ISJ 6: S58-S66, 2009 ISSN 1824-307X

REVIEW

Inflammatory hemocytes in *Ciona intestinalis* innate immune response

V Arizza, D Parrinello

Laboratory of Marine Immunobiology, Department of Animal Biology, University of Palermo, Palermo, Italy

Accepted March 13, 2009

Abstract

In the present paper an attempt is carried out to revise *Ciona intestinalis* inflammatory hemocytes according to their morphology as formerly observed by light and electron microscopy, and taking in account recent reports on innate immunity gene expression. We also examine hemocyte morpho-functional aspects as derived from previous papers that refer to the tunic and body wall inflammatory responses challenged by corpusculate or soluble agents. LPS inoculation into the body wall or treating hemocytes *in vitro* with LPS have also been taken in account. LPS inoculation stimulated the expression of *Ci*TNFα, *Ci*FACIT-αchain collagen, *Ci*C3a, *Ci*CD94 and enhanced phenoloxidase activity. These reports allow us to distinguish two main hemocyte types categories: 1. agranular hemocytes, including hemoblasts, circulating lymphocyte-like cells, hyaline amebocytes; 2. granular hemocytes including granulocytes with small granules, granulocytes with large granules, unilocular refractile granulocytes and morula cells. Compartment cells and signet ring cells could be intermediate or terminal states presumably involved in releasing inflammatory factors or tunic matrix components. We suggest that the various hemocyte shapes, as shown by light and electron microscopy, could represent functional states as disclosed in inflamed tissues. Although it cannot be excluded that a same cell expresses multiple activities, it is likely that several populations of a same cell type can exert distinct roles.

Key words: tunicate; innate immunity, inflammation, hemocyte, *Ciona intestinalis*

Evolutionary relevance of ascidian immunology studies

The developmental plan, the tadpole-like larva as well as molecular phylogenesis analysis support that tunicates are primitive members of the phylum Chordata. Ascidians (Urochordata) occupy a critical position in the phylogenetic line leading to the vertebrates (Swalla *et al*., 2000; Zeng and Swalla, 2005). Recently Delsuc *et al.* (2006) suggested that ascidians and not cephalochordates are the sister group of vertebrates, consequently urochordates have attained further importance for evolutionary immunology studies.

Ciona intestinalis is the representative species of the solitary ascidians generally retained a basic model for comparative biology research. The whole genome has been sequenced and several vertebrate homologous genes have been annotated (Dehal *et al.*, 2002; Satou, 2002, 2003). Bioinformatic approach and extensive *in silico* search have concerned immunorelevant molecules, gene expression patterns and some immune properties (Davidson and Swalla, 2002; Fujita, 2002; Nonaka and Miyazawa, 2002; Azumi *et al.*, 2003; Shida *et al.*, 2003; Terajima *et al.*, 2003; Du Pasquier, 2004; Fujita *et al.*, 2004; Kasahara *et al.*, 2004).

Inflammatory reaction in the body wall of *C. intestinalis,* is a key evolutionary innate immunity model as well as can disclose hemocyte morpho functional aspects and pro-inflammatory products. Soluble or particulate materials injected into the body wall are competent in challenging an inflammatory response including encapsulation and, in some cases, a tissue damage (Parrinello, 1981; Parrinello *et al.* 1984; Parrinello and Patricolo, 1984). In a variable time-course, these reactions can be visible through the transparent tunic, and microscopy observations show that in few hours the tunic matrix appears to be densely populated with hemocytes infiltrated through the epidermis(Di Bella and De Leo, 2000), presumably coming from the pharynx and connective tissue close to the epidermis. Hematogenic sites (crypts or nodules in the pharynx and emopoietic cells clusters) in the

Corresponding author:
Vincenzo Arizza
Department of Animal Biology
University of Palermo
via Archirafi, 18, 90123 Palermo, Italy
E-mail: arizza@unipa.it

pharynx and connective tissue (Ermak, 1976) as well traits of proliferating epidermis have been shown (Di Bella and De Leo, 2005). The nature of the used inflammatory agent as well as seasonal or environmental effects on the naïve ascidian populations could explain the variability in the time-course and strength of the response. Usually, in a few days, corpuscolate materials cause an intense heightened hemocyte populations density in the pharynx vessels, connective tissue, and tunic matrix where undergo differentiation, degranulation, necrosis, apoptosis, contributing in tunic matrix remodelling phase. The inflammatory cell-types show various shapes that could be related to activation and responding state, their products surround and isolate the host tunic containing the foreignness. In a significant number of individuals a degenerative process provokes a tunic wound which, successively, can be repaired (Parrinello *et al.*, 1977, 1984).

Although the timing sequence of the tunic reaction, after a second-set injection, is characterized by a heightened non-specific response, the chronic inflammation due to the first inoculation could maintain high hemocyte number and inflammatory factors level allowing an accelerate secondary response. Therefore the presence of committed immunocompetent hemocytes may be excluded (Parrinello *et al.*, 1977).

Morphologically distinguishable hemocytes and their transitional forms, including cells that release their contents, have been observed. Few stem cells, granular amebocytes and numerous hemocytes with large granules, signet ring cells and unilocular refractile granulocytes (URGs) have been found in the inflamed tunic. Granulocytes degranulate, signet ring cells release their content, and numerous URGs express phenoloxidase (PO) activity and release the active enzyme and/or pathway products. In the inflamed tunic matrix, a great amount of free granules and vacuoles, including lysosome particles, appear to be discharged by hemocytes. Cellular debris and inflammatory molecules amplify the process also leading to tissue damage. After infection with *E. coli*, also circulating hemocytes promptly phagocytise the bacteria and excrete lysosome particles, while granular amebocytes liberate a lot of particles (Liu *et al.*, 2006).

Recently, we have examined *C. intestinalis* body wall inflammatory responses challenged by lipopolysaccharide (LPS) (see below). LPS is a component of the pathogen-associated molecular patterns **(**PAMPs). In organisms, ranging from invertebrates to humans, immune cells bear Toll-like receptors (TLRs) that bind PAMPs (Vasselon and Detmers, 2002). Three TLR distinct genes and the corresponding signal transduction cascades have been recognized in *C. intestinalis* draft genome (Kimbrell and Beutler, 2001; Azumi *et al.* 2003; Khalturin *et al.*, 2004; Roach *et al.*, 2005). Therefore it is presumable that LPS-TLR binding induces responses including phagocytosis and release of inflammatory agents initiating the inflammatory response.

Ascidian hemocytes involved in immunity

Several approaches have been attempted to identify *C. intestinalis* hemocyte-types by using their morphological features under light or electron microscopy (Rowley, 1981, 1982; De Leo, 1992). In the last few years, interest in the mutual relationships between ascidians hemocyte types and products of innate immunity gene repertoire has led to a more clear-cut knowledge of these cells and their roles in immunity. This approach could also disclose that differentiation of activated cells yield to morpho-functional shapes of inflammatory hemocyte populations.

A technique to classify *C. intestinalis* hemocytes is to look for the presence of granules, which allows to distinguish cells into two wide categories as agranulocytes (hemoblast, lymphocyte-like cells, hyaline amebocytes), or granulocytes (granulocytes with small or large granules, unilocular refractile granulocytes, morula cells). Signet ring cells and compartment cells could be intermediate or final differentiation shapes following a challenge. Variable frequency of circulating hemocyte types have been reported, presumably due to the variability within distinct ascidian populations as well as to seasonal factors and sea coastal environmental conditions. Although transitional hemocytes have been identified in the hemolymph, they can be mainly found in inflamed tissues.

Hemoblasts and lymphocyte-like cells

Hemoblasts in hemopoietic nodules, are considered stem cells (about 3.0 - 5.0 µm) with a typical high nucleus/cytoplasm *ratio* (Ermak, 1976, 1982). Small hematogenic nodules are abundant in the pharyngeal wall and around the gut-loop; a few clusters also occur where the pharynx is attached to the body wall, under the endostyle and mesenteries. In the pharynx, hematogenic clusters are plentiful in transverse bars, spread in longitudinal bars, along the endostyle, and associated with the connective tissue. Few hemoblasts with a lesser nucleus/cytoplasm *ratio* have been found in the circulating hemolymph (Rowley, 1981; Wright 1981; De Leo, 1992). As shown by fine structure observations, the round nucleus contains condensed chromatine adherent to the nuclear envelope, and a characteristic prominent nucleolus. The cytoplasm presents free ribosomes and polyribosomes, mitochondria, occasional rough endoplasmic reticulum, rare Golgi cisternae and a few lipid droplets. In different ascidian species, after an allogeneic challenge, circulating hemoblasts could proliferate and/or differentiate diverse hemocyte types (Raftos and Cooper, 1991).

Lymphocyte-like cells (LLCs) (4.0-5.0 µm) in the hemolymph are similar to the circulating hemoblasts but present a lesser nucleus/cytoplasm *ratio*, the nucleus does not present a nucleolus, and the basophilic cytoplasm contains few small vesicles. Light microscopy studies of unstained circulating cells do not allow a precise distinction between

hemoblasts and LLCs, and they are put on a pair with stem cells. Frequently, structures recognized in the LLCs presumably disclose initial differentiation steps, and some LLCs wider in size are similar to small amebocytes containing numerous mitochondria and lysosome-like granules (Warr *et al,.* 1977; Fuke and Fukumoto, 1993).

Various LLCs frequency have been reported, presumably dependent on environmental conditions and the life cycle phase. Frequency ranging from few cells up to about 20 % have been calculated (Rowley 1981; Wright 1981).

Recently Liu *et al.* (2006) reported that some LLCs can be marked by anti-CD34 monoclonal antibodies in agreement with the potential role of a pluripotent cell able to differentiate cell types. CD34 is a transmembrane protein expressed in mammalian hemopoietic cells (Furukawa, 1998) and widely adopted as a marker of the human hemopoietic stem cells. Although circulating hemoblasts were not distinguished, the LLCs frequency slightly but significantly increases after *in vivo* bacterial (*Escherichia coli*) challenge suggesting that they could proliferate in the circulatory system or in discrete body wall sites. LLCs have been retained a primordial form of vertebrate lymphocyte (Peddie and Smith, 1995). Although the enhanced proliferative activity may be related to their role in immunity, unequivocal evidences on *C. intestinalis* immunocompetent LLCs in allorecognition have not been reported. In other ascidian species, LLCs have been linked to non-self recognition and allograft rejection, also claimed as depositary of an adaptive histocompatibility-dependent cellular response including immunocompetent cell proliferation and differentiation of inflammatory hemocytes (Raftos *et al.* 1987; Raftos and Cooper 1991).

Hyaline amebocytes

In the hemolymph, hyaline amebocytes have been distinguished by electron microscopy as non-vacuolar or vacuolar hyaline amebocytes (Rowley, 1982; De Leo, 1992). In the inflammatory response, especially examined by light microscopy, immunocytochemistry and *in situ* hybridization methods, non-vacuolar and vacuolar amoebocytes cannot be distinguished, thus they are considered together as hyaline amoebocytes.

Electron microscopy observations show the cytoplasm with several vesicles of a variable size containing electron-lucid or a diffuse fibrous material, microtubules and microfilaments. Vacuoles contain finely electron-dense granular inclusions, poorly developed endoplasmatic reticulum and Golgi cisternae can also be observed. Large and small vacuoles have been estimated as derived from expanded endoplasmic reticulum and Golgi cisternae. Morphologically some vesicles resemble the primary lysosomes of mammalian macrophages, while AcPase positivity suggests phagolysosome formation (Rowley, 1982).

Hyaline amebocytes are the most common cell type with phagocytic activity. Within few minutes, they attach and, after the formation of pseudopodia, ingest formalinized sheep erythrocytes (more than 3/cell) (Rowley, 1981), *E. coli* (Liu *et al.*, 2006) and yeast (personal observations), whereas they are not able to phagocytise polystyrene latex beads (Zucchetti *et al.*, 2008). After *E. coli* inoculation, secondary lysosomes can be formed and lysosomal enzyme particles secreted on the bacteria surface. Part of the infected cells undergo cell death, either necrosis or apoptosis. Electron microscopy observations show that the necrosed cells lose their membrane integrity, and broke completely. Another portion of hemocytes which present integral membrane showed characteristics of apoptotic cells that promptly appear after *in vivo* infection. The phagocyte activity against yeast can be enhanced when the targets were opsonized with lectins (Parrinello *et al.,* 2007) suggesting that a lectin recognition mechanism characterizes these cells.

Hyaline amebocytes, with the above characters described for circulating cells, have not been observed in the inflamed tunic tissue, although the possibility exists that infiltrated cells undergo morpho-functional differentiation.

In a recent paper Parrinello *et al.* (2008) showed that, at 4 h after LPS inoculation, circulating hyaline amoebocyte populations contain and presumably release the *Ci*TNFα cytokine (cloned and sequenced) as shown by *in situ* hybridization analysis that identified the mRNA mainly in the nucleus.

Granulocytes

Several circulating granulocytes have been described by light and electron microscopy, and distinguished by the granule size and abundance, shape and electron density of their content (De Leo, 1992). In some cases, the granules contain a low electron-dense material and they have been referred as “vesicles” or “vacuoles”. Rod-like and refractile granules have been observed upon phase contrast microscopy (Rowley, 1981).

Light and electron microscopy showed that inflamed tissues including pharynx vessels, hemolymph and tunic were enriched with granulocytes containing granules of various shape, size, content density and fine structure organization.

Granular amebocytes with small granules

Following an inflammatory challenge numerous granular amebocytes promptly populate the tunic tissue, degranulate (Parrinello *et al.,* 1990) and release inflammatory factors. Circulating granular amebocytes with small granules challenged *in vitro* and *in vivo* by *E. coli* (Liu *et al*., 2006) lose their membrane integrity and degranulate, and electron microscopy shows small holes in the plasma membrane. After *in vivo* infection, apoptosis promptly appeared in the granular amebocytes, and massive density of necrotic hemocytes and degranulating granulocytes were also found in the inflamed tunic. In the inflamed tunic, cell functional states could be characterized by various electron-density of the granular content that in some case appears heterogeneous in its fine structure. Some granules present an electron-dense area surrounded with microtubules, whereas small

electron-transparent granules are AcPase positive. Rowley (1981) reports that these cells did not usually spread out to the same extent as the hyaline amebocytes and their cytoplasmatic extensions were often less evident.

Contrasting data have been reported on their phagocytic activity. Although circulating granular amebocytes can fix bacteria, they do not phagocytised *E. coli* (Liu *et al.*, 2006), and it has been shown that after bacterial challenge the activated cells secrete *in vitro* numerous granules while typical apoptosis bodies emerge in ameboidic granulocytes. On the contrary several reports concern their phagocytic activity. Smith and Peddie (1992) showed that granular amebocytes collected from a Percoll density band, ingest *in vitro Psychrobacter immobilis* and their activity increases when the bacteria were pre-treated with hemocyte-lysate supernatant. However they were not able to distinguish between granular amebocytes and hyaline amebocytes both contained in the separated band. Zucchetti *et al.* (2008) report that granular amebocytes are able to phagocytise polystyrene latex beads, and, after LPS inoculation or *in vitro* treatment, these cells become more effective (up to 80 % granulocytes) in phagocytising the target. Finally, Rowley (1981) showed that these hemocytes promptly ingest formalinized sheep erythrocytes, showing indistinct ruffled membranes or spike-like pseudopodial extensions. The attachment of erythrocytes not always results in their internalization presumably due to differences in the recognition pattern. These various behaviours could be in accordance with granular amebocyte populations provided of different target specificity.

LPS inoculation puts in evidence that granular amebocytes, presumably distinct populations, are engaged in *Ci*C3a production and *Ci*CD94-1 expression. Two C3-like genes, *Ci*C3-1 and *Ci*C3-2, from hemocyte total RNA have been cloned and sequenced (Marino *et al.*, 2002). As in mammals, anaphylotoxin *Ci*C3a peptide is generated by proteolytic cleavage of the C3α-chain and it may exert proinflammatory activity including hemocyte recruitment (Pinto *et al.*, 2003). Following LPS inoculation, an increased number of these cells contain *Ci*C3a fragment with chemotactic activity (Pinto *et al.*, 2003), and also constitutively express a *Ci*C3a receptor (Melillo *et al.*, 2006) supporting the recruitment and activation of granular amebocytes and other effector cells in inflammation (C3aR positive circulating hyaline amebocytes identified by immunostaining could be granular amebocytes). Inhibition experiments with the antibodies revealed that a *Ci*C3a-*Ci*C3aR binding is requested for exerting *Ci*3a chemotactic activity on hyaline and granular amebocytes.

A granular amebocyte population constitutively expresses *Ci*CD94-1 (homolog to vertebrate CD94 that marks NK cells), involved in the phagocytic activity, as a self-non-self recognition receptor with a C-type lectin domain exposed on the cell surface (Zucchetti *et al.*, 2008). After *in vitro* LPS treatment 80% amebocytes express the *Ci*CD94 transcript.

Granulocytes characterized by the size and number of their granules

They are large cells (ranging from 5.0 to 11.0 µm) with a cytoplasm, partially or almost entirely occupied by great granules containing materials of various density and refractile properties (Rowley, 1981). The size and number of these granules can be very different (0.5-2.5 µm). Granule number and size characterize granulocytes with many large granules, whereas other granulocytes contain a variable number of globules filled with material of low, moderate or high density. Granules tend to fuse, and in the inflammatory reaction globular material appear to be released. An unique large granule occupies almost the whole cytoplasm and identify the unilocular refractile granulocytes. Finally compartment and signet ring cells could be terminal hemocyte forms that release their content. The inflammatory response allows to distinguish the following hemocytes.

Granulocytes with large granules

These granulocytes are abundant in the inflamed tunic, their inflammatory role is mainly indicated by phenoloxidase (PO-2)-positive granules contained in the cytoplasm (Parrinello *et al.*, 2003). The enzyme was identified by dopa-MBTH cytochemical reaction and anti-*Ci*PO-2 specific antibodies (immunoistochemistry) raised against a peptide designed from cloned and sequenced *C. intestinalis* PO-2 (Immesberger and Burmester, 2004). The density of the PO-positive granulocyte population as well as the enzyme activity increased in the tunic inflamed by LPS inoculation, reaching the highest level within a few hours post injection (Cammarata *et al.*, 2008). *In vitro* assay of inflamed tissue and PO assay of the tunic lysate supernatant supports that cells containing prophenoloxidase (proPO) are activated. Quinones, promptly (8 h) produced as an effect of cellular proPO activation (proteolysis activated by LPS), are distributed in the tunic matrix and presumably contribute to the inflammatory reaction. These granulocytes could have relationships with PO-positive unilocular refractile granulocytes and morula cells.

Fine structure observations showed that numerous granulocytes with large granules undergo degranulation and granule contents (amorphous and granular) are released into the inflamed tunic matrix (De Leo *et al*., 1992).

Unilocular refractile granulocytes (URGs)

The cells present an unique large granule, filled with electrondense material, nearly occupies the whole cell and confines the nucleus at the periphery close to the cell membrane. This hemocyte-type has been identified in the hemolymph, the granule content appears to be refractile under phase contrast microscopy (Rowley, 1981), provided with homogeneous fine granular content [electron microscopy (TEM) observations] arranged in flocculent

structures or with loose or condensed fibrogranular material that forms masses aggregated into two or more foci often flowing together. The URGs are promptly involved in the tunic inflammatory reaction and are numerous in the inflamed tissue.

The unique granule contains phenoloxidase (Parrinello *et al.*, 2003), cytochemical reaction with dopa-MBTH shows a strong enzyme activity and the presence of *Ci*PO2 was revealed by specific antibodies. Following LPS injection (Cammarata *et al.*, 2008) numerous PO-positive URGs occupy the tunic and presumably release products of PO pathway. It is noteworthy that the circulating URGs exert cytotoxic activity, presumably CD94-independent, when assayed *in vitro* with erythrocytes (Parrinello *et al.*, 1996). Plaque forming cell assay demonstrated that cytotoxic molecules can be released following effector-target contacts (Parrinello *et al.*, 1996).

An URG population from naïve ascidians expresses an antimicrobial peptide (*Ci*PAP-A) that exerts a potent antimicrobial activity against a variety of bacteria and the yeast *Candida albicans* (Fedder and Leippe, 2008). Depending on the cell differentiation state, either the cytoplasm or the inclusion inside the large compartment contain *Ci*PAP-A. Although an assay on hemocytes from LPS injected ascidians was not performed, it is reasonable that *Ci*PAP-A can exert its antimicrobial activity in inflamed tissue injured by the injection procedure as well as by the inflammatory wound.

Multilocular granulocytes

Some large globular granules, tightly packed and close to the cell membrane, occupy the most cytoplasm. Electron dense or electron transparent granule contents have also been used to distinguish two hemocyte types, morula cells and compartment cells respectively.

Morula cells

They are spherical (8 - 16 µm), present variable number of tightly packed symmetrically arranged large globules (2 - 3 µm, max. 7). The nucleus, devoid of nucleolus, eccentrically located and compressed into an angular mass, is usually obscured by the globules. The globules are filled with high density homogeneous material, in some case the content appears electron-transparent with dense inclusion which may be granular or filamentous. It is peculiar that globules have a high refractive index (Rowley, 1981). To one side of the nucleus there is a well-developed Golgi apparatus containing irregularly shaped masses and an endoplasmic reticulum whose cisternae enclose dense granules (Wright, 1981). Cellular activity may be indicated by endoplasmic reticulum cisternae containing dense granules, and by irregularly-shaped masses in the Golgi vesicles. When allowed to stand they assume a berry-like or morular appearance. Presumably distinct morula cells populations could be distinguished.

Although phenoloxidase (dopa-MBTH reaction and immunohistochemistry stain) was mainly found in granulocytes with numerous large granules and in URGs, it is noteworthy that, both in the hemolymph and in inflamed tissue (*in vitro* observations), some morula cells show globular granules with a faint PO positivity.

In addition these cells express (*in situ* hybridization) and contain (immunocytochemistry) *Ci*TypeIX-like (FACIT) collagen α-chain (Parrinello *et al.*, 2008). We cloned and sequenced a FACIT collagen (1α-chain) which is constitutively expressed in *C. intestinalis* tissues (Vizzini *et al.*, 2002). A prompt (4 h) expression of this collagen was shown by real-time PCR in the pharynx of LPS inoculated ascidians as well as in circulating hemocytes challenged *in vitro* (flow cytometry analysis). Collagens are major structural components of extracellular matrix in tissues of vertebrates and invertebrates, also involved in defence and reparative processes (Singer and Clark, 1999). In acute inflammatory reactions cellular, humoral, and molecular events are activated resulting in a regulated pattern of tissue repair with collagen fibres bundles organized during the remodelling (Nwomeh *et al.*, 1998).

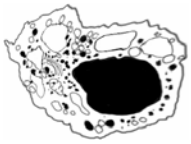
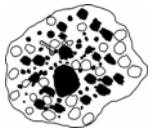
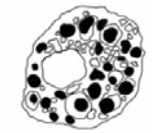
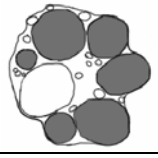
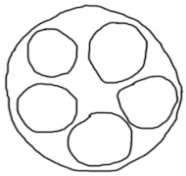
Intermediate or fully developed hemocytes

The possibility exists that compartment cells and signet ring cells may be intermediate or fully developed hemocytes.

Compartment cells

Spherical cells (8 - 12 µm) that present a variable number of large round globules containing electron transparent material with electron-dense granules of variable size on the inside. The nucleus contains no nucleolus and is centrally located, surrounded on one side by mitochondria and numerous profiles of dense rough endoplasmic reticulum and ribosomes (Wright, 1981). Rowley *et al.* (1984) report that compartment cells are usually far less common (5 % max.) than morula cells, moreover X-ray microanalysis results suggest inter-relationship between these two cell types. We have found that, like morula cells they contain *Ci*-FACIT collagen α-chain and are involved in tissue inflammatory response (Vizzini *et al.*, 2008). Compartment cell-like cells releasing their globule content can be recognized in the inflamed tunic (De Leo *et al.*, 1992), suggesting they could be regarded as a fully developed hemocyte engaged in discharging inflammatory factors. In addition, they can express and contain inducible *Ci*TNFα-chain in the cytoplasm lining the globules (Parrinello *et al.*, 2008). Although both collagen chain and *Ci*TNFα are constitutively expressed more numerous positive cells can be identified in the body wall after the LPS challenge. The *Ci*TNF enhanced time course expression is fast whereas that of the collagen increases after few days. Finally, compartment cells express *Ci*C3a fragment (Pinto *et al.*, 2003) but not *Ci*C3a-Receptor has been found (Melillo *et al.*, 2006). It is difficult to identify hemocyte types by using immunostaining method, and some *Ci*C3aR positive cells could be compartment cells. In additon, *Ci*C3aR positive granular amebocytes (presumably granulocytes with

Table 1 Features, activities and innate immunity genes expression of *C. intestinalis* inflammatory hemocytes observed in the inflamed tunic matrix or hemolymph after inoculation with LPS. Hemocyte drawings from light and electron microscopy observations

Agranulocytes	Hemoblast LLC		Hematogenic tissue • Steam cells Circulating hemolymph • Proliferation • CD34 positive • Allograft reaction.	Liu *et al.,* 2006; Reddy *et al.,* 1975; Peddie *et al.*, 1995
	Circulantin hyaline amebocyte		Circulating hemolymph • Express Ci-TNF (LPS) • Fagocytosis FSRBC *E. coli, Saccharomices cerevisiae* • Necrosis/apoptosis.	Parrinello *et al.*, 2008; Rowley 1981; Liu *et al*., 2006
Granulocytes	Granulocyte with small granule		Inflamed tunic • Degranulation • *Ci*C3-1 expression • *Ci*C3a-R expression • *Ci*CD-94 expression Circulating hemolymph • Necrosis/apoptosis • Phagocytosis: *Psicobacter immobilis*, polystirene latex beads, FS RBC (not always), fix *E. coli* • Degranulation following LPS inoculation • *Ci*CD-94 expression.	Parrinello *et al.,* 1990; Liu *et al.,* 2006; Pinto *et al.,* 2003; Melillo *et al.,* 2006; Zucchetti *et al.,* 2008; Cammarata *et al.,* 2008
	Granulocyte with large granules		Inflamed tunic • A few granules PO-2 positive.	Cammarata *et al.,* 2008
	MC refractile globules		Inflamed tunic • Same MCs contain PO-2 positive globules • *Ci*-FACITα-chain collagen.	Vizzini *et al.,* 2008
	URG Unique refractile granulocyte		Inflamed tunic • PO-positive unique large granule Circulating hemolymph • PO-positive unique large granule • *Ci*CD-94-independent cytotoxic activity (RBC, K562) • Plaque forming cell • *Ci*PAP-A expression against a variety of bacteria and *Candida albicans.*	Zucchetti *et al.*, 2008; Parrinello *et al.,* 1996; Fedden and Leippe, 2008; Cammarata *et al.,* 2008
Intermediate and/or terminal forms	Compartment		Inflamed tunic (LPS) • *Ci*-FACITα-chain collagen expression • *Ci*-TNFα expression • *Ci*-C3a • No *Ci*-C3a-R.	Vizzini *et al.,* 2008 Parrinello *et al.,* 2008 Pinto *et al.,* 2003
	Signet ring cell		Inflamed tunic • Encapsulation • Release of electron transparent content.	Parrinello, 1981, 1990; De Leo *et al.,* 1992.

small granules) and some large round non ameboidic cells can be distinguished with difficulty from the negative cells identified as morula cells.

Signet ring cells

These cells have been mainly described in the inflamed tunic (Parrinello and Patricolo, 1984; De Leo *et al.*, 1992), their frequency in the hemolymph may be very low (about 2 %), or they are absent. A large electron-transparent granule occupies almost entirely the cytoplasm and confines the nucleus at a peripheral site.

Electron microscopy observations show that these hemocytes (De Leo *et al.,* 1992) are very numerous in the inflamed tunic matrix where they discharge their granular or amorphous content as an effect of membrane dissolution. (Parrinello 1981, 1990; De Leo *et al.*, 1992). The nature of the discharged granule materials is unknown, but they seem to contribute in encapsulation response. According to Rowley (1981) presumably these cells represent the secretive state of challenged hemocytes.

Conclusions

Electron and light microscopy morphological features of *C. intestinalis* hemocytes allowed controversial classifications of the circulating cells. Insights have been obtained by examining both cellular aspects of the inflammatory response and innate immunity genes expression (Table 1). Corpusculate and soluble inflammatory agents, bacteria and LPS inoculation into the ascidian body wall, as well as *in vitro* challenge of circulating hemocytes, contribute to distinguish two main categories, agranulocytes (hemoblasts, lymphocyte-like cells, hyaline amebocytes) and granulocytes (granulocytes with small granules, granulocytes with large granules, unilocular refractile granulocytes, morula cells). Compartment cells and signet ring cells could be intermediate or fully developed hemocytes presumably involved in releasing inflammatory factors or tunic matrix components. Gene expression studies, immunohistochemistry and immunocytochemistry assays disclose that hyaline amebocytes, mainly phagocytes, contain and presumably release *Ci*TNFα-like cytokine, and show that granular amebocytes express *Ci*C3-1, *Ci*C3aR, *Ci*CD94 receptor and produce chemotactic *Ci*C3a supporting their inflammatory role.

Granulocytes with large granules show a weak PO activity of a few granules, and could have relationships with PO-positive unilocular refractile granulocytes and morula cells. URGs are the main components of the inflamed tissue where presumably release products of the PO pathaway, whereas circulating URGs exert cytotoxic activity when assayed against erythrocytes and K562 tumor cell line. Cytolysins cannot be related to the Ca^{2+}-independent *Ci*CD94 NK-like receptor, they are released and exert Ca^{2+}-dependent activity. On the contrary, *Ci*CD94 NK-like receptor is involved in phagocytic activity. Finally, an URG population from naïve ascidians expresses an antimicrobial peptide (*Ci*PAP-A) with a potent antimicrobial activity. It is reasonable that *Ci*PAP-A exerts its antimicrobial activity in inflamed tissue injured by the injection procedure and by the inflammatory wound.

Morula cells contain globules filled with high density homogeneous material showing high refractive index, and some of them are provided with faint PO activity. In addition these cells express *Ci*TypeIX-like (FACIT) collagen α-chain following an LPS challenge. Some similarities suggest inter-relationship between morula and compartment cells which contain a variable number of large round globules with electron transparent content. However, circulating compartment cells appear to be engaged in releasing inflammatory factors including *Ci*TNFα-chain and *Ci*FACIT α-chain, *Ci*C3a fragment. These hemocytes have been also found in inflamed tissues. Finally signet ring cells could be a fully developed hemocytes discharging their granular or amorfous content as an effect of membrane dissolution, representing the secretive state of challenged hemocytes.

We suggest that morpho-functional studies on challenged inflammatory hemocytes could contribute in establishing a more precise classification of the *C. intestinalis* hemocytes, taking in account that several populations of a same cell type can exert distinct roles.

Aknowledgments

Research on the *C. intestinalis* gene expression (*Ci*TNF, *Ci*FACIT collagen), PO and cytotoxic activities of inflammatory hemocytes, as well as the present paper, have been supported by the PRIN-Cofin 2006 grant to Prof N Parrinello.

References

Azumi K, De Santis R, De Tomaso A, Rigoutsos I, Yoshizaki F, Pinto MR, *et al*. Genomic analysis of immunity in a Urochordate and the emergence of the vertebrate immune system: "waiting for Godot". Immunogenetics 55: 570-581, 2003.

Cammarata M, Arizza V, Cianciolo C, Parrinello D, Vazzana M, Vizzini A, *et al*. The prophenoloxidase system is activated during the tunic inflammatory reaction of *Ciona intestinalis*. Cell Tissue Res. 333: 481-492, 2008.

Davidson B, Swalla BJ. A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. Development 129: 4739-4751, 2002.

De Leo G, Parrinello N, Di Bella MA. Structural changes in granulocytes involved in the lysis of the tunic during inflammatory-like reaction induced in *Ciona intestinalis* (Tunicata, Ascidiacea). Eur. Arch. Biol. 103: 113–119, 1992.

De Leo G. Ascidian hemocytes and their involvement in defense reactions. Boll. Zool. 59: 195–213, 1992.

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan BA, De Tomaso B, *et al*. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins Science 298: 2157–2167, 2002.

Delsuc F, Brinkmann H, Chourrot D, Hervé P. Tunicate and not cephalochordates are the clotest living relatives of vertebrates Nature letter. 439: 965-968, 2006.

Di Bella MA, and De Leo G. Hemocyte Migration during Inflammatory-like Reaction of *Ciona intestinalis* (Tunicata, Ascidiacea) J. Invertebr. Pathol. 76: 105-111, 2000.

Di Bella MA, Carbone MC, De Leo G. Aspects of cell production in mantle tissue of *Ciona intestinalis* L. (Tunicata, Ascidiacea) Micron 36: 477-481, 2005.

Du Pasquier L. Speculations on the origin of the vertebrate immune system. Immunol. Lett. 92: 3-9, 2004.

Ermak TH. The hematogeneic tissues of tunicates. In: Wright RK, Cooper EL (eds), Phylogeny of thymus and bone marrow-bursa cells, Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands, pp 45-55, 1976.

Ermak TH. The renewing cell populations of ascidians. Am. Zool. 22: 795-805, 1982.

Fedders H, Leippe MA. Reverse search for antimicrobial peptides in *Ciona intestinalis*: Identification of a gene family expressed in hemocytes and evaluation of activity Dev. Comp. Immunol. 32: 286-298, 2008.

Fujita T, Endo Y, Nonaka M. The lectin-complement pathway its role in innate immunity and evolution. Immunol. Rev. 198: 185-202, 2004.

Fujita T. Evolution of the lectin-complement pathway and its role in innate immunity. Nature Rev. Immunol. 2: 346-353, 2002.

Fuke M, Fukumoto M. Correlative fine structural, behavioral, and histochemical analysis of ascidian blood cells. Acta Zool. 74: 61-71, 1993.

Furukawa, Y. Cell cycle regulation of the hematopoietic stem cells. Hum. Cell 11: 81-92, 1998.

Immesberger A, Burmester T. Putative phenoloxidases in the tunicate *Ciona intestinalis* and the origin of the arthropod hemocyanin superfamily. J. Comp. Physiol. B 174: 169-80, 2004.

Kasahara M, Suzuki T, Du Pasquier L. On the origins of the adaptive immune system: novel insights from invertebrates and cold-blooded vertebrates. Trend Immunol. 25: 105-111, 2004.

Khalturin K, Pancer Z, Cooper MD, Bosch TCG. Recognition strategies in the innate immune system of ancestral chordates. Mol. Immunol. 41: 1077-1087, 2004.

Kimbrell DA, Beutler B. The evolution and genetics of innate immunity. Nature Rev. Genet. 2: 256-267, 2001.

Liu L, Wu C, Chen T, Zhang X, Li F, Luo W, *et al*. Effects of infection of EGFP-expressing *Escherichia coli* on haemocytes in *Ciona intestinalis* J. Exp. Mar. Biol. Ecol. 332: 121-134, 2006.

Marino R, Kimura Y, De Santis R, Lambris JD, Pinto MR. Complement in urochordates: cloning and characterization of two C3-like genes in the ascidian *Ciona intestinalis*. Immunogenetics 53: 1055-1064, 2002.

Melillo D, Sfyroera G, De Santis R, Graziano R, Marino R, Lambris JD, *et al*. First identification of a chemotactic receptor in an invertebrate species: structural and functional characterization of *Ciona intestinalis* C3a receptor. J. Immunol. 177: 4132-4140, 2006.

Nonaka M, Miyazawa S. Evolution of the initiating enzymes of the complement system, Genome Biol. 3: 1-5, 2002.

Nwomeh BC, Yager DR, Cohen IK. Physiology of the chronic wound. Clin. Plast. Surg. 25: 341-356, 1998.

Parrinello N, Arizza V, Chinnici C, Parrinello D, Cammarata M. Phenoloxidases in ascidian hemocytes: characterization of the pro-phenoloxidase activating system Comp. Biochem. Physiol. 135B: 583-591, 2003.

Parrinello N, Arizza V, Vazzana M, Cammarata M, Giaramita FT, Di Bella ML, *et al*. Separated hemocyte populations from the ascidian *Ciona intestinalis* contain and release in vitro opsonizing Ca^{++}-independent and β-galactoside specific lectins. Inv. Surv. J. 4: 55-64, 2007.

Parrinello N, Cammarata M, Arizza V. Univacuolar refractile hemocytes from the tunicate *Ciona intestinalis* are cytotoxic for Mammalian erythrocytes in vitro. Biol. Bull. 190: 418-425, 1996.

Parrinello N, De Leo G, Di Bella MA. Fine structural observation of the granulocytes involved in the tunic inflammatory-like reaction of *Ciona intestinalis* (Tunicata). J. Invert. Pathol. 56: 181-189, 1990.

Parrinello N, Patricolo E, Canicatti C. Inflammatory-like reaction in the tunic of *Ciona intestinalis* (Tunicata). I. Encapsulation and tissue injury. Biol. Bull. 167: 229-237, 1984.

Parrinello N, Patricolo E, Canicattì C. Tunicate immunobiology. I. Tunic reaction of *Ciona intestinalis* L. to erythrocyte injection. Boll. Zool. 44, 373–381, 1977.

Parrinello N, Patricolo E. Inflammatory-like reaction in the tunic of *Ciona intestinalis* (Tunicata). II. Capsule components Biol. Bull. 167: 238-250, 1984.

Parrinello N, Vizzini A, Arizza V, Salerno G, Parrinello D, Cammarata M, *et al*. Enhanced expression of a cloned and sequenced *Ciona intestinalis* TNFα-like (*Ci*TNFα) gene during the LPS-induced inflammatory response Cell Tissue Res. 334: 305-17, 2008.

Parrinello N. The reaction of *Ciona intestinalis* L. (Tunicata) to subcuticular erythrocyte and protein injection Dev. Comp. Immunol. 5: 105-110, 1981.

Peddie CM, Smith VJ. Lymphocyte-like cells in ascidians: Precursors for vertebrate lymphocytes? Fish Shellfish Immunol. 5: 613-629, 1995.

Pinto RM, Chinnici C, Kimura Y, Melillo D, Marino R, Spruce LA, *et al*. *Ci*C3-1a-Mediated chemotaxis in the deuterostome invertebrate *Ciona intestinalis* (Urochordata). J. Immunol. 171: 5521-5528, 2003.

Raftos DA, Cooper EL. Proliferation of lymphocyte-like cells from the solitary tunicate, *Styela clava*, in response to allogeneic stimuli. J. Exp. Zool. 260: 391-400, 1991.

Raftos DA, Tait NN, Briscoe DA. Cellular basis of allograft rejection in the solitary urochordate, *Styela plicata*. Dev, Comp. Immunol. 11: 713-725, 1987.

Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, *et al*. The evolution of vertebrate Toll-like receptors Proc. Natl. Acad. Sci. USA 102: 9577-9582, 2005.

Rowley, AF. The blood cells of the sea squirt *Ciona rntestinali*s. morphology, differential counts, and in vitro phagocytic activity. J. Invertebr. Pathol. 37: 91-100, 1981.

Rowley AF. Ultrastructure and cytochemical studies on the blood cells of the sea squirt *Ciona intestinalis*. 1. Stem cells and amoebocytes. Cell Tissue Res. 223: 403-414, 1982.

Rowley AF, Rhodes CP, Ratcliffe A. Protochordate leucocytes: a review. Zool. J. Linn. SK 80: 283-295, 1984.

Satoh N. The ascidian tadpole larva: comparative molecular development and genomics. Nature Rev. Genet. 4: 285-295, 2003.

Satou Y, Yamada L, Mochizuki Y, Takatori N, Kawashima T, Sasaki A, *et al*. A cDNA resource from the basal chordate *Ciona intestinalis*. Genesis 33: 153-154, 2002.

Shida K, Terajima D, Uchino R, Ikawa S, Ikeda M, Asano K, *et al*. Hemocytes of *Ciona intestinalis* express multiple genes involved in innate immune host defense. Biochem. Biophys. Res. Commun. 302: 207-218, 2003.

Singer AJ, Clark RAF. Mechanisms of desease: cutaneous wound healing. N. Engl. J. Med. 341: 738-743, 1999.

Smith VJ, Peddie CM. Cell cooperation during host defence in the solitary tunicate *Ciona intestinalis* (L), Biol. Bull. 183: 211-219, 1992.

Swalla BJ, Cameron CB, Corley LS, Garey JR. Urochordates are monophyletic within the deuterostomes. Syst. Biol. 49: 52-64, 2000.

Terajima D, Yamada S, Uchino R, Ikawa S, Ikeda M, Shida K, *et al*. Identification and sequence of seventy-nine new transcripts expressed in hemocytes of *Ciona intestinalis*, three of which may be involved in characteristic cell–cell communication, DNA Res.10: 203–212. 2003.

Vasselon T, Detmers PA. Toll receptors: a central element in innate immune responses. Infect. Immun. 70: 1033-1041, 2002.

Vizzini A, Arizza V, Cervello M, Cammarata M, Gambino R, Parrinello N. Cloning and expression of a type IX-like collagen in tissues of the ascidian *Ciona intestinalis*. Biochim. Biophys. Acta 1577: 38-44, 2002.

Vizzini A, Pergolizzi M, Vazzana M, Salerno G, Di Sano C, Macaluso P, *et al*. FACIT collagen (1α-chain) is expressed by hemocytes and epidermis during the inflammatory response of the ascidian *Ciona intestinalis*. Dev. Comp. Immunol. 32: 682-692, 2008.

Warr GW, Decker JM, Mandel TE, De Luca D, Hudson R, Marchalonis JJ. Lymphocyte-like cells of the tunicate, *Pyura stolonifera*: binding of lectins, morphological and functional studies. Aust. J. Exp. Biol. Med. Sci. 55: 151-164, 1977.

Wright RK. Urochordates In: Ratcliff NA, Rowley AF (eds), Invertebrate blood cells, Academic Press, New York, vol 2, pp 565-626, 1981.

Zeng L, Swalla BJ. Molecular phylogeny of the protochordates: chordate evolution. Can. J. Zool. 83: 24-33, 2005.

Zucchetti I, Marino R, Pinto MR, Lambris JD, Du Pasquier L, De Santis R. *Ci*CD94-1, an ascidian multipurpose C-type lectin-like receptor expressed in *Ciona intestinalis* hemocytes and larval neural structures Differentiation 76: 267-282, 2008.