Sweet Tunicate Blood Cells: A Glycan Profiling of Haemocytes in Three Ascidian Species

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Abstract: Ascidians are invertebrate chordates and may reveal parallels to vertebrate traits including cellular immunity, tissue rejection, and self-renewal, all functions executed by ascidian blood cells. Understanding their individual properties, functional plasticity, and lineage resemblances among ascidian species is, however, limited by a lack of cytochemical and molecular markers. We performed a lectin-based glycan profiling of haemocytes in three selected ascidian species to compare different blood cell populations and mirror their relatedness. We found differing repertoires of species-specific glycans for blood cells believed to be homologous in their function. Within species, characteristic glycans or glycan combinations mark haemocyte types and support their hematopoietic relatedness or distinguish maturation stages. Strikingly, *Ciona* and *Phallusia* haemoblasts have few carbohydrate decorations and drastically differ from differentiated cells, likewise phagocytes from cytotoxic cells, as compared with *Botryllus*, where a complex role of haemocytes in asexual self-renewal and allorecognition may involve carbohydrates. Cytotoxic cells generally carry most decorations. Within cell types, specific carbohydrates reside on the cell surface including amoeboid extensions, while others are within granules possibly marking molecules important in cytotoxicity and crosslinking. Taken together, these carbohydrate biosensors should further the molecular and functional characterisation of the outstanding properties of the different haemocytes in genetically accessible ascidian species.

1. Introduction

Tunicates are the closest relatives to vertebrates and include the ascidians that form swimming larvae with a chordate body plan. Understanding their blood cells is essential for understanding the evolution of the vertebrate immune system and notably the origins of adaptive immunity. Ascidians possess a simple form of allelic self–non-self recognition, particularly important for colonial ascidians to reject non-allelic mates or to fuse with those of allelic resemblance. Genome sequencing of model tunicates and molecular surveys have revealed a clear lack of an orthologous highly polymorphic major histocompatibility complex (MHC) locus used for adaptive immunity in vertebrates but found a less polymorphic fusibility locus (fuhc/BHF)

that contains several genes discussed in non-self reactions (reviewed in Taketa and Tomaso 2015). Interestingly, the immune receptors of innate immunity inherent to all metazoans and which normally cooperate with MHC molecules in vertebrates are more numerous in tunicates, and the variable products of the less polymorphic immune loci identified in tunicates may support a primitive resemblance of the immune system to the vertebrate condition (Azumi et al. 2003; Mueller and Rinkevich 2020). It remains a domain of active research to understand how the various immune molecules mediate specific immune functions in tunicates (reviewed in Franchi and Ballarin 2017; Parrinello et al. 2018; Rosental et al. 2020).

As in most metazoans, the immune functions in tunicates are overly executed by haemocytes, and their immune receptors are important for both the evolutionary ancient innate (defensive, non-specific) immunity and the more sophisticated allogeneic immune response (reviewed in Rosental et al. 2020). While tunicate blood cells resemble their vertebrate counterparts in many aspects, their striking features as stem cells attract more attention recently, in parallel to other invertebrates (reviewed in Ballarin et al. 2021a). Of note is their outstanding functional plasticity and regenerative potential, well evident in colonial tunicates where haemocytes can reconstitute an entire animal (reviewed in Manni et al. 2019; Ferrario et al. 2020; Alié et al. 2021). Mature haemocytes exert specialised functions such as immune recognition, phagocytosis, or cytotoxicity, but as a highly dynamic cell population with various differentiation and activation stages, it remains challenging to clearly group them into functional subtypes. Morphological criteria were used to distinguish the different haemocytes, and their resembling characteristics to vertebrates' blood cells were used to categorise their functions (Hartenstein 2006; Arizza and Parrinello 2009; Franchi and Ballarin 2017; Blanchoud et al. 2017; Rosental et al. 2020). When comparing blood cells among tunicates, clear similarities, but also notable differences, are observed between species, both in their morphological diversity and number of prominent subtypes with several questions about their functional homologisation and origin within the hematopoietic lineage remaining open (reviewed in Cima et al. 2016; Parrinello et al. 2018).

Toward understanding the functions of tunicate blood cells at a molecular level, the isolation of distinguishing molecular markers is instrumental to characterise and subgroup them. Such markers have been limiting in the study of haemocytes and for their functional comparisons (Rosental et al. 2020). Classically, proteins are considered major effectors of cellular functions, including immune recognition, and are detected at the level of their coding mRNA or by antibodies for differential expression to be associated with haemocyte functions. An independent but overlapping functional category includes carbohydrate modifications occurring on glycoproteins and glycolipids that strongly influence their maturation, structure, and function.

Furthermore, sugar epitopes often extend far in the extracellular space, giving these structural decorations great relevance to molecular and cellular interactions.

We have previously performed extensive lectin profiling of ascidian (*Ciona intestinalis)* larvae focusing on their sensory adhesive organs (papillae, palps) and have detected interesting similarities in three model organisms (*Ciona*, *Phallusia,* and *Botryllus*), suggesting a possible functional conservation of certain sugar residues, at least related to their papillary function (Zeng et al. 2019a, 2019b). Since we also observed specific lectin binding to migratory cell types, which were suggested to include haemocytes (Cloney and Grimm 1970; Sotgia et al. 1993; Sato et al. 1997; Davidson and Swalla 2002; Jimenez-Merino et al. 2019), we here aimed to profile and compare lectin patterns of the well accessible migratory haemocytes and provide useful markers and tools to access the molecules behind their epitopes.

In an attempt to provide an array of biosensors for tunicate blood cells of *Ciona intestinalis*, *Phallusia mammillata,* and *Botryllus schlosseri*, we fingerprinted their carbohydrate decorations in three model ascidian species using a collection of sixteen biotinylated plant lectins. We obtained glycan patterns of typical combinations in the three species that allow for haemocyte distinction and for mirroring their hematopoietic relatedness. Uniquely binding lectins will further the identification and functional characterisation of the interacting immune receptors within the highly dynamic haemocyte populations. Our glycophenotyping notably identifies the multiple sugar reactive sites for endogenous ascidian lectins on their natural target counterreceptors present on haemocytes. This knowledge is relevant to deciphering the intricate haemocyte functions and crosstalk in simpler chordates.

2. Materials and Methods

2.1. Animal Husbandry

The three ascidian species selected (two solitary and one colonial species) provide well-developed genomics tools and will be amenable for further molecular profiling and functional testing. *Ciona intestinalis* and *Phallusia mammillata* adults were purchased and shipped from the Roscoff Marine Station, France, and kept in aquaria with circulating and oxygenated artificial seawater at 16 °C. *Botryllus schlosseri* colonies were from the Venice Lagoon (provided by A. P. and L. B., Padova, Italy). The colonies were grown on glass slides and maintained in aerated aquaria (temperature 17 °C, salinity 35‰) and fed with Interpet Liquifry Marine (Dorking, UK).

2.2. Haemocyte Preparations from Three Species

Solitary ascidian *Ciona intestinalis* and *Phallusia mammillata* were tissue dried for any excess seawater, then dissected with scissors to expose their hearts. By a small incision, the haemocytes were released and collected into ice-cold Eppendorf tubes with 0.38% Na–citrate in filtered ASWH, pH 7.5 to prevent haemocyte aggregation,

then centrifuged at 750× *g* for 10 min and resuspended in FSW at a final concentration of 5×10^6 cells/ml. 80–100 µL of this haemocyte suspension were placed in the centre of Superfrost glass slides to which they were adhered for 20–30 min, to generate haemocyte monolayers for later lectin staining. For the colonial ascidian, *Botryllus schlosseri*, zooids were torn using a fine tungsten needle causing blood cells leakage, and haemocytes were collected and prepared as above.

2.3. Lectin Staining of Haemocytes from Three Species

Haemocyte monolayers of *Ciona intestinalis*, *Phallusia mammillata,* and *Botryllus schlosseri* were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature, transferred, and kept in 1× phosphate-buffered saline (PBS) until use. Lectin labelling was performed as described previously (Zeng et al. 2019a). Biotinylated lectins (GSL I, DBA, SBA, PNA, RCA I, SJA, ECL, GSL II, PHA-E, PHA-L, LEL, STL, DSL, PSA, LCA and UEA I, Vector Laboratories, Burlingame) were incubated at a final concentration of 15–25 µg mL^{-1} in 3% BSA–TBS, followed by washes with TBS, then fluorescently labelled with Streptavidin dye light 488 (Vector Laboratories, Burlingame) at 1:300 dilution in 3% BSA–TBS with DAPI (Merck/Sigma, D9542, Darmstadt) added at 0.035 µg mL^{-1} for nuclear staining. Samples were imaged by Leica fluorescent microscopy with filter cube L5 (wavelength 488), DAPI, and DIC; images were analysed with ImageJ (version 1.52 h).

3. Results

3.1. A Common Standard to Compare the Ascidian Haemocytes

The description of the ascidian haemocytes shows considerable variations among species and may exhibit 5–11 morphologically distinguishable cell types (Nette et al. 1999; Hartenstein 2006; Arizza and Parrinello 2009; Blanchoud et al. 2017; Cima et al. 2017; Gutierrez and Brown 2017). Therefore, to gain comparable information on the differing glycans of blood cells, we adopted a common standard for the haemocyte classification in ascidians, with three main groups: haemoblasts, immunocytes, and storage cells (Figure 1).

Figure 1a shows the main groups and subtypes of ascidian blood cells. Haemoblasts (Hbs) are uniquely small haemocytes, with a high nucleus/cytoplasm ratio, considered multipotent cells involved in regeneration and budding phenomena. Among immunocytes described in the three species, the phagocytes (Phcs) may encompass hyaline amoebocytes (HAs), macrophage-like cells (MLCs), and granular amoebocytes (GAs), while cytotoxic cells may include granulocytes with small granules (GSs), with large granules (GLs), morula cells (MCs), and unilocular refractile granulocytes (URGs). Storage cells may include pigment cells (PCs) or bivacuolated cells (BCs) and signet ring cells (SCs).

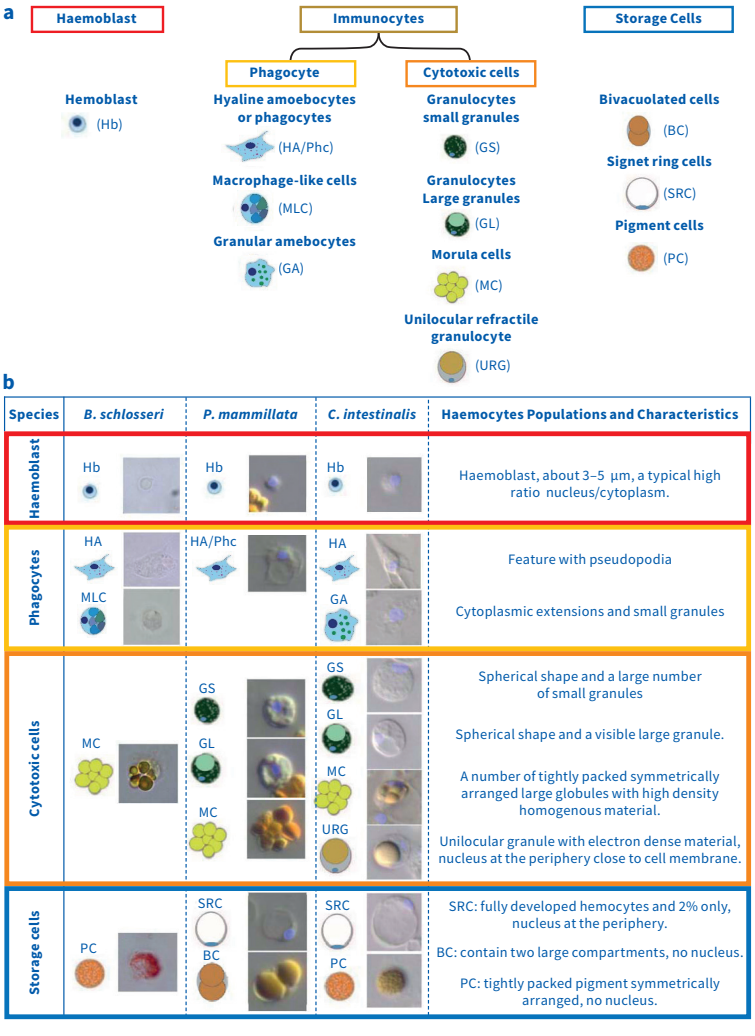


Figure 1. Ascidian blood cell types. (**a**) Scheme of ascidian blood cells: haemoblasts (Hb), immunocytes and storage cells. Immunocytes divide into phagocytes (containing hyaline amoebocytes or phagocytes, HA/Phc, macrophage-like cells, MLC and granular amebocytes, GA) and cytotoxic cells (encompassing granulocytes with small granules, GS, granulocytes with large granules, GL, morula cells, MC, and unilocular refractile granulocyte, URG). Storage cells include bivacuolated cells (BC), signet ring cells (SRC) and pigment cells (PC). (**b**) Haemocyte populations and characteristics in three ascidian species, *Botryllus schlosseri*, *Phallusia mammillata* and *Ciona intestinalis*. Haemocyte colors code: haemoblasts red, phagocytes yellow, cytotoxic cells orange, storage cells blue. Haemocyte schemes are modified from: (Nette et al. 1999, Hartenstein 2006, Arizza and Parrinello 2009, Blanchoud et al. 2017 and Cima et al. 2017). Source: Graphic by authors.

In Figure 1b, according to the three main categories of ascidian blood cells listed above (haemoblast, immunocytes, and storage cells), we grouped the haemocyte subtypes described in each of the three species, *Botryllus schlosseri*, *Phallusia mammillata*, and *Ciona intestinalis*, and summarised their typical characteristics. The blood cells of the colonial *Botryllus* encompass Hbs, phagocytic HAs, and MLCs, while MCs are only cytotoxic cells, and PCs are only storage cells. In contrast, in the solitary *Phallusia* and *Ciona*, several additional cell types are distinguished. They both feature additional cytotoxic granulocytes (GSs, GLs), and *Ciona* has a fourth cytotoxic cell type, the URGs, and the second type of phagocytic GA. As storage cells, they both have SCs and PCs, while *Phallusia* has BCs instead of PCs.

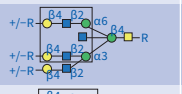
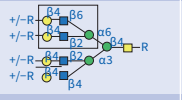
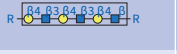
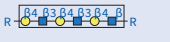
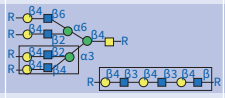
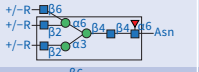
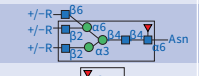
The scheme of ascidian blood cells includes haemoblasts (Hbs), immunocytes, and storage cells. Immunocytes divide into phagocytes, (containing hyaline amoebocytes or phagocytes (HAs/Phcs), macrophage-like cells (MLCs), and granular amoebocytes (GAs)) and cytotoxic cells (encompassing granulocytes with small granules (GSs), granulocytes with large granules (GLs), morula cells (MCs), and unilocular refractile granulocytes (URGs)). Storage cells include bivacuolated cells (BC)s, signet ring cells (SRCs), and pigment cells (PCs). Haemocyte schemes are modified from Blanchoud et al. (2017).

3.2. Carbohydrate Profiling of Haemocytes in Three Model Ascidian Species

The diversity of haemocytes is considered here as a function of their carbohydrate modifications since it was previously shown to play an important role in haemocyte recognition and interactions for immune activation. Plant lectins, in addition to being well-defined biosensors recognising specific carbohydrate moieties, can also be considered as biochemical tools to access the corresponding glycosylated receptors.

To obtain a more complete picture of the carbohydrate moieties in haemocytes of the three ascidian species, we screened 16 lectins featuring various sugar specificities listed in Table 1 (key recognition structures from lectins, Chapters 25 and 45: Yasuda et al. 2014; Kobayashi et al. 2014). We used lectins that recognise derivatives of galactose, glucose, mannose, and fucose. The first group comprises *GSL I*, *SBA*, *DBA*, *PNA*, *SJA,* and *RCA I* recognising galactose (Gal)/N-acetylgalactosamine (GalNAc) and N-acetyllactosamine (LacNAc, Gal-GalNAc)/GalNAc for *ECL*, with *PNA* and several others in this group known to bind O-linked sugars. A second group comprises lectins recognising sugars often found in N-linked protein glycosylation including *N*-acetylglucosamine (GlcNAc), recognised by *GSL II*, Gal/GlcNAc/mannose (Man) by *PHA-L* and *PHA-E* (the latter only for bisections of core Man), chitin (poly-GlcNAc)/GlcNAc/LacNAc by *LEL*, *STL*, *DSL*, and fucosylated glycans containing D-mannose/D-glucose/GlcNAc interacting with *PSA* and *LCA*, or with *UEA I* when in terminal position.

Table 1. Lectin bound glycosylations and key recognition structures.

Lectins	Acro-nym	Preferred Sugar Specificity	General Binding Motif	Key recognition structures *(as of, Lectins' ch. 25 and 45)*
Griffonia (Bandeiraea) simplicifolia I	GSL I	αGal, αGalNAc		αGalNAc, GalNAcα-Ser/Thr (Tn) and α-Gal
Dolichos biflorus	DBA	αGalNAc		GalNAcα-Ser/Thr (Tn) and GalNAcα1-3GalNAc
Soybean	SBA	α > βGalNAc		Terminal GalNAc, especially GalNAcα1-3Gal of O–linked glycopeptides
Peanut agglutinin	PNA	Galβ3GalNAc		Galβ1-3GalNAcα-Ser/Thr (T) of O-glycans and glycolipids
Ricinus communis I	RCA I	Gal		LacNAcβ, GalNAcβ, Galβ, Lacβ of O- and N-glycans
Sophora japonica	SJA	βGalNAc		
Erythrina cristagalli	ECL	Galβ4GlcNAc		Galβ1-4GlcNAc- > Lac > GalNAc > Gal
Griffonia (Bandeiraea) simplicifolia II	GSL II	α or βGlcNAc		GlcNAc and agalactosylated N-glycans
Phaseolus vulgaris Erythroagglutinin	PHA-E	Galβ4GlcNAcβ2Manα6 (GlcNAcβ4) (GlcNAcβ4Manα3) Manβ4		Bisecting GlcNAc and biantennary N-glycans
Phaseolus vulgaris Leucoagglutinin	PHA-L	Galβ4GlcNAcβ6 (GlcNAcβ2Manα3)		Tetraantennary complex-type N-glycans
Lycopersicon esculentum	LEL	$(GlcNAc)_{2-4}$		(GlcNAcβ1-4)n, (Galβ1-4GlcNAc)n (polylactosamine)
Solanum tuberosum	STL	$(GlcNAc)_{2-4}$		(GlcNAc-)n, (GlcNAc-4MurNAc)n (peptidoglycan backbone)
Datura stramonium	DSL	$(GlcNAc)_{2-4}$		N-linked (Galβ1-4GlcNAc-)n polyLacNAc and branched LacNAc
Pisum sativum	PSA	αMan, αGlc		Fucα1-6GlcNAc (core fucose) and α-Man
Lens culinaris	LCA	αMan, αGlc		Fucα1-6GlcNAc (core fucose) and α-Man, α-Glc
Ulex europaeus I	UEA I	αFuc		Fucα1-2Galβ1-4GlcNAc

Gal GalNAc GlcNAc Man Fuc

Source: Table created by authors.

Overall, we isolated circulating blood cells of the three ascidian species and performed stainings using conjugates of biotinylated lectins and fluorescent streptavidin. The haemocyte stainings are summarised for the three species in Table 2 and shown for the individual species in Figures 2–4, respectively. The staining pattern of each of the 16 lectins is shown in Appendix A Figures A1–A16 as a comparison in the three species.

Table 2. Summary of lectin profiling for ascidian blood cells: *Botryllus schlosseri*, *Phallusia mammillata*, and *Ciona intestinalis* blood cells lectin fluorescent labelling intensity: (–) no staining, (+) very weak, + weak, ++ intermediate, +++ strong labelling, (?) no corresponding cells identified; colour code: haemoblasts—red; immunocytes—yellow; phagocytes or cytotoxic cells—orange; storage cells—blue.

	Lectin	Haemocytes					Sugar Specificity
		Haemoblast	Immunocytes			Storage cells	
		Hb	HA	MLC	MC	BC	
B. schlosseri	GSL I	+	+++	+	++	+	αGal, αGalNAc
	DBA	++	+++	+++	+++	+++	αGalNAc
	SBA	++	+++	++	+++	+++	α > βGalNAc
	PNA	–	–	–	++	–	Galβ3GalNAc
	RCA I	?	?	–	–	–	Gal
	SJA	–	+	–	+	–	βGalNAc
	ECL	?	–	–	++	–	Galβ4GlcNAc
	GSL II	?	–	+	–	–	α or βGlcNAc
	PHA-E	?	++	+	+	–	Galβ4GlcNAcβ2Manα6 (GlcNAcβ4) (GlcNAcβ4Manα3) Manβ4
	PHA-L	–	+	–	++	–	Galβ4GlcNAcβ6 (GlcNAcβ2Manα3) Manα3
	LEL	+	?	+++	–	?	(GlcNAc)2–4
	STL	?	–	–	–	?	(GlcNAc)2–4
	DSL	++	+	+++	+++	+	(GlcNAc)2–4
	PSA	–	–	–	++	+	αMan, αGlc
	LCA	–	?	–	++	–	αMan, αGlc
	UEA I	+/–	?	–	+	+	αFuc

	Lectin	Haemocytes							Sugar Specificity
		Haemoblast	Immunocytes				Storage cells		
		Hb	Phc	GS	GL	MC	SRC	BC	
P. mammillata	GSL I	–	–	–	++	++	–	–	αGal, αGalNAc
	DBA	–	–	–	–	+++	–	–	αGalNAc
	SBA	–	++	++	–	+++	–	–	α > βGalNAc
	PNA	–	++	–	–	++	–	–	Galβ3GalNAc
	RCA I	–	–	–	–	–	–	–	Gal
	SJA	–	+	–	–	+	–	–	βGalNAc
	ECL	–	+++	–	–	++	–	–	Galβ4GlcNAc
	GSL II	+	+	+	+	–	++	+	α or βGlcNAc
	PHA-E	–	–	–	++	+	–	–	Galβ4GlcNAcβ2Manα6 (GlcNAcβ4) (GlcNAcβ4Manα3) Manβ4
	PHA-L	–	–	–	+	++	–	–	Galβ4GlcNAcβ6 (GlcNAcβ2Manα3) Manα3
	LEL	–	–	–	+	–	–	–	(GlcNAc)2–4
	STL	–	–	–	–	–	–	–	(GlcNAc)2–4
	DSL	–	–	+	–	+++	–	–	(GlcNAc)2–4
	PSA	–	–	+/–	++	++	–	–	αMan, αGlc
	LCA	–	–	–	+	++	–	–	αMan, αGlc
	UEA I	–	–	–	+	+	–	–	αFuc

	Lectin	Haemocytes								Sugar Specificity
		Haemoblast	Immunocytes					Storage cells		
		Hb	HA	GA	GS	GL	MC	URG	SRC	PC	
C. intestinalis	GSL I	–	–	–	+	–	–	+	–	–	αGal, αGalNAc
	DBA	–	–	–	–	++	–	–	–	–	αGalNAc
	SBA	–	–	–	–	++	–	–	–	–	α > βGalNAc
	PNA	–	–	–	–	+++	++	+	–	–	Galβ3GalNAc
	RCA I	–	–	–	–	–	++	+	–	–	Gal
	SJA	–	–	–	–	–	+	++	–	+	βGalNAc
	ECL	–	–	–	+	+	–	++	–	+	Galβ4GlcNAc
	GSL II	–	–	–	++	+++	–	++	–	+	α or βGlcNAc
	PHA-E	–	–	–	–	–	+	+/–	–	–	Galβ4GlcNAcβ2Manα6 (GlcNAcβ4) (GlcNAcβ4Manα3) Manβ4
	PHA-L	–	–	–	+	–	+	–	–	+	Galβ4GlcNAcβ6 (GlcNAcβ2Manα3) Manα3
	LEL	–	–	–	–	+++	–	++	–	+	(GlcNAc)2–4
	STL	–	–	–	+++	–	–	–	–	–	(GlcNAc)2–4
	DSL	–	+	+	–	+++	–	+	–	–	(GlcNAc)2–4
	PSA	–	–	+++	+	+	–	–	–	–	αMan, αGlc
	LCA	–	–	+/–	++	+++	–	–	–	+/–	αMan, αGlc
	UEA I	–	–	++	+	++	–	–	–	–	αFuc

Source: Table created by authors.

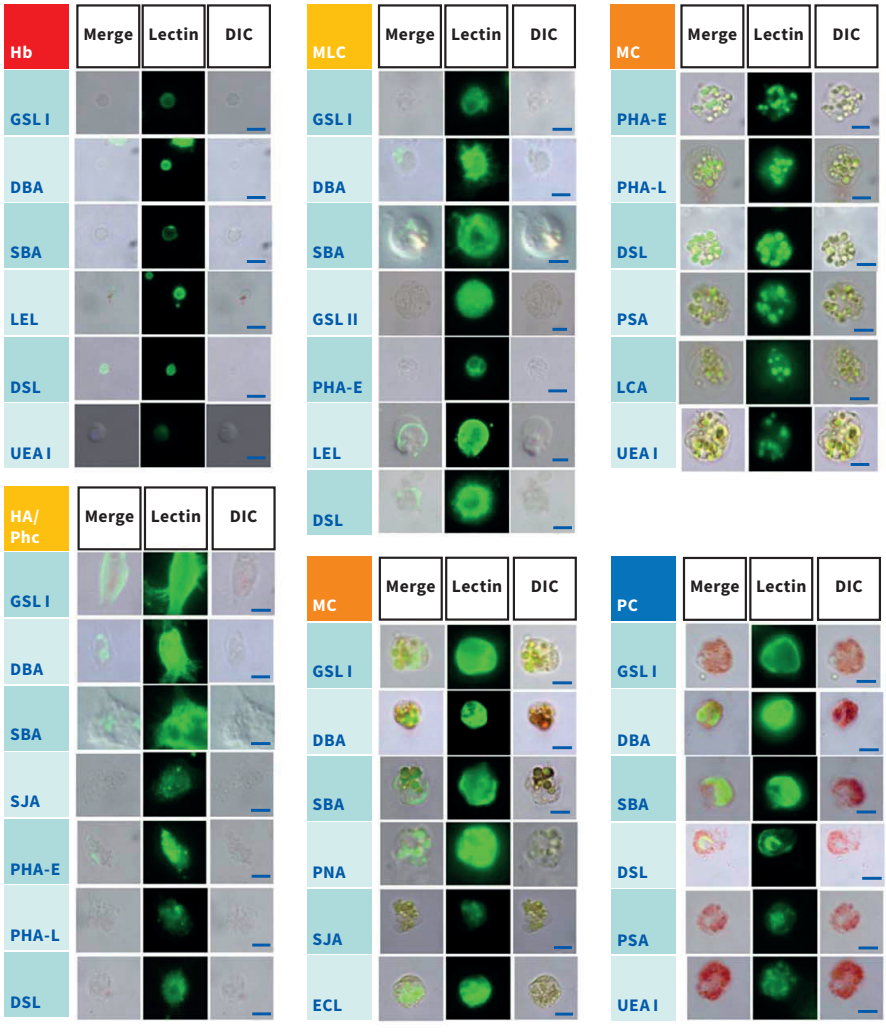


Figure 2. *Botryllus schlosseri* haemocyte types labelled with lectin probes. Lectin fluorescent labelling of *B. schlosseri* haemocytes: haemoblasts (Hbs) with *GSL I*, *DBA*, *SBA*, *LEL*, *DSL*, *UEA I*; hyaline amoebocytes or phagocytes (HAs/Phcs) with *GSL I*, *DBA*, *SBA*, *SJA*, *PHA-E*, *PHA-L*, *DSL*; macrophage-like cells (MLCs) with *GSL I*, *DBA*, *SBA*, *GSL II*, *PHA-E*, *LEL*, *DSL*; morula cells (MCs) with *GSL I*, *DBA*, *SBA*, *PNA*, *SJA*, *ECL*, *PHA-E*, *PHA-L*, *DSL*, *PSA*, *LCA*, *UEA I*; pigment cells (PCs) with *GSL I*, *DBA*, *SBA*, *DSL*, *PSA*, *UEA I*. Each lectin staining compares fluorescence images (green), the merged overlap (lectin with DIC), and DIC (bright field), scale bar: 5 μm. Source: Graphic by authors.

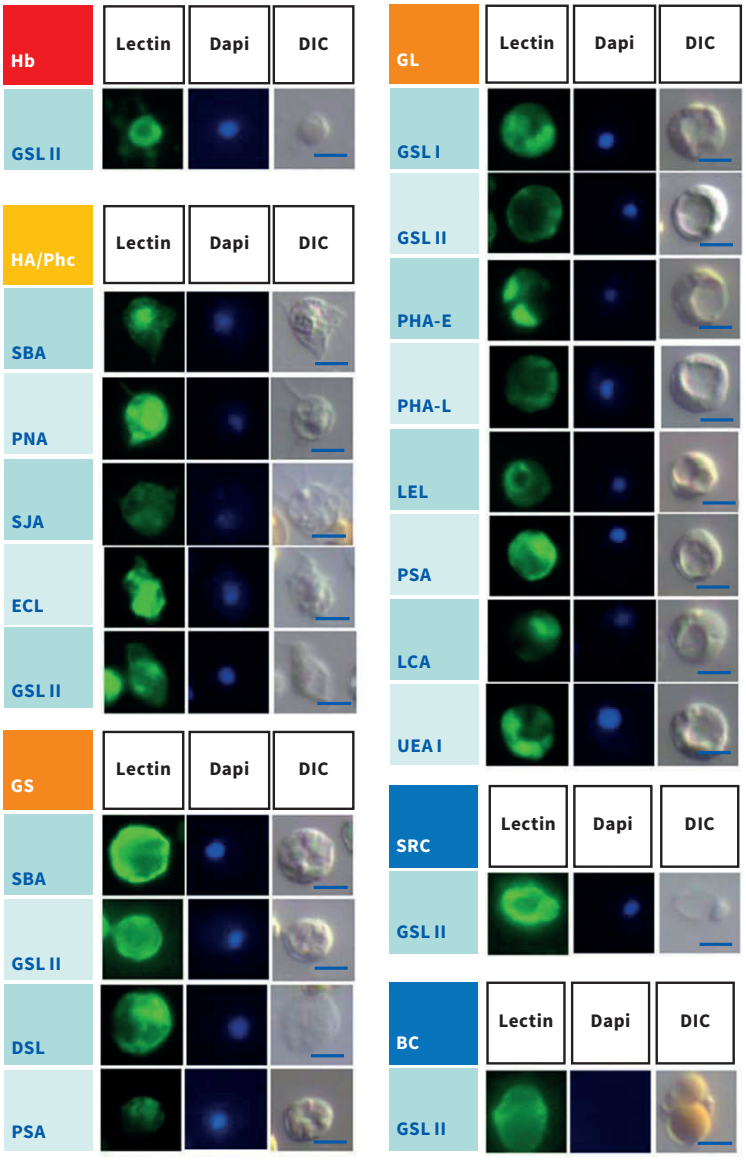


Figure 3. *Phallusia mammillata* haemocyte types labelled with lectin probes. Lectin fluorescent labelling of *P. mammillata* haemocytes: haemoblasts (Hbs) with *GSL II*; hyaline amoebocytes or phagocytes (HAs/Phcs) with *SBA*, *PNA*, *SJA*, *ECL*, *GSL II*; granulocytes with small granules (GSs) with *SBA*, *GSL II*, *DSL*, *PSA*; granulocytes with large granules (GLs) with *GSL I*, *GSL II*, *PHA-E*, *PHA-L*, *LEL*, *PSA*, *LCA*, *UEA I*; signet ring cells (SRCs) with *GSL II*; bivacuolated cells (BCs) with *GSL II*. Each lectin staining has fluorescence images (green), Dipa (blue), and DIC (bright filed), scale bar: 5 μm. Source: Graphic by authors.

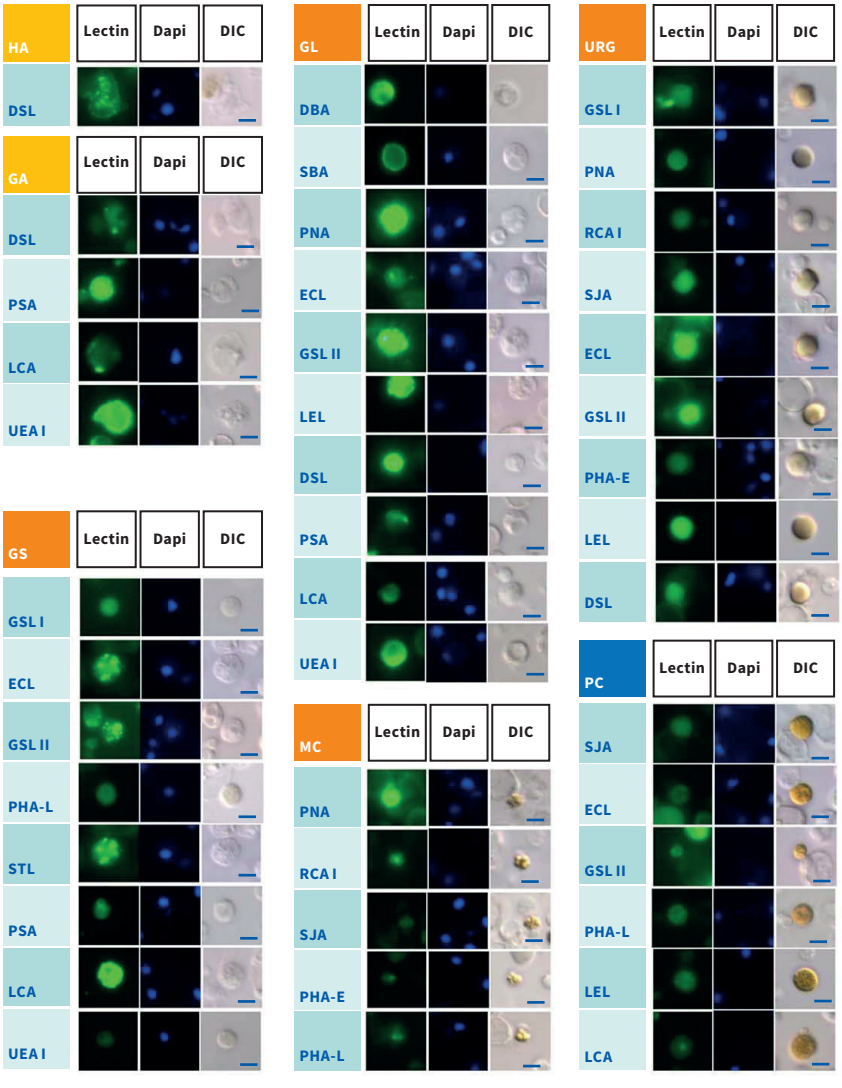


Figure 4. *Ciona intestinalis* haemocyte types labelled with lectin probes. Lectin fluorescent labelling of *C. intestinalis* haemocytes: hyaline amoebocytes (HAs) with *DSL*: granular amoebocytes (GAs) with *DSL*, *PSA*, *LCA*, *UEA I*; granulocytes with small granules (GSs) with *GSL I*, *ECL*, *GSL II*, *PHA-L*, *STL*, *PSA*, *LCA*, *UEA I*; granulocytes with large granules (GLs) with *DBA*, *SBA*, *PNA*, *ECL*, *GSL II*, *LEL*, *DSL*, *PSA*, *LCA*, *UEA I*; morula cells (MCs) with *PNA*, *RCA I*, *SJA*, *PHA-E*, *PHA-L*, unilocular refractile granulocytes (URGs) with *GSL I*, *PNA*, *RCA I*, *SJA*, *ECL*, *GSL II*, *PHA-E*, *LEL*, *DSL*; pigment cells (PCs) with *SJA*, *ECL*, *GSL II*, *PHA-L*, *LEL*, *LCA*. Each lectin staining has Lectin in fluorescence images (green), Dipa (blue), and DIC (bright filed), scale bar: 5 μm. Source: Graphic by authors.

3.2.1. *Botryllus schlosseri* Haemocytes Are Richly Carbohydrated

Circulating *Botryllus schlosseri* blood cells have multiple and diverse carbohydrate decorations on all of their haemocytes (Table 2 and Figure 2). Hbs and PCs bound 6 lectins, phagocytes (HAs and MLCs), 7 lectins each, and cytotoxic cells (MCa) 12 lectins of the 14 positively reacting, of the overall 16 tested lectins.

Four lectins labelled all of the *Botryllus* haemocytes: three were αGalNAc specific (*GSL I*, *DBA*, and *SBA*) and one GlcNAc specific (*DSL*). Interestingly, the first group of sugars (notably *GSL I*) was more abundant in the cellular periphery and well visible on membrane extensions, while the latter was rather enriched in the cytoplasm or in inclusions.

Several shared carbohydrate epitopes were found for haemocyte subgroups: All of the immunocytes (phagocytes and cytotoxic cells) carried the *PHA-E* epitope for complex bisecting N-linked sugars. Among phagocytes, the hyaline amoebocytes (HA) can be distinguished by *SJA* and *PHA-L* staining (complex N-linked sugars) but are negative for *GSL II* and *LEL* which, in turn, label macrophage-like cells (MLCs). Interestingly, the *SJA* and *PHA-L* epitopes of HAs are shared with cytotoxic MCs, while the *LEL* epitope (GlcNAc oligomers) of MLCs is shared with the immature haemoblasts. *UEA I* (fucosyl modifications) occur on Hbs, MCs, and PCs.

Cytotoxic MCs of *Botryllus* were most diversely carbohydrated and bound almost all of the lectins, also recognising other haemocytes, except for *GSL II* and *LEL*. Pigment (storage) cells bound (in addition to the four common lectins) also *PSA* and *UEA I*, both shared with MCs and the latter epitope (fucosylation) with haemoblasts.

Unique sugar specificities associated with a single haemocyte type occurred only on two cell types: only MC-bound *PNA*, *ECL*, and *LCA* on the terminal (O-linked) galactoses and core-fucosylated complex N-linked sugars, while macrophage-like cells (MLCs) uniquely bound *GSL II* for *N*-acetylglucosamines (GlcNAc).

3.2.2. *Phallusia mammillata* Haemocytes Are Sparsely Carbohydrated

The sugar decorations of *Phallusia mammillata* haemocytes showed the lowest diversity of the three species analysed, particularly for haemoblasts and storage cells (Table 2 and Figure 3). Hbs and storage cells carried a single sugar decoration, common also to all other haemocytes except MCs. Within immunocytes, the phagocytes (Phcs) bound five lectins, while cytotoxic GSs and GLs bound four and eight lectins, respectively. Strikingly, the MCs of *Phallusia* were devoid of any carbohydrate decorations tested.

All blood cells (with the exception of MCs) bound one universal lectin, *GSL II* (for terminal Gal free GlcNAc), and that was also the only one for haemoblasts and storage cells (both BCs and SRCs).

Few overlapping sugars were found on *Phallusia* haemocyte subgroups, while several unique lectins bound to a single haemocyte type within immunocytes:

Phagocytes uniquely featured terminal galactoses on GalNAc or GlcNAc of likely O-linked sugars recognised by *PNA*, *SJA,* and *ECL*. In contrast, cytotoxic granulocytes presented mostly N-linked sugars on mannose, interacting with *PSA* common to GSs and GLs, which were more complex in GLs, with longer side chains (*LEL*) and terminal decorations including galactose (*PHA-L*), fucose (*UEA I*), or even bisections (*PHA-E*). Finally, GSs uniquely bound *DSL* (GlcNAc chains) and shared *SBA* (terminal GalNAc) with phagocytes only, while GLs uniquely bound *PHA-E*, *PHA-L*, *LEL*, *LCA*, and *UEA I* (bisecting complex and fucosylated N-linked sugars).

3.2.3. *Ciona intestinalis* Haemocytes Are Selectively Carbohydrated

The most diversified haemocyte subtypes of all three ascidian species are reported in *Ciona intestinalis* (Figure 1b, Table 2 and Figure 4). However, only selective subgroups are richly decorated with glycans—namely, all of the cytotoxic cell types (GSs and GLs bound 8 and 10 lectins; MCs and URGs 5 and 9 lectins, respectively) but also the pigment cells (six lectins), while phagocytes bound only one (HA) or four (GA) lectins, respectively). In contrast, Hbs and the storage SRCs did not bind any of the tested lectins.

Sugar residues common to the major haemocyte groups were scarce: Only the storage subtype of PC shared all of their six epitopes with different cytotoxic cells (*SJA*, *ECL*, *GSL II*, *PHA-L*, *LEL*), representing GalNAc and/or GlcNAc residues on likely core N-linked glycans. Within the group of immunocytes, only a few epitopes (four lectins) were shared between phagocytes and cytotoxic cells, while extremely many (all tested lectins) were variably distributed among the four cytotoxic cell types. More precisely, *Ciona* phagocytes (HAs and GAs) and cytotoxic GSs and GLs commonly featured GlcNAc chains (*DSL* lectin, shared with URGs), but only the GA shared fucosylated N-linked sugars (*PSA*, *LCA*, *UEA I* lectins) with the cytotoxic GSs and GLs.

Interestingly, different combinations of all of the 16 tested lectins are found among the four cytotoxic subtypes. The GSs and GLs stained with 8 and 10 lectins, respectively, while MC and URG bound 5 and 9 lectins. Only GS and GL carried fucosylated, N-linked sugars (*PSA*, *LCA*, and *UEA I*), as mentioned above. Only GLs uniquely bound *DBA* and *SBA* (αGalNAc) but shared Gal-GalNAc (*PNA*) with MCs and URGs. The GSs uniquely bound *STL* but also *PHA-L*-marking Gal-GlcNAc on complex N-linked sugars, which are not shared with GLs. The GLs, instead, stained with *LEL* and *DSL*, for Gal-GlcNAc oligomers but possibly not on complex branches (*PHA-L* negative). Consistently, GSs and GLs, both stained with *ECL* and *GSL II* (GlcNAc lacking Gal). The simpler GL epitopes (*ECL*, *GSL II*) are shared with URGs, while only the complex N-linked GS epitope (*PHA-L*) is also found on MC. Interestingly, MCs and URGs uniquely share the complex bisecting N-linked sugars (*PHA-E* epitope). In addition, MC and URG were uniquely recognised by *RCA I* and

SJA (galactose and GalNAc), while staining by *PNA* (Gal-GalNAc) was also shared with GLs. URGs, but not MCs, bound *GSL I* (Gal in α-position) shared with GS cells only. Overall, URGs featured no unique markers but significantly overlapped with MCs and GLs, but also PCs, as mentioned above.

Unique sugar decorations on single haemocyte types of *Ciona* were, therefore, only for *STL* on GSs, and *DBA* and *SBA* on GLs, while all the other epitopes were variably shared among the haemocytes.

4. Discussion

Our carbohydrate profiling of circulating haemocytes in three ascidian species revealed that sugar decorations largely differ in composition and complexity between species but also feature some notable commonalities. Interestingly, the circulating haemocytes of the colonial *Bortyllus* are richly sugar modified throughout all subtypes of haemocytes, which contrasts with the solitary *Phallusia* and *Ciona* that, although carrying various decorations on cytotoxic cells, are more scarcely or not glycosylated otherwise. *Botryllus* haemocytes are also the only ones to carry multiple glycan epitopes shared among all of their blood cells. As glycan residues are involved in the molecular interactions of their carriers such differences point to a divergent functional complexity of circulating blood cells in colonial versus solitary ascidians but also to important differences among solitary ascidians.

4.1. Comparing Sugar Decorations on Blood Cells of Different Ascidians

The presented sugar profiling constitutes a more detailed and sensitive carbohydrate fingerprinting, as compared with previous records (Schlumpberger et al. 1984; Cima et al. 2017; Rosental et al. 2018), with unique and overlapping specificities of 16 plant lectins combined to increased signal amplification through biotinylated lectins crosslinking streptavidin of multiple fluorescent residues. The detection of weaker lectin binding and a wider reactivity is expected and observed.

A comparative summary of the lectin profiling for the three species is presented in Table 2, with colour codes for haemocyte subtypes consistently grouped into haemoblasts (red), immunocytes (yellow and orange, for phagocytes and cytotoxic cells, respectively), and storage cells (blue), and further subdivided according to their known morphological and functional relatedness (Figure 1). The plant lectin probes are roughly ordered according to their similar specificities (elaborated in Table 1) with the upper vs. lower groups preferably recognising Gal residues on often O-linked sugars vs. rather N-linked glycans, respectively, separated by the bisecting N-linked modification (*PHA-E*), and at the bottom mostly fucose recognition.

Haemoblasts (Hbs, red in Figures 1–4, Table 2) are small stem cells believed to give rise to all of the other blood cells and are also capable of whole-body regeneration in colonial ascidians (reviewed in Ballarin et al. 2021a, Ballarin et al. 2021a, 2021b).

Interestingly, the Hbs of colonial *Botryllus* carry much richer carbohydrate decorations (6 of 16 tested lectins), as compared with solitary *Phallusia* or *Ciona* (0 or 1 lectin). Such striking difference may reflect the differing degree of importance and variability in functions of Hbs, notably in the circulatory system of colonial versus solitary ascidians. It will be interesting to compare the Hbs of other colonial (vs. solitary) species and consider the bound immune receptors possibly with functions in stemness, regeneration, or plasticity.

Immunocytes (yellow and orange in Figures 1–4, Table 2) constitute the largest and most diverse haemocyte group with a moderate variability in phagocytes and greater diversity defined in cytotoxic cell subtypes. Two types of phagocytes (Figure 1, Table 2, yellow) are presented in *Botryllus* and *Ciona*, but only one in *Phallusia*. The sugar residues of phagocytes differ in the three ascidians with *Phallusia* presenting mostly O-linked sugars, while overly N-linked or bisecting in *Botryllus* and fucosylated in *Ciona.* For *Botryllus* and *Ciona* the phagocyte decorations significantly overlapped with those on cytotoxic cell types.

Cytotoxic cells are the most diverse and richly glycosylated haemocyte subgroup among immunocytes. The considerable differences between species are reflected by a variable presentation of maturation stages in the circulating blood cell populations (Figure 1, Table 2, orange): MCs exist in all three species, the two solitary species also comprise many earlier-stage GSs or GLs and, in *Ciona*, an additional URG. As of plant lectin binding, cytotoxic MCs of *Botryllus* carry abundant O- and N-linked sugars, while those of *Phallusia* are rather N linked (and devoid of sugar for MCs). *Ciona* presents an interesting situation where the various cytotoxic morphotypes carry a combination of all of the 16 lectin epitopes, with typical combinations for subtypes: most strikingly, GSs likely lack O-linked sugars, and GSs and GLs share fucosylation on N-linked sugars that in MCs and URGs lack the fucose but are core bisecting, in turn.

Storage cells (blue in Figures 1–4, Table 2) are much less diverse, and some differences among species exist: PCs are present in *Botryllus* and *Ciona* but absent in *Phallusia*, while they are represented by BCs; only the solitary species feature SRCs. In all three species, their sugar modifications overlap with those of cytotoxic cells. In *Phallusia*, however, their single sugar epitope also occurs on all of the other haemocytes (except the non-glycosylated MCs). In *Botryllus*, the PCs carry fucosylations such as MCs (but also the Hbs), and *Ciona* presents rather only N-linked cores, as shared with many cytotoxic subtypes.

4.2. Functional Implications from Haemocyte Glycophenotyping

The diversity of tunicate blood cells is strikingly amplified by their variable glycan modifications and the idea suggests that they were important drivers of

tunicate evolution. Our plant biosensors can 'sugar phenotype' the haemocytes and 'phenocopy' the sugar binding of endogenous lectins to haemocytes.

To understand the enormous combinatorial possibilities offered by carbohydrate residues in biological systems, a defined 'sugar code' is proposed for sugar recognition (Solis et al. 2015). Sugars can be combined in three 'dimensions': linear, branching, and conformational variants. The presentation and recognition of the sugars are rigid on both sides, with conserved residues and little conformational effects upon binding (key-lock principle). Specifically, tailored protein domains, the carbohydrate recognition domains (CRDs), are common denominators of lectins to recognise specific sugars, and 14 different folds are described for animals/humans. Evolutionary diversification of lectins occurred via domain duplications and multiple events are paralleled by a secondary loss of non-functional domains. Lectins may present tandem CRDs and often contain different domains to produce the actual biological effect. The CRDs furthermore read sugar encoded 'postal codes' to reach desired destinations, and haemocytes are thusly targeted, attracted, or activated by endogenous lectins.

Our sugar profiling thus pinpoints to various haemocytic targets for endogenous tunicate lectins. Glycan recognition, indeed, plays a prominent role in both tunicate and vertebrate innate immunity, notably via the group of so-called pattern recognition receptors (PRRs) containing CRD domains to recognise foreign and endogenous carbohydrate residues, elicit interactions with downstream signalling components and trigger a network of crosstalks for a proper inflammatory immune response (Franchi and Ballarin 2017; Parrinello et al. 2018). These include lectins with conserved CRDs such as galectins (binding galactoside residues), RBLs (rhamnose or galactoside binding) or VCBPs (variable chitin-binding proteins, binding poly-GlcNAc) or more variable sugar-binding domains such as C-type lectins, including the collectins MBL/GBL and ficolins (for mannose/glucose and GlcNAc binding, respectively). Such endogenous lectins may have multiple distributions and functions, may be soluble, membrane bound, or intracellular, and may trigger the activation of haemocytes for release of cytokines, phagocytosis, or cytotoxicity, cause their crosslinking, recruitment to specific locations of inflammation, or interact intracellularly in glycan metabolism and proliferation (summary table in Franchi and Ballarin 2017). Not unexpectedly, many endogenous lectins are themselves expressed and also secreted by haemocytes.

Various subcellular locations of sugar epitopes could be detected, pointing to the variable functions of carbohydrates in cell compartments of the haemocytes. Epitopes in the cell periphery were particularly well visible on membrane extensions of phagocytes (Figures 2–4). Extracellular residues are likely involved in cell interactions, migration, and triggering the immune response. Intriguingly, these peripheral epitopes include several sugars shared among all of the *Botryllus* haemocytes (Table 2), and it is tempting to speculate that these are present on membrane-associated

molecules that are crucial in the synchronised, weekly generation change in *Botryllus* orchestrated by haemocytes (Cima et al. 2010). Interestingly, these share galactoside residues, suggesting a possible role for galectins or RBLs targeting common haemocyte receptors. Notably, RBLs are known to play important roles in colonial generation change, phagocytic clearance of apoptotic haemocytes, and termination of the blastogenetic cycle, while ascidian galectins are known to play pivotal roles in haemagglutination, as well as the recruitment of migrating haemocytes (reviewed in Ballarin et al. 2013).

In contrast, several sugar epitopes seem enriched in the cytoplasm or in inclusions, well visible within cytotoxic cells (Figures 2–4). These are often N-linked sugars with fucosylations in all three species and bisecting sugars in *Botryllus*. Such inclusions may be degradation products from ingested microbes (pathogen-associated molecular patterns (PAMPs)) often containing high mannose contents. Alternatively, they may represent stored molecules to be released upon inflammatory activation including cytokines, enzymes, or toxic material. It is well known that the vacuolar localisation in haemocytes of inactive phenoloxidase (proPO, related to tyrosinase) causes the oxidation of polyphenol substrates (including the tunichromes, representing L-DOPA and L-TOPA peptides) into microcidal chinones upon release in the seawater/physiologic pH. Intriguingly, the major PO activity and release of microcidal components such as polyphenols is exerted by different cytotoxic subtypes in the three tunicates: MCs in *Botryllus*, URGs in *Ciona*, and GLs in *Phallusia*, rather than MCs (reviewed in Franchi and Ballarin 2017; Parrinello et al. 2018). Strikingly, such distribution roughly coincides with the resembling sugar epitopes in their vacuolar compartments. It will be interesting to identify their molecular carriers. Nevertheless, *Ciona* granulocytes (GSs and GLs) also contain intracellular glycans and could represent glycoproteinaceous maturation stages and targets for intracellular lectins, such as collectins, involved in the complement pathway (Franchi and Ballarin 2017).

Overall, it became evident that the same sugar residues can be followed throughout several related morphotypes, and thus, inversely, haematopoietic lineage relatedness may be concluded by the shared sugar epitopes, thus representing lineage markers. Such lineage identity considerations are of interest when haemocytes are analysed in a tissue context other than the circulating haemolymph, such as microbial-induced inflammation in the pharynx, but also if the host tissue is damaged or stressed (reviewed in Parrinello et al. 2018). Damage-associated molecular patterns (DAMPs) may cause an inflammation-like gene activation repertoire, as was shown for heat-shock proteins or during larval metamorphosis. It will be interesting to determine whether the shared sugar epitopes on DAMPs represent identical molecules or may resemble each other by mere coincidence.

In the future, our sugar profiling and the specificity of the individual biosensors will promote the analysis of glycoproteinaceous interactions of haemocytes and, notably, the various haemocytic targets for the endogenous tunicate lectins. Hybrid synthetic lectin probes prepared from CRDs (Dishaw et al. 2016) of the various lectin families will confirm the binding specificities and the differences detected in the three ascidians. Finally, such probes may also give direct biochemical access to the bound counterreceptors and will enrich our understanding of the intricate life of tunicate haemocytes.

Author Contributions: Conceptualisation, U.R. and F.Z. methodology, F.Z. and A.P.; validation, F.Z., U.R., L.B., and A.P.; formal analysis, F.Z. and U.R.; writing—original draft preparation, U.R. and F.Z.; writing—review and editing, L.B.; funding acquisition, U.R. and F.Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

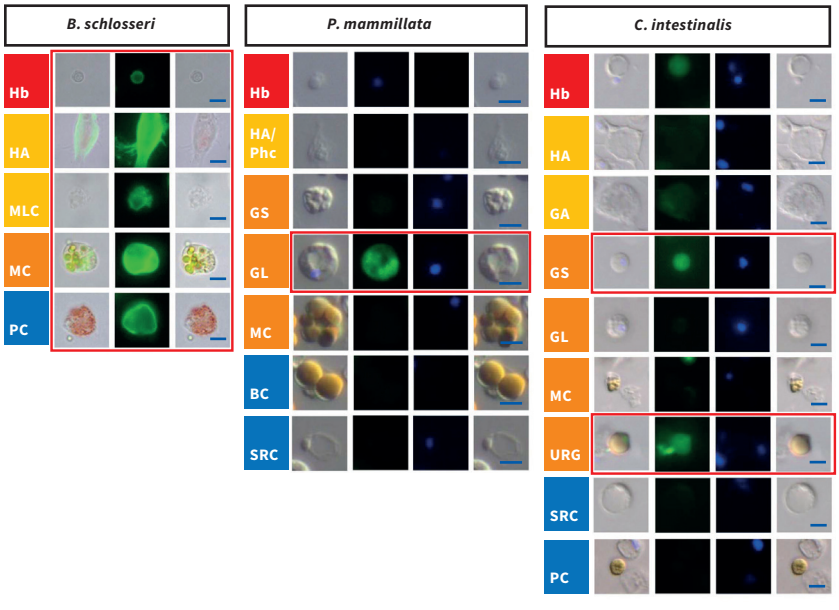

Figure A1. Individual lectin stainings for ascidian blood cells, GSL I. GSL I [αGal, αGalNac]. Brown frames indicate positively stained haemocytes. Scale bar, 5 µm.

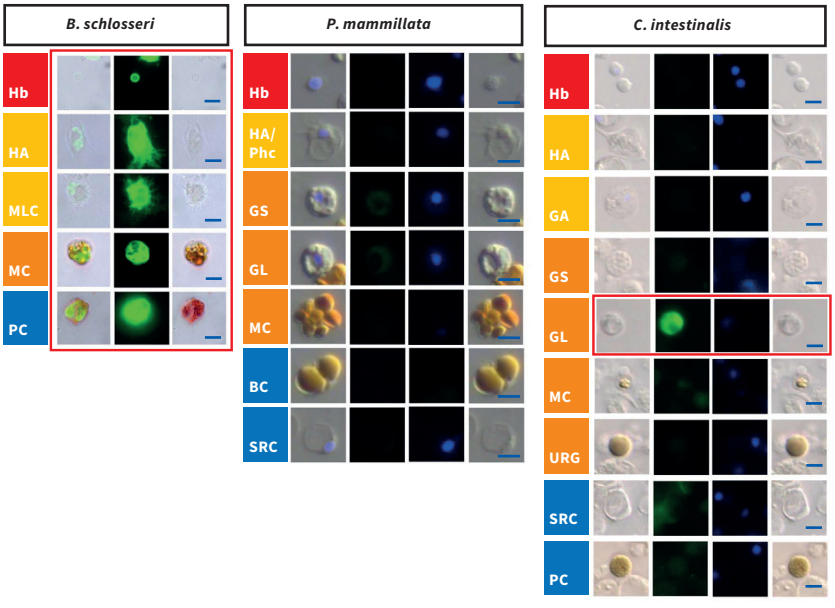

Figure A2. Individual lectin stainings for ascidian blood cells, DBA. DBA [αGalNac]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

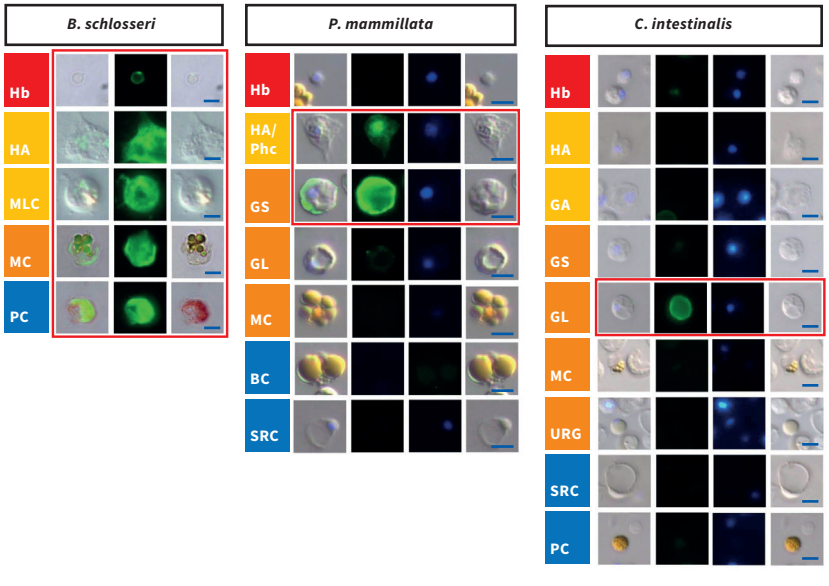

Figure A3. Individual lectin stainings for ascidian blood cells, SBA. SBA [α > βGalNac]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

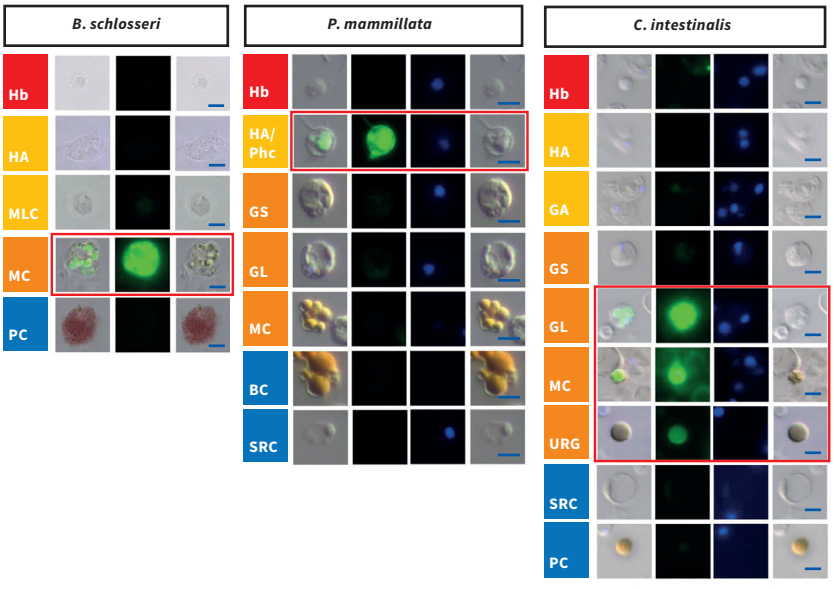

Figure A4. Individual lectin stainings for ascidian blood cells, PNA. PNA [Galβ3GalNac]. Brown frames indicate positively stained haemocytes. Scale bar, 5 µm.

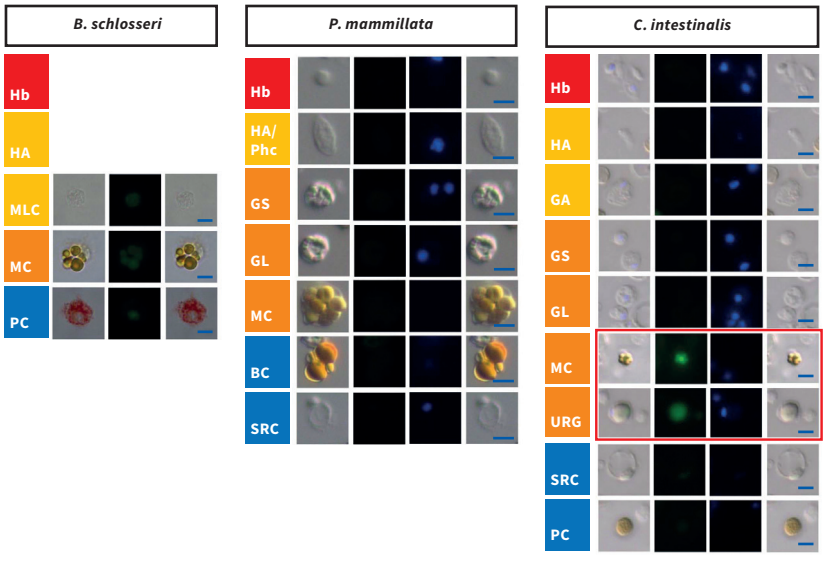

Figure A5. Individual lectin stainings for ascidian blood cells, RCA I. RCA I [Gal]. Brown frames indicate positively stained haemocytes. Scale bar, 5 µm.

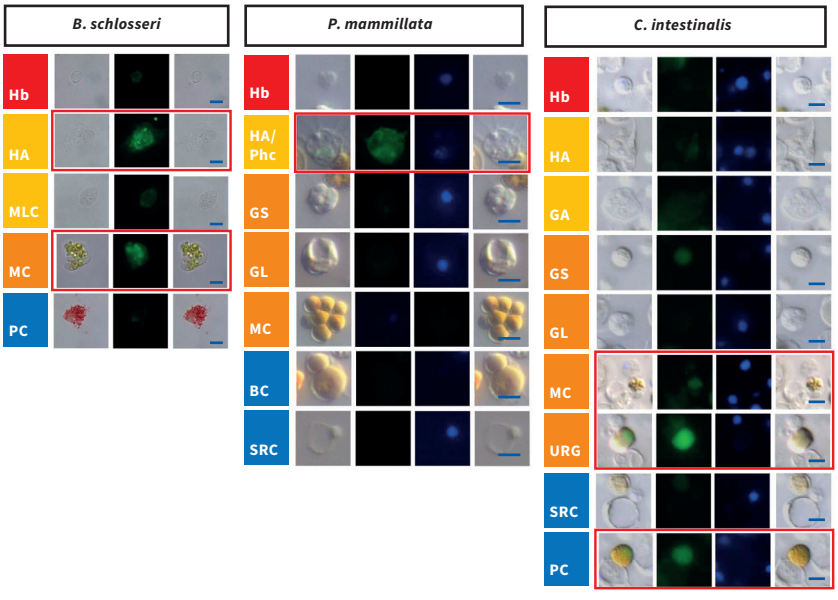

Figure A6. Individual lectin stainings for ascidian blood cells, SJA. SJA [βGalNac]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

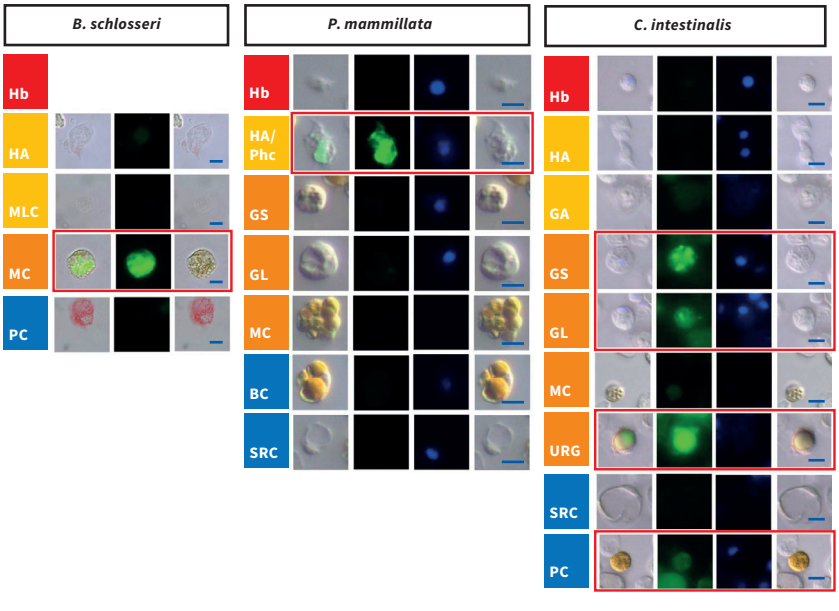

Figure A7. Individual lectin stainings for ascidian blood cells, ECL. ECL [Galβ4GlcNAc]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

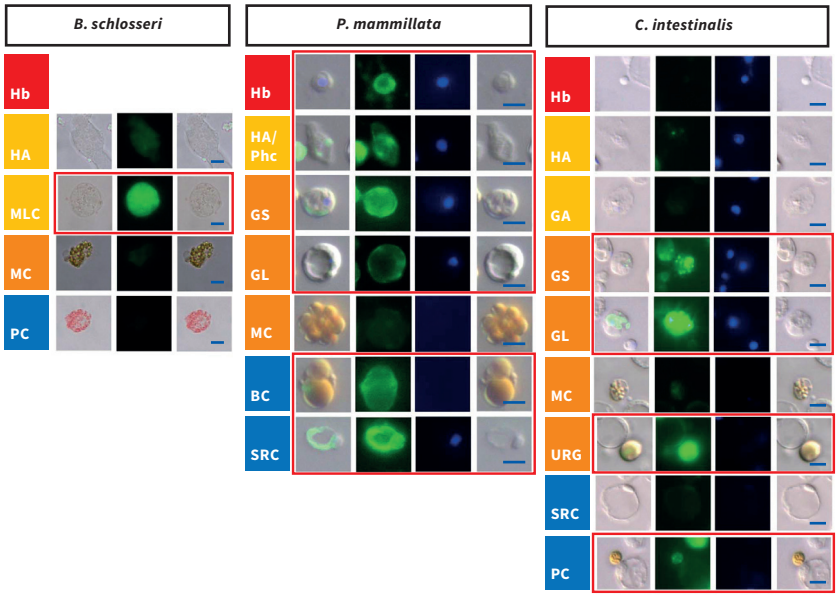

Figure A8. Individual lectin stainings for ascidian blood cells, GSL II. GSL II [α or β GlcNAc]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

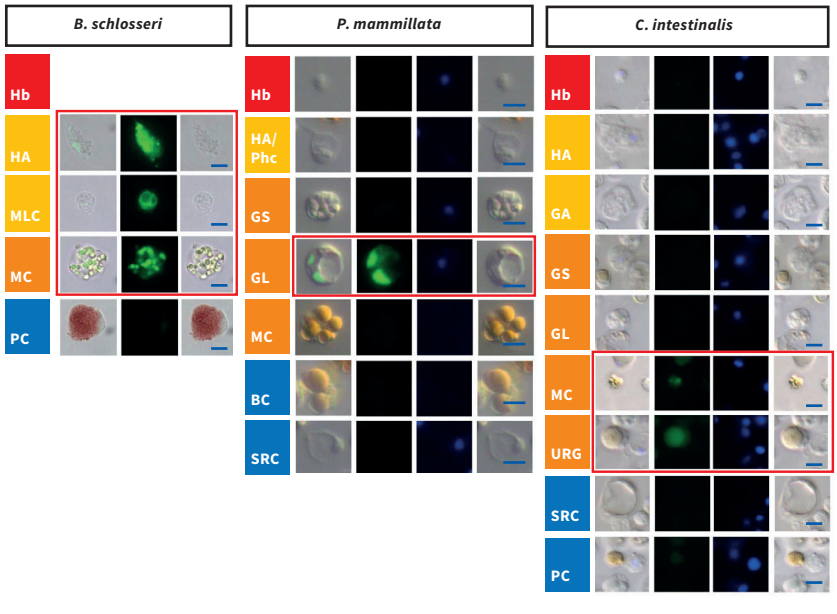

Figure A9. Individual lectin stainings for ascidian blood cells, PHA-E. PHA-E [Galβ4GlcNAcβ2Manα6 (GlcNAcb4) (GlcNAcb4Manα3) Manβ4]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

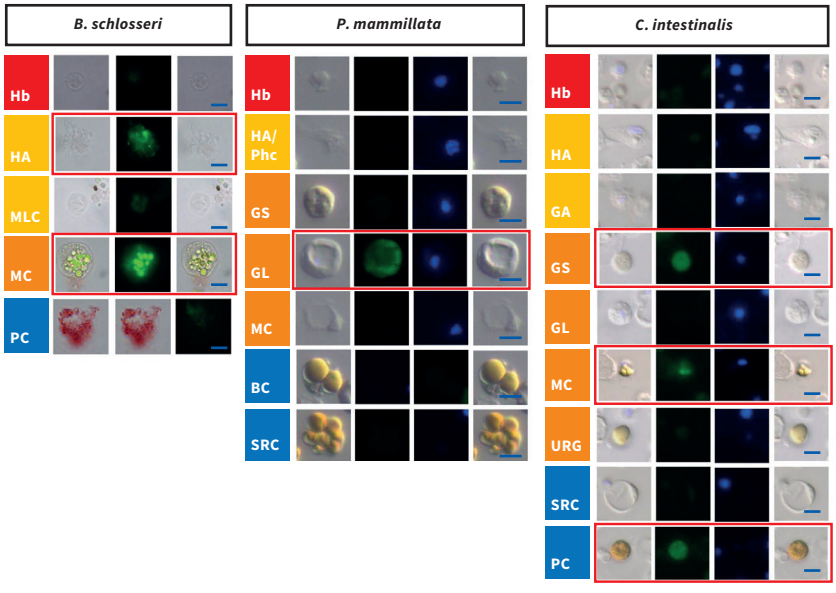

Figure A10. Individual lectin stainings for ascidian blood cells, PHA-L. PHA-L [Galβ4GlcNAcβ6 (GlcNAcβ2Manα3)Manα3]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

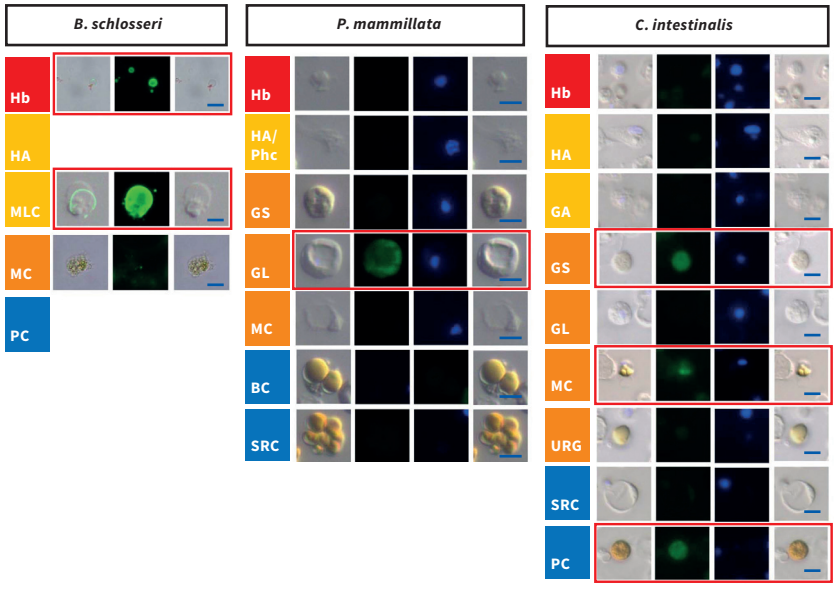

Figure A11. Individual lectin stainings for ascidian blood cells, LEL. LEL [$(GlcNAc)_{2–4}$]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

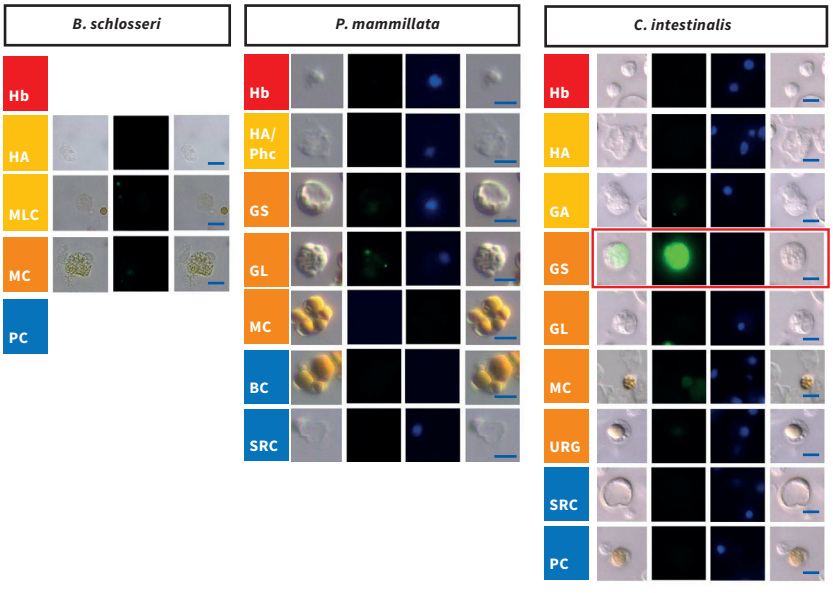

Figure A12. Individual lectin stainings for ascidian blood cells, STL. STL [$(GlcNAc)_{2–4}$]. Brown frames indicate positively stained haemocytes. Scale bar, 5 µm.

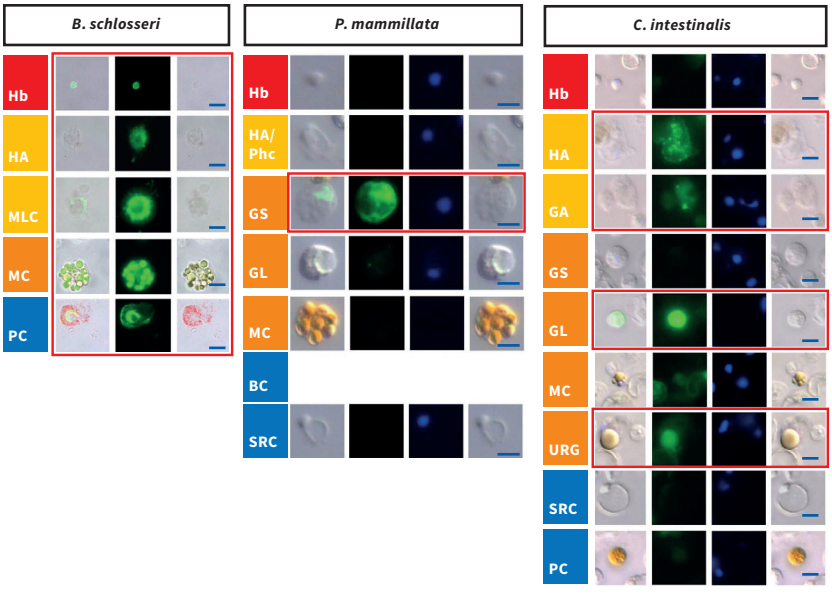

Figure A13. Individual lectin stainings for ascidian blood cells, DSL. DSL [$(GlcNAc)_{2–4}$]. Brown frames indicate positively stained haemocytes. Scale bar, 5 µm.

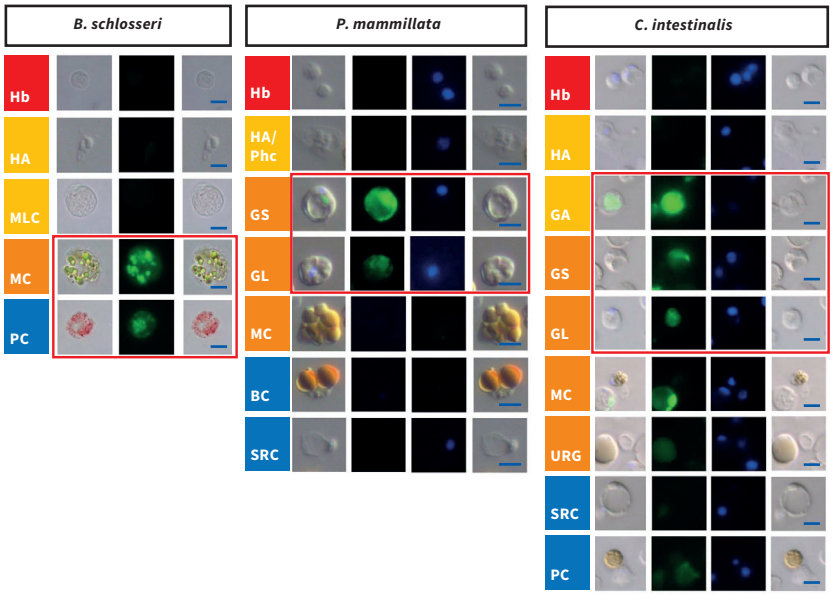

Figure A14. Individual lectin stainings for ascidian blood cells, PSA. PSA [αMan, αGlc]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

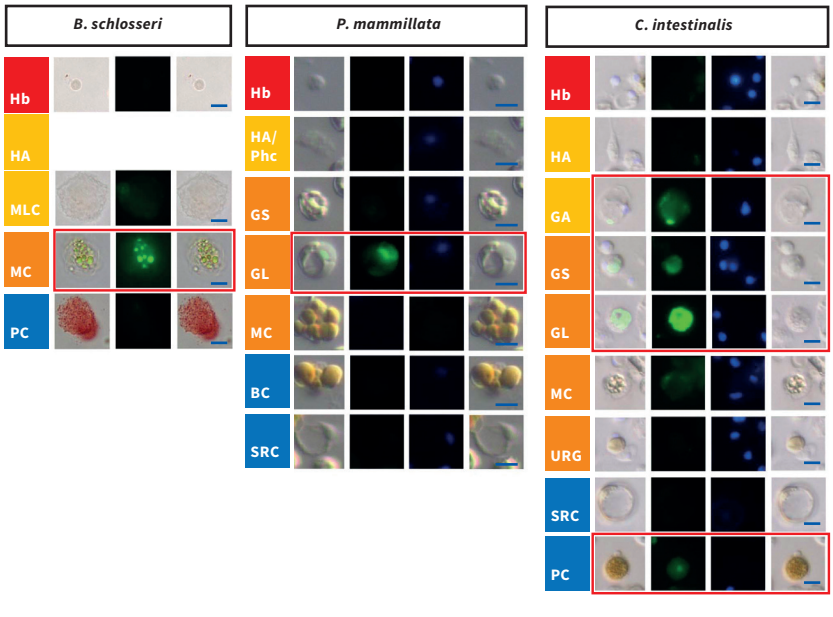

Figure A15. Individual lectin stainings for ascidian blood cells, LCA. LCA [αMan, αGlc]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

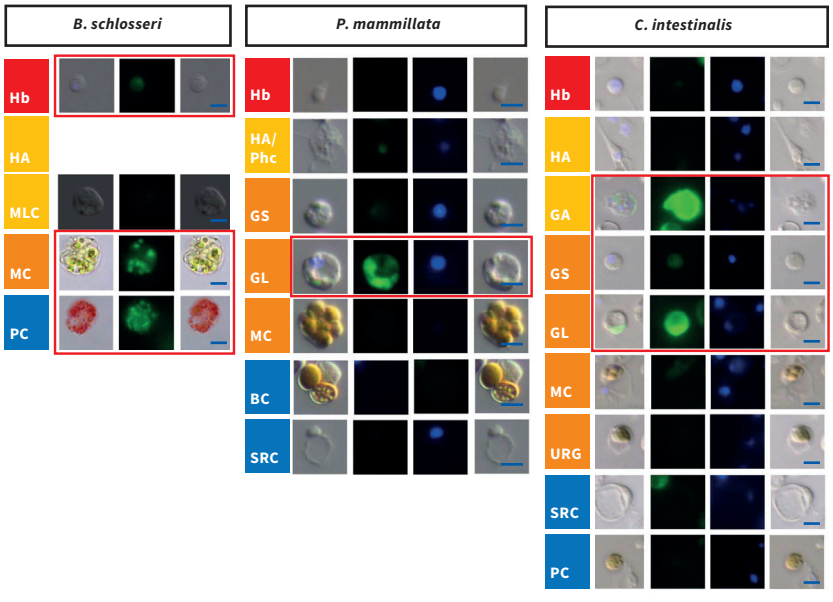

Figure A16. Individual lectin stainings for ascidian blood cells, UEA I. UEA I [αFuc]. Brown frames indicate positively stained haemocytes. Scale bar, 5 μm.

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