therefore it includes the invertebrate chordate homolog of the vertebrate thyroid gland (36). It exhibits unique expression patterns with site-specific factors that are linked to developmental regulation and stem cell maintenance (37–38). In 2008 we identified a somatic stem cell niche in the anterior subendostylar sinus (37). Recently, we discovered that the specific somatic stem cells that harbor and home to this endostyle niche are hematopoietic stem cells (Figure 1f; 22). In adult mammals and reptiles, the bone marrow is the main center of hematopoiesis, and during embryogenesis the yolk sac, dorsal aorta, placenta, and later fetal liver are centers; but the site of hematopoiesis in other chordates reveals a list of diverse organs and tissues. Hematopoiesis takes place in the head kidney in bony and cartilaginous fish, T cell development in gill tips, and B cell development takes place in the bursa of Fabricius in birds (39). In adult amphibians, hematopoiesis takes place in the bone marrow, but during the larval stage, the head kidney is the hematopoietic tissue (40–41). A bone marrow-like organ, the protovertebral arch, and the kidney are the sites of hematopoiesis in jawless vertebrates (42), and in cephalochordates it is suggested that the aorta-gonads-mesonephros is the site of hematopoiesis (43). Cellular and molecular comparison of these organs and tissues will reveal conserved elements that were maintained throughout evolution essential for hematopoiesis.

Cellular and Molecular Mechanisms of Histocompatibility

The ability to tolerate self and reject a foreign transplant is a critical element of the immune system in all animals. Although this is well characterized in mammals, the cellular and molecular details are not well understood in other groups. Morula cells, cells that contain phenoloxidase and accumulate in rejection points, have been identified as cytotoxic cells that mediate the execution of the rejection reaction in *B. schlosseri* (44–47). We further identified *BHF*, a single gene that determines self-recognition in *B. schlosseri* colonies, and begun characterizing the basic mechanisms of this process (16, 22).

In mammals, the Major Histocompatibility Complex (MHC) encodes the linked genes [haplotype] for tissue-antigens that allows the immune system to recognize and tolerate itself. Histocompatibility is governed by two types of cytotoxic cellular recognition: cytotoxic T-cells and Natural Killer (NK) cells. A subset of T-cells will recognize non-self MHC as foreign, and will be activated to eliminate cells that express this MHC. On the other hand, NK cells are inhibited by self-MHC through Killer Inhibitory Receptors (KIRs), and will eliminate cells that do not express self-MHC. Both the T-cells, which are activated by non-self, and NK cells that are activated by “missing-self” go through an educational process as they mature that allows them to make this distinction (48). Similar to NK

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inhibitory mechanisms, the allogeneic functional assays with blocking of BHF in *B. schlosseri*, reveal that the self-BHF recognition is a major inhibitory mechanism of cytotoxicity in allorecognition (22). These assays and the observation that colonies sharing at least one *BHF* allele fuse (16), demonstrate that the cellular cytotoxicity allorecognition mechanism in *B. schlosseri* is based on “missing self” and comparable to NK recognition in mammals. Similar to NK mediated cellular recognition through its KIR receptor repertoire we hypothesize that recognition between inhibitory receptors expressed on the effector cells and different BHF alleles is the mechanism that mediates self-recognition in *B. schlosseri*.

All cell populations sequenced express *BHF* RNA, however the *B. schlosseri* cell population that is enriched for the cytotoxic Morula Cells (MC)does not express BHF protein on the membrane (as detected by antibodies), suggesting the existence of an inhibitory receptor on cytotoxic cells that can recognize self-BHF. Interestingly, one of the genes which is differentially expressed by cytotoxic MC is *sFuHC*. This polymorphic gene was previously suggested to be the *Botryllus* histocompatibility gene (49), and is located in close proximity to the *BHF* locus (16). Considering the inhibition mechanism of the BHF and the significantly high expression of *sFuHC* in the *B. schlosseri* cytotoxic cells, it is possible that the *sFuHC* plays the role of an inhibitory recognition receptor for BHF. These observations suggest that there is an educational mechanism in *B. schlosseri* cytotoxic cells for selection for the inhibitory receptors, similar to NK cell education (50). Further studies will be needed to understand whether the colonial life history of *B. schlosseri* led to this complex immunity recognition system or if it is shared by all tunicates, both solitary (that do not form chimeras) and colonial. Although the specific molecules are not homologous to mammalian histocompatibility recognition, the features underlying this recognition are similar.

Evolution of the Cellular Immune System

The two best characterized aspects of cellular immunity are cytotoxicity and phagocytosis. *B. schlosseri* has a myeloid lineage including cells that take part in phagocytosis similar to those found in vertebrates (22). It also contains amoebocytes and large phagocytes with morphologies resembling invertebrate cell types found in Arthropods and Echinoderms (51–57). Jawed vertebrates’ cytotoxic cells belong to the lymphoid lineage and includes NK cells and cytotoxic T cells (58). It has been proposed that the lymphoid lineage VLRA and VLRC In jawless vertebrates also carries cytotoxic activity (32). While *B. schlosseri* cytotoxic MC share some genes (15%; 22) with the cytotoxic lymphocytes of vertebrates, they mainly express a tunicate specific gene repertoire (85%; 22). Cytotoxic MC have a large granular lymphocyte-like morphology, resembling NK cells, which originally were characterized as large granular lymphocytes (60). Moreover, the activated MC gene expression has resemblance to mouse lymphocytes as analyzed by Geneset Activity Analysis (22). While this activity resemblance is not significant, it raises the question of whether there is a homologous lineage to lymphocytes in colonial chordates. Several studies describe morula-like cells that carry enzymatic activity of phenoloxidase in other invertebrate species (51, 54–56, 61) We hypothesize that the *B. schlosseri* granular morula cells originate from the same hematopoietic lineage as other invertebrate MC (Figure 3).

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In a recent work on MC and phagocytic cells in *B. schlosseri*, only the MC expressed the *B. schlosseri* C3 gene (62), a gene that is exclusively expressed in the mammalian myeloid lineage (Figure 2c). An additional phagocytosis marker, the MFGE8, is also upregulated in the *B. schlosseri* cytotoxic MC (22). These examples suggest that MC have some commonality with the myeloid lineage (Figure 3). Moreover, in teleosts and later in mammals it was demonstrated that specific cellular immune function is not restricted by cell lineage (63–64). These studies proved that B cells can perform phagocytosis and antigen presentation. Moreover, the same group now suggests a cytotoxic ability of B cells (65). These studies suggest that there might be greater plasticity in function within the different immune lineages. Comprehensive characterization of immune cells from diverse species / clades will be necessary to determine lineage homologies.

In summary, here we limit our discussion of the co-evolution of histocompatibility and migratory stem cells to protection against intra-species ‘pathogens’, primitive germline stem cells that use the bodies of siblings or kin to mature the germline cells that will determine the genotypes for the next reproductive generation. We find parallel blood lineage cells involved in innate immune recognition in a colonial tunicate and mammals, sharing homologous genes restricted to hematopoiesis and immunity. The cytotoxic and phagoptotic cell deaths that prevent germline stem cells from non-kin invasions appear to share common cellular, and some molecular homologues with the vertebrates.

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and found to be homologous to human hematopoietic bone marrow. This study revealed significant conservation between the gene expression profiles of the B. schlosseri and mammalian HSC and blood progenitor populations. Immune functional assays revealed that cellular rejection between genetically incompatible colonies is mediated by cytotoxicity that is inhibited by histocompatibility, a mechanism similar to inhibition of NK cell cytotoxicity by MHC in mammals.

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Highlights

- *Botryllus schlosseri* HSC have been isolated and their niche identified.
- Mammalian and *B. schlosseri* HSCs and their respective niches share many homologous transcripts.
- Cytotoxicity is the mechanism of immune allogeneic cellular rejection.
- Botryllus Histocompatibility Factor (BHF) determines fusion or rejection of colonies.
- Allogeneic rejection by cytotoxic cells is inhibited by BHF, similar to NK cells.

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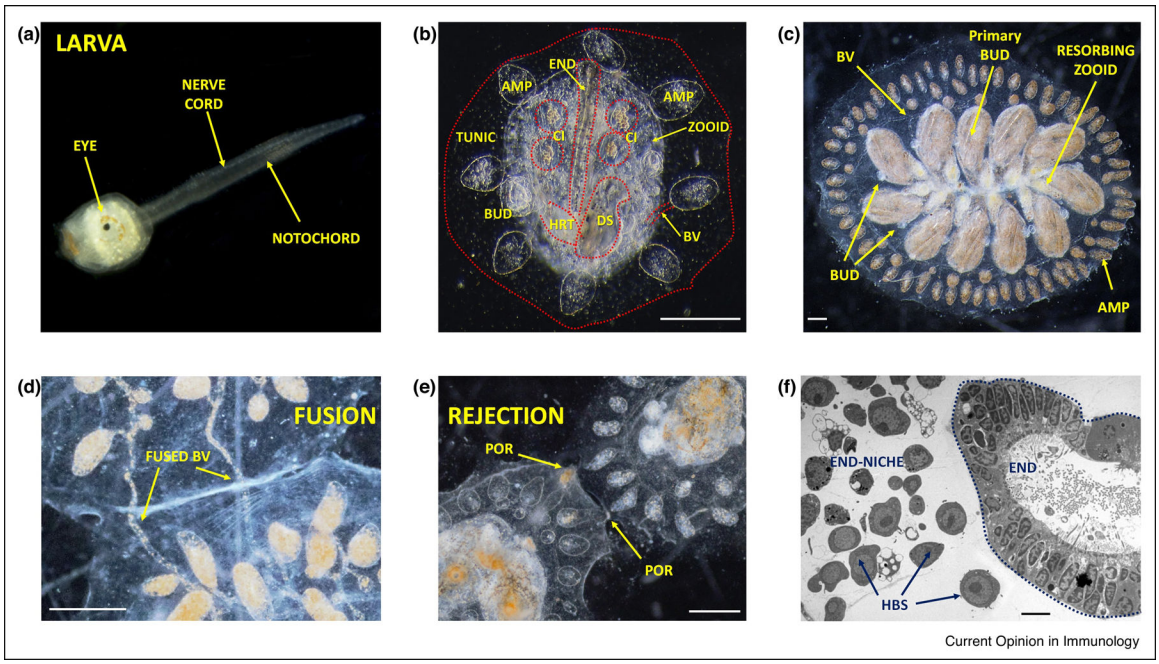

Figure 1: B. schlosseri larva, colony anatomy, and allogeneic reactions.

a, The larval tadpole phase of the *B. schlosseri* early lifecycle (adopted from 21). **b**, An image of the invertebrate stage zooid; its primary buds (BUD), blood vessels (BV) connected to an ampulla (AMP), endostyle (END), cell islands (CI), digestive system (DS), heart (HRT) and the surrounding tunic (TUNIC) are marked. **c**, A colony during the take-over stage, where the old generation zooids are resorbed and replaced by buds that completed their development. The colony's individuals are connected to each other by vasculature which extend outward with ampullae (AMP). **d-e**, Live imaging of colonies undergoing fusion (**d**) and rejection (**e**); arrows point to fused vasculature (**d**; BV), with apparent pigment cells in the fused blood vessels and points of rejection (**e**; POR). **f**, Electron microscopy cross section of the endostyle and the sub-endostyle area (endostyle-niche; END). Cells with hemoblast (HBS; candidate HSC) morphology are enriched within the endostyle-niche (adopted from 22). Scale bar b-c, d-e: 0.2 mm; f: 5 μm.

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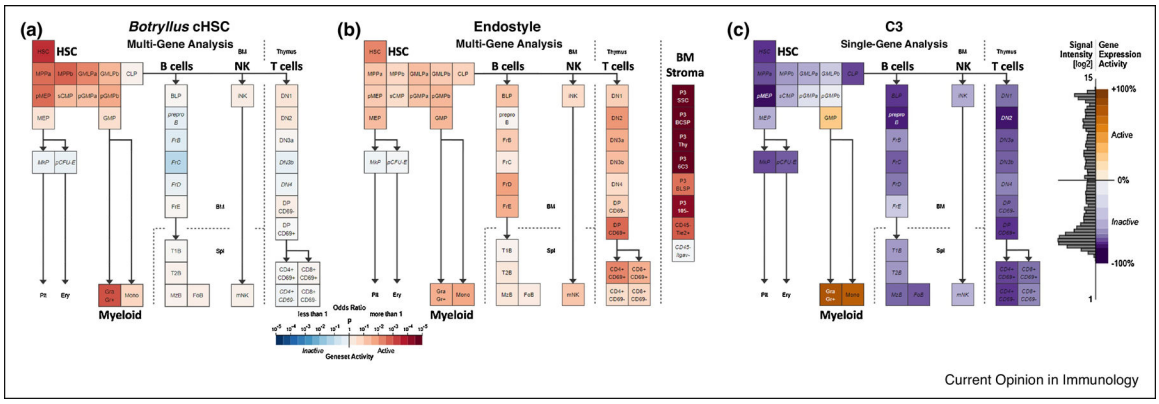

Figure 2: Gene expression analysis of genes whose expression is upregulated in the Botryllus cHSC and endostyle as compared to the expression of their homologues (based on sequence similarities) in the mouse hematopoiesis lineages.

a. Multi-gene analysis (235 genes) using Geneset Activity Analysis of the genes significantly upregulated by the candidate HSCs cell population of *B. schlosseri*. The enriched populations are HSCs and the myeloid lineage (adopted from Ref. [22]). **b**. Multi-gene analysis using Geneset Activity Analysis (https://gexc.riken) of top 200 genes significantly upregulated in the *B. schlosseri* endostyle associated with the blood system, on a mouse hematopoiesis model including the bone marrow (BM) stromal cells. This analysis revealed significant activity between the mouse bone marrow stromal cells and the *B. schlosseri* endostyle (adopted from Ref. [22]). **c**. Expression of C3 (complement component 3) gene, as determined by microarray analysis, in 39 different mouse hematopoietic populations. The C3 expresses in the phagocytic myeloid lineage, highest levels of the gene expression activity are detected in the granulocytes (Gra) and monocytes (Mono), and some activity is detected in their common progenitor (GMP).

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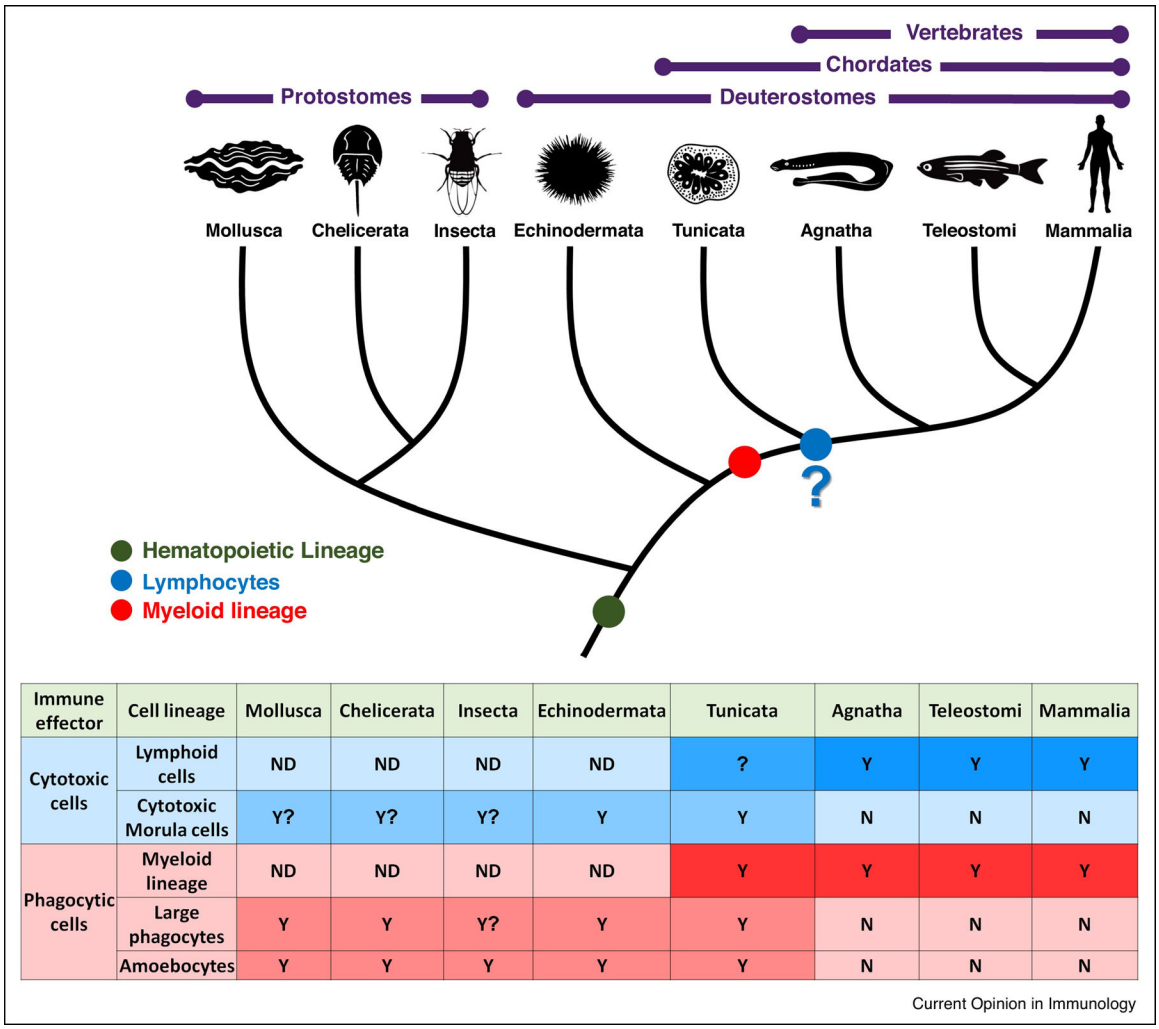

Immune effector	Cell lineage	Mollusca	Chelicerata	Insecta	Echinodermata	Tunicata	Agnatha	Teleostomi	Mammalia
Cytotoxic cells	Lymphoid cells	ND	ND	ND	ND	?	Y	Y	Y
	Cytotoxic Morula cells	Y?	Y?	Y?	Y	Y	N	N	N
Phagocytic cells	Myeloid lineage	ND	ND	ND	ND	Y	Y	Y	Y
	Large phagocytes	Y	Y	Y?	Y	Y	N	N	N
	Amoebocytes	Y	Y	Y	Y	Y	N	N	N

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Figure 3: Proposed evolution of cellular immune effector lineages.

Proposed evolutionary perspective of the cellular immune system, mainly cytotoxic and phagocytic cell lineages. Table at the bottom describes the type of presumed immune associated cells found in each animal group. It appears that myeloid lineage evolved before the branching of the vertebrata from tunicates (red). Amoebocytes and large phagocytes can be found in *B. schlosseri* and other invertebrate species (light red). While there are some populations and molecular markers resembling lymphoid lineage, whether this lineage evolved in the common ancestor of tunicates and vertebrates is still to be deciphered (blue). On the other hand the cytotoxic morula cells are characterized in tunicates and likely exist in other invertebrates (light blue).Y- Yes, cell lineage present, N- cell lineage Not present, ND- No data, "?"- insufficient data (adopted from 22).