ISJ 6: S46-S57, 2009 ISSN 1824-307X

REVIEW

Focusing on *Ciona intestinalis* (Tunicata) innate immune system. Evolutionary implications

N Parrinello

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

Accepted March 13, 2009

Abstract

Phylogenetic analyses based on molecular data provide compelling evidence that ascidians are of critical importance for studying chordate immune system evolution. The *Ciona intestinalis* draft genome sequence allows searches for phylogenetic relationships, gene cloning and expression of immunorelevant molecules. Acidians lack of the pivotal components of the vertebrate recombinatory adaptive immunity, i.e., MHC, TCRs and dimeric immunoglobulins. However, bioinformatic sequence analyses recognized genic elements indicating the essential features of the Ig superfamily and ancestor proto-MHC genes, suggesting a primitive pre-duplication and pre-recombination *status*. *C. intestinalis* genes for individuality in the absence of MHC could encode diverse molecular markers, including a wide panel of complement factors that could be responsible for self-nonself discrimination. Genome analysis reveals a number of innate immunity vertebrate-like genes which encode Toll-like and virus receptors, complement pathways components and receptors, CD94/NK-receptor-like, lectins, TNF, IL1-R, collagens. However, pure homology seeking for vertebrate-specific immunorelevant molecules is of limited value, and functional screening methods may be a more promising approach for tracing the immune system evolution. *C. intestinalis,* which displays acute and chronic inflammatory reactions, is a model organism for studying innate immunity genes expression and functions.

Key words: immunoevolution; genome; *Ciona intestinalis*; ascidians; innate immunity; inflammatory response; gene expression

Evolutionary relevance of ascidian immunity studies

Ascidians (Urochordata), including cosmopolitan compound and non-colonial species, occupy a key position in the phylogenetic line leading to the vertebrates (Swalla *et al.,* 2000; Zeng and Swalla, 2005; Delsuc *et al.,* 2006), therefore they have attained importance for immunity evolution studies recently promoted by available genome sequences (Dehal *et al.*, 2002b; Satou *et al.* 2002; Satoh, 2003; Yokobori *et al.*, 2003; Kasahara *et al.*, 2004; Litman and Cooper, 2007; Ben-Shlomo, 2008). Bioinformatic approach and extensive *in silico* search of immunorelevant genes have been in part validated by expression patterns and biological properties of their products (Davidson and Swalla, 2002; Nonaka and Miyazawa, 2002; Fujita, 2002; Azumi *et al.*, 2003; Shida *et al.*, 2003; Terajima *et al.*, 2003; Fujita *et al.*, 2004; Du Pasquier, 2004; Kasahara *et al.*, 2004; Litman and Cooper, 2007).

Botryllids provided of allorecognition reaction (De Tomaso and Weissman, 2004; De Tomaso *et al.* 2005; Ballarin , 2008; Gasparini *et al.*, 2008) and *Ciona intestinalis* which displays acute inflammatory responses (Parrinello, 1981; Parrinello and Patricolo, 1984; Parrinello *et al.,* 1984) are model organisms for studying chordate evolution. The whole genome of *C. intestinalis* has been sequenced and analyzed, genes have been annotated and some expression studies carried out, whereas adequate botryllid genome sequencing is lacking.

Corresponding author:
Nicolò Parrinello
Laboratory of Marine Immunobiology
Department of Animal Biology
University of Palermo
via Archirafi 18, 90123 Palermo, Italy
E-mail: nicpar@unipa.it

Adaptive immunity pivotal genes

In vertebrates, the emergence of the adaptive immune system is linked to the acquisition of the enzyme machinery encoded by the recombination activating genes (RAG) that provide to the rearrangement of immunoglobulin (Ig) and T cell receptor (TCR) genes. Both B and T cells carry

receptor molecules to recognize and respond to the antigens. B cell receptor is a prototype of the antibody, and TCR on the surface of T lymphocytes is responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. These receptors are composed of two polypeptides of the Ig superfamily (Ig heavy H and light L chains; TCR α and β or γ and δ chains) with recognizable constant (C) and variable (V) domains. The C domain of C1-set characterizes only Ig, TCR, and MHC class II, class I and class I-related molecules, whereas the C2 set domain occurs in both vertebrates and invertebrates (see Kasahara *et al.*, 2004). The domain provided with variability is generated by somatic recombination of multiple elements scattered in the genic *locus*. In lymphocytes, RAGs cut DNA at the recombinant signal sequence (RSS) for mediating recombination of V(D)J regions through the joining gene segments. Somatic hypermutation, RAGs and activation-induced cytidine deaminase (AID), regulators of secondary Ig diversification, are crucial components of vertebrate adaptive immunity (Manis *et al.,* 2002; Bransteitter *et al.*, 2003) producing an unlimited repertoire of diversity that enables the cells to recognize and respond to unpredictable pathogens.

Analysis of *C. intestinalis* genome sequences did not reveal the pivotal genes and molecules for adaptive immunity, such as MHC genes, TCRs, or dimeric Igs (Dehal *et al.* 2002b; Azumi *et al.,* 2003; Shida *et al.,* 2003). Nevertheless, bioinformatic sequence analyses have recognized two Ig domain-containing regions, key V regions, the essential feature of an Ig superfamily VC1-like core trait, presumptive proto-MHC regions scattered throughout the genome, and three types of genes with receptor-like V-C architecture (Du Pasquier, 2004). In addition, there are indicators of V domains that could be targets for RAG-mediated transposition. These genes belong to two families with recognizable homologs in vertebrates: the cortical thymocyte marker of *Xenopus*/Junctional adhesion molecules family (*Ci*-CTX/JAM) and the nectin *Ci*-Nec2 family.

Signal of ancestor proto-MHC genes can be drawn from the human MHC genome paralogy (Kasahara, 2000). More than 100 genes are located in the human leukocyte complex (HLA) and about 40 of them have paralogous copies on 1, 9 and 19 chromosomes. *C. intestinalis* genome contains a single copy gene with features of a precursor of multiple human MHC paralogous suggesting the existence of a pre-duplication region (Bodmer, 1972; Kasahara *et al.*, 2004). Chromosome duplications could be took place before the emergence of a common ancestor of vertebrates as indicated by MHC paralogous emergence. MHC-based allorecognition seems to be a unique feature of jawed vertebrates, and finding of variable lymphocyte receptors in agnates (Pancer *et al.*, 2004) indicate that different groups of molecules could be responsible for similar functions. Accordingly, allorecognition machinery in urochordates has nothing in common with the MHC-based histocompatibility reaction of vertebrates (De Tamaso *et al.,* 2005; Klein, 2006), and observations indicate that different ascidian species could have evolved their own independent allorecognition strategies. In example, the hystocompatibility Fu/Hc receptor of *B. schlosseri* has no direct homologs neither in vertebrates nor in *C. intestinalis*, indicating that self-nonself discrimination systems have branched off into a variety of unique and specialized systems during evolution.

In the absence of vertebrate-like adaptive immunity, *Ciona*'s genes reflect a primitive pre-duplication and/or pre-recombination *status* leading to the genesis of MHC, TCR and Ig (Yu *et al.*, 2005). Moreover, it is generally accepted that *C. intestinalis* activating and inhibitory receptors of immunocytes have MHC-independent functions.

Presumptive molecular codes for sef-nonself discrimination in the absence of MHC

Although most animals are able to distinguish self from non-self (Buss, 1987; Chadwick-Furman and Rinkevich, 1994; Cadavid *et al.,* 2004), allorecognition molecular mechanisms do not seem of monophyletic origin and remain to establish if they evolved independently. Different groups of invertebrates could have developed their own histocompatibility system and could express taxon-specific self-nonself determinants.

Ciona is a valuable model for deepening the question. It is hermaphrodite producing eggs and sperm simultaneously, but self-fertilization normally does not occur because the follicle cells accept only allogeneic sperm (Rosati and De Santis, 1978; Pinto *et al.*, 1995; Murabe and Hoshi, 2002). It can be assumed that a receptor, potentially involved in self-incompatibility, should have sequence and expression variable in oocytes from different individuals. Based on this assumption, novel models have been proposed upon a *C. intestinalis* molecular code for individuality in the absence of MHC.

To search for molecular markers of individuality, a suppression subtractive hybridisation approach has been used for comparing the somatic transcriptomes of two *Ciona* individuals and for identifying individually variable cDNAs (Khalturin *et al.,* 2005). The results show that two genes, *Ci*META2 and *Ci*S7, encode two classes of soluble proteins which exhibit high degree of inter- and intra-individual variability and contain multiple domains suitable for protein-protein interaction. Both classes of individually variable proteins are coded in the genome by several gene copies organized in clusters. One class consists of secreted protein thrombospondin type 1-like domains (thrombospondin type 1 repeat, TSR superfamily), the second one consists of secreted proteins with multiple epidermal growth factor (EGF)-like domains. Although the functional significance is unknown, the authors suggest that these gene loci may participate in controlling non-self recognition. *Ci*S7 and *Ci*META2 isoforms with a high degree of aminoacid sequence variations are expressed in hemocytes and gametes which are mediators of recognition events. Individuals have several genes and transcribe several mRNAs coding for similar but

not identical proteins of each gene family. Apparently each individual carries an unique repertoire (haplotype) in each *locus*, probably established by crossing over events during gamete maturation and/or fertilization. Individuality may be encoded by the haplotype and the individual-specific combination of genes in the genome *locus*. This phenomenon resembles the variability of vertebrate killer cell Ig-like receptors (KIR), and fit with the predictions of self-nonself recognition molecules provided with individual variability, allorecognition and block of self-fertilization.

To shed light on *C. intestinalis* specific receptors for allorecognition and self fertilization avoidance, Kurn and colleagues (2007) subctracted gonadal cDNA from three genetically unrelated *C. intestinalis* individuals by suppression subtractive hybridisation. Individual-specific genes encoding variable transmembrane complement receptor-like protein (vCRL) have been identified. Interestingly, they contain several complement controlling protein domains (SCR/CCP). One of these genes reveals a high degree of inter-individual vCRL amino acid variation, and it is expressed in follicle cells and hemocytes. Diverse vCRL1s, highly variable between individuals, show sequence similarity varying from 70 % to 93 %. Each animal has its own version of vCRL1, therefore cells in different individuals are marked by non-overlapping receptors and corresponding ligands. Intra-individual variants most likely are due to the two alleles of a single *locus*, and several alternative intracellular vCRL1 domains may be produced by alternative splicing. The cells within one individual are appropriately marked and will be referred to as "self" whereas any cell of genetically different individual will be distinguished as "non self".

In mammals, the complement components are not variable between individuals. In *Ciona,* the number of genes encoding complement system components is greatly expanded compared to mammals and, together the vCRL high variability, allow to suppose that the *Ciona* allorecognition reactions involve complement-related receptors in a "missing self" mechanism. Intriguingly, complement receptors are also expressed in human gametes and are involved in sperm/oocyte interaction (Seya *et al.*, 1999).

The *Ci*FLRT transmembrane receptor gene expressed in hemocytes (Kurn *et al.,* 2007) was also analyzed. The corresponding protein consists of a signal peptide, nine leucine-rich repeats (LRRs, protein interaction modules), fibronectin type III domain (mammalian FNIII repeats on cell surface and extracellular) and a transmembrane domain (Khalturin *et al.* 2005). The *Ci*FLRTs from two individuals were amplified, sequenced and compared to sequences reported by Khalturin and colleagues (2005). The distance between the *Ci*FLRT proteins of the four individuals is much smaller than that between vCRL1. The high degree of cCRL1 variation clearly exceeds the naturally occurring variations normally found in *Ciona* genes.

Toll-like receptors

The Toll-like receptor (TLR) multigene family encodes recognition receptors of innate immune system conserved in invertebrates and vertebrates (Kaisho and Akira, 2000; Medzhitov and Janeway, 2000; Imler and Hoffmann, 2001; Vasselson and Detmers, 2002). TLRs recognize a variety of endogeneous and exogeneous ligands, they are pattern recognition receptors that bind molecules broadly shared by pathogens collectively referred to as PAMPs, many of which are conserved molecules essential for pathogen survival (Zhong and Kyriakis, 2007). *C. intestinalis* genome sequence analysis disclosed three distinct TLRs expressed genes and the corresponding signal transduction cascades (Azumi *et al.*, 2003; Terajima *et al.,* 2003; Roach *et al.*, 2005; Shida *et al.,* 2005). In invertebrates, Toll-like receptors mediated antibacterial, antifungal and antiviral systems.

Recently, *C. intestinalis* inflammatory responses challenged by LPS have been reported. The triggering mechanism presumably includes Toll-like receptors (TLRs) which bind LPS (a component of the PAMPs) initiating the inflammatory reactions. Responses stimulated by LPS including complement activation and products, galectin-cytokine-like and CD94-NK-C-type lectin-like receptor genes expression, and collagen synthesis have been reported (see below).

Genes for intracellular immunoreceptors, tyrosine-based inhibition motifs (*Ci*ITIMs) and tyrosine-based activation motifs (*Ci*ITAMs) may be responsible of the cell signaling pathways (Azumi *et al.* 2003; Shida *et al.,* 2003). Vertebrate ITIMs and ITAMs are short conserved sequences in the cytoplasmic tails of many immune cells inhibitory and activating receptors (including TCRs), respectively.

Virus receptors

In *C. intestinalis* genome, homologs to vertebrate adhesion molecules members of membrane Ig superfamily show ancestral features of antigen receptors (Du Pasquier, 2004). They include the junction adhesion molecule (reovirus receptor) and the cortical thymocyte marker of *Xenopus* (adenovirus receptor) which are members of *Ci*CTX/JAM family, and the poliovirus receptor (PVR) members of the *Ci*Nec family. PVR genes contain one distal V domain followed by two C domains, transmembrane and cytoplasmic segments. Vertebrate PVRs are known to allow endocytosis of different viruses through the interaction with V domain. Since in humans 4 paralogous groups exist, the *Ciona* set of genes could correspond to a preduplication *status*. It has been suggested that the virus binding property of the members of this family were recruited in the vertebrate immune system following the introduction of the somatic rearrangement machinery.

Complement system

The innate complement system evolved long

before the origin of vertebrate somatically re-arranging antibodies. In vertebrates, there are about 30 complement protein components and three distinct pathways by which the complement system can be activated (Nonaka and Yashizaki, 2004a, b): 1. classical, antibody-mediated pathway; 2. lectin mediated pathway; 3. alternative pathway, triggered the pathogen surface. All the activation mechanisms converge to the C3 component proteolysis, and generate a same set of activation products. C3 is the central component, equipped with an unique intramolecular thioester bond which, upon activation, is exposed to the molecular surface and forms a covalent bond with invading microorganisms (Lambris, 1990). C3 proteolysis enhances the phagocytosis (opsonin) of pathogens through some C3b fragment and the chemotaxis by the C3a-fragment which binds to macrophage and leucocyte receptors, furthermore C3b contribute in forming C5 convertase that activates C5-C9 late complement components leading to the formation of a cytolytic complex (membrane attack complex or MAC). A large number of complement components are conserved between higher vertebrates and urochordates.

In *C. intestinalis*, a wide and detailed architecture of the primitive complement system has been described (Nonaka and Yoshizaki, 2004a, b; Fujita *et al.,* 2004), and complement-like genes, most of which are transcriptionally active, indicate potential activity of lectin and alternative pathways.

The *Ciona* lectin mediated complement pathway has been revealed by genome sequence analysis, EST, cloning and expression studies that identified nine mannose-binding lectins (*Ci*MBL), nine ficolins and four *Ci*MBL-associated serine proteases (*Ci*MASPs C1r/C1s-like). The carbohydrate-binding collectin pathway (*Ci*MBLs/ficolins as pattern recognition proteins), initiated by recognition of PAMPs and activated by *Ci*MASPs, leads to *Ci*C4, *Ci*C2 and *Ci*C3 cleavage. Several soluble components and receptors (four *Ci*MASPs, *Ci*C3 and corresponding *Ci*CR3/CR4 receptors) have been recognized in the genome, and ESTs disclosed transcripts in hemocytes (Fujita, 2002; Azumi *et al.*, 2003; Fujia *et al.*, 2004; Wakoh *et al.*, 2004). *Ci*MASPs could exert trypsin-like activity, and the identified *Ci*MBL collectin genes encode proteins composed of the collagen and lectin-like domains.

Recently a *Ci*MBL has been cloned and sequenced, and its expression was promptly enhanced by LPS (Bonura *et al.,* submitted). The deduced amino acid sequence (221 aa) showed a N-cysteine-rich terminal domain, a type 2 collagen domain, and a C-type mannose/glucose-specific CRD. Comparative analysis reveals 56.5 % similarity and 37.8 % identity with human MBL. Marino and colleagues (2002) cloned two *Ci*C3-like genes (*Ci*C3-1 and *Ci*C3-2 cDNA) from *Ciona* hemocyte mRNA. The deduced aminoacid sequences of both *Ci*C3 proteins show an overall similarity to the C3 molecules from vertebrate species, and exhibit a canonical processing site for α- and β-chains, including a typical thioester site with the His residue required for nucleophilic activation.

LPS activates the complement, presumably *via* *Ci*MBL and *Ci*C3-1 proteolysis leading to the *Ci*C3a fragment which is a pro-inflammatory peptide akin to the vertebrate anaphylatoxin (Pinto *et al.,* 2003). The inflammatory challenge upregulates the *Ci*C3-1 expression in hemocytes and *Ci*C3-1a production. The recombinant *Ci*C3-1a exerts *in vitro* chemotactic effect on hemocytes interacting with a receptor molecule *Ci*C3aR coupled with G_i protein and homologous to the mammalian receptor (Melillo *et al.,* 2006).

An ancestor of the two *Ci*C3 seems to have diverged from a common ancestor of vertebrate C3/C4/C5 and has duplicated in two genes. Accordingly, sequence phylogenetic analysis indicates that *Ci*MASPs have diverged from the common ancestor of vertebrate MASP/C1r/C1s. In addition, the genome presents two α2Macroglobulin-like (*Ci*α2M) genic elements (Azumi *et al*., 2003). Mammalian α2M is able to inactivate an enormous variety of proteinases, inhibits complement activation by MASP and it is conceivable that it plays a regulatory role in MBP-derived complement activation (Terai *et al.*, 1995). C3/C4/C5 and α2M form a molecular family different from other complement components and they do not show clear domain structure.

In mammals, the factor B (Bf) is the central serine esterase of the alternative pathway of complement activation, and in the active form (Bb) is component of C3 convertase. In *Ciona* three Bf-like genes, supported by cDNA evidence, have also been identified and they, presumably responsible for the alternative pathway, encode predicted proteins with domain structures similar to the vertebrate Bf/C2 gene family basic domain structure (Fujita, 2002; Azumi *et al.* 2003; Fujita *et al.* 2004). Bf and C2 are catalytic subunits of the C3 convertases of the alternative and vertebrate classic pathways respectively. The *Ci*Bf genes are longer than those of jawed vertebrates, the deduced amino-acid sequences of *Ci*Bf1-3 contain the usual domains of Bf and, in addition, three extra domains at the N-terminus. Similarly to vertebrate MASP-2, MASP-3 and C1r/C1s, *Ci*Bf presents one additional short consensus repeat domain, and two low-density lipoprotein receptor (LDLR) domains. Overall deduced amino-acid identity between *Ci*Bf-1 and *Ci*Bf -2 was 88 %, whereas *Ci*Bf-3 showed 49 % identity to both *Ci*Bf-1 and *Ci*Bf-2. The *Ci*Bf serine protease domain shared characteristic features with the complement components C1r/C1s, MASP-2 and MASP-3, and the active site appears to be of the AGy codon type for the catalytic serine residue, and lacks of a histidine loop disulfide bridge (Fumiko *et al.,* 2005; Yoshizaki *et al.,* 2005).

Phylogenetic analysis indicates that *Ci*Bf genes could be the result of duplication and gene conversion occurred after the divergence of the urochordate lineage from the vertebrate subphylum as also shown by genomic organization and intron/exon composition. Presumably they have diverged from the common ancestor of vertebrate Bf

and C2 before the divergence of Bf and C2.

These results indicate that complement genes have evolved through extensive exon shuffling events in the early stage of chordate evolution. Exons 3 and 5 of the three *Ci*Bf genes show an extremely high degree of nucleotide identity, indicating duplication and gene conversion, since its divergence from the vertebrate Bf/C2 gene.

The *Ciona* genome analysis also allowed the identification of eleven presumptive genes with MAC/perforin domain, nine of them exhibit domain structures similar to those of late complement components. Although, activation mechanism and a functional linkage between *Ci*C3 and the possible lytic components have not been demonstrated, the lytic function is strongly suggested by the presence of the MAC/perforin-like domain. Combination of several domains assign the lytic components to the C6-C9 family. However, *Ci*C6 and *Ci*C7 lack the several C-terminal domains of the corresponding human components, and their functional link to *Ci*C3 remain unclear.

Finally, a group of 132 presumptive genes with complement control module (SRC domain) have been identified (Azumi *et al.,* 2003). In mammals, regulators for inhibiting undesirable complement activation against self cells are composed of repeat of short consensus repeats SRC domain.

In brief, three functional units may be distinguished in activating *Ci*C3 and active factors production: 1. collectins, composed of collagen and lectin-like domains which recognize miscroorganisms, associated with serine proteases that presumably activate *Ci*C3 leading to a chemotactic product; 2. *Ci*Bf and *Ci*C3, components of the vertebrate alternative pathway, and the activation product may have an opsonic activity; 3. presumptive *Ci*C6-C9 molecules forming the MAC/Perforin complex with cytolytic activity.

Cytokine-like molecules and receptors

Pleiotropic and multifunctional proinflammatory cytokines (tumour necrosis factor TNF, interleukins IL1 and IL6) play a pivotal role in innate immune responses, in cell proliferation, differentiation, apoptosis, and stimulation of the collagen synthesis in wound healing and tissue repair. In invertebrates, cytophilic humoral molecules with functional similarities to vertebrate cytokines have been reported (Beck, 1998; Beshin *et al.*, 2001; Ottaviani *et al.,* 2008).

In ascidians, cytokine-like molecules active in stimulating cell proliferation, phagocytosis and opsonisation, have been revealed by immunological and biochemical methods (see Beshin *et al.*, 2004). Genome sequencing, cDNA/EST derived from *C. intestinalis* hemocytes, and identification of the corresponding genes in genome sequences (Shida *et al.*, 2003; Terajima *et al.*, 2003) revealed the existence of *Ci*IL1 receptor, *Ci*IL17 receptor genes and an ectodysplasin/TNF-like multigene family. The presumptive IL6 gene was also identified.

Three interleukine-1-recepor-like (*Ci*IL-1R) genes present an extracellular Ig and an intracellular TIR domain which is the conserved Toll/IL-1 receptor (TIR) domain of the two families of receptors (Tong, 2005). This domain was first characterized due to the homology between the intracellular region of the mammalian IL-1Rs and the *Drosophila* TLRs. Like TLRs, IL-1R signaling pathways are key mediators of the innate immune response to bacteria (LPS), fungi, cytokines and growth factors.

In mammals, the signalling pathways mediated by TLR, IL-1R and TNFR share common components. The possibility exists that these signalling cascades are indeed functioning in *C. intestinalis* hemocytes.

Hemocyte *Ci*TNFα gene expression is challenged by LPS

Vertebrate TNFα is a component of a wide TNF family, it is a type II transmembrane protein with an extracellular homotrimeric C-terminal domain. A membrane-bound form may be cleaved, and the mature cytokine may be released as soluble form by a variety of cell types including macrophages, monocytes, granulocytes, NK-cells. TNFs are promptly expressed, and regulate inflammatory reactions by interacting with other pro-inflammatory cytokines recruiting and activating inflammatory cells (Arika *et al.*, 1990). In the *Ciona* genome, one *Ci*TNF-like and three *Ci*TNF-receptors–like genes have been identified (Terajima *et al.,* 2003).

The *C. intestinalis* TNFα-like cDNA (*Ci*TNFα) has been cloned from the pharynx excised at 4 h after LPS inoculation (Parrinello *et al.,* 2008). Comparative analysis of the deduced aminoacid sequence discloses that the cloned *Ci*TNFα-like clusters at a phylogenetic position close to vertebrate TNFα, whereas a considerable distance separates *Ci*TNFα from *Drosophila melanogaster* TNF-related Eiger isoforms and earthworm CCF. Like the vertebrate TNFα, *Ci*TNFα is constitutively expressed in the hemocytes and it is promptly (4 h) increased by LPS both in the pharynx after *in vivo* inoculation and in hemocytes challenged *in vitro*. Western blot analysis with monoclonal antibodies specific for human recombinant TNFα, showed a cell bound form (43 kDa) in hemocytes and a 15 kDa soluble form in the serum suggesting the role of this cytokine in both local and systemic responses to inflammation. Densitometry analysis of these bands confirms the gene upregulation. In particular the cell bound form is enhanced at 2 h *post* LPS injection, whereas later (4 h pi.) the soluble form appears to be enhanced in the serum in accordance with the gene expression disclosed by real-time PCR analysis of the pharynx. The anticipated expression of the cell-bound form in hemocytes challenged *in vitro* appears to be congruent with the presumed maturation process of the soluble one. Similarly to vertebrates, different cell types can secrete the same cytokine. At 4 h after LPS inoculation, amebocytes with large granules, contained in the pharynx vessels and in the connective tissue lining the tunic, as well as circulating hyaline amebocytes and granulocytes express the *Ci*TNFα mRNA as revealed by *in situ*

hybridization and immunohystochemistry with anti-human rTNFα monoclonal antibody.

Hemolymph galectins with IL1α epitopes are modulated by LPS

The direct homologue of mammalian IL-1 has not been found in the *Ciona* genome. In mammals, IL-1α and -β interleukines as well as galectins are pro-inflammatory molecules, furthermore many cytokines are bifunctional molecules containing a receptor-binding domain and an evolutionary conserved carbohydrate recognition domain (CRD) that is typical of lectins. The carbohydrate binding is requested for the cytokine biological activity (Beschin *et al.,* 2004). In this regard, IL1α and β can be considered as lectins (Cebo *et al.,* 2001, 2002) directly interacting and contributing to pathogen elimination *via* opsonization and/or leukocyte activation.

Recently, inducible galectin-like molecules with human recombinant IL-1 epitopes and opsonic properties have been found. Parrinello and colleagues (2007) have shown that Ca^{2+}-independent *Ci*galectin-like molecules, specific for D-galactose and D-galactosides, present human rIL1α epitopes. The LPS inoculation challenges a promptly (4 h) enhanced serum concentration of this lectin that has been related to the augmented serum opsonizing and hemagglutinating activities assayed with yeast and rabbit erythrocytes respectively. Furthermore, human IL-1 epitopes are involved in the opsonizing and hemagglutinating processes which are blocked by anti-human recombinant ILα monoclonal antibodies. The western blot pattern showed that, within the initial phase of the inflammatory response (4 h), several serum proteins (59, 37, 30, 23, 15 kDa) cross-reacted with the antibody suggesting an oligomerization process of the opsonin/lectin.

***Ci*IL-17 receptor**

Hemocytes express an interleukine *Ci*IL-17R gene that encode a predicted polypeptide of 769 aminoacid residues (Dehal *et al.*, 2002b; Shida *et al.*, 2003). The C-terminal half of this protein shows homology to the cytoplasmic region of mammalian IL-17R (27 % identity/40 % similarity). The central portion is rich in hydrophobic aminoacid residues presumably correspondent to a trans-membranous region, and 22 residues at the extreme N-terminal portion could be a signal peptide sequence. The N-terminal portion, probably an extracellular region, shows no homology to the corresponding region of mammalian IL-17R. IL-17 is produced by mammalian T-lymphocytes whereas its receptor is expressed in a variety of cell types such as fibroblasts and stromal cells disclosing a wide spectrum of activity. A possible ligand of *Ci*IL17R has not been predicted.

Emergence of NK cells receptors

Natural killer (NK) cells are critical in the evolution of the innate immune system being active in discriminating and killing “normal” and virus-infected, tumor or allogeneic cells. In mammals, NK cells monitor MHC class I expression on target cells by means of inhibitory NK cell receptors (NKRs) (Lanier, 2000; Vivier *et al.*, 2002). The NKRs transmit an inhibitory signal that cancels a program for cytotoxic action previously triggered by the target cell contact. NKRs belong to two distinct groups of molecules: Ig-like receptors or C-type lectin receptors including human CD94 (Boyington, 1999). C-type lectin receptors are type II transmembrane glycoproteins with a C-type lectin domain (CTLD) in the extracellular region that bind proteins in a Ca^{2+}-independent manner rather than sugars. They are known to be a hallmark of surface markers for NK cells (Biassoni *et al.,* 2007). C-type lectin superfamily includes carbohydrate-binding proteins (lectins) involved in pathogen recognition and neutralization, leukocyte trafficking, phagocytosis, antigen uptake and processing, and apoptosis. The Ca^{2+}-dependent binding of their carbohydrate recognition domain (CRD) characterized the CTDL. However, many C-type lectins included in the superfamily lack critical amino acid residues required for CRD to bind carbohydrates (Rogers and Wong, 2003). In this respect it has been hypothesized that divergent evolution, acting on the CTLD fold, has generated the lectin-like natural killer (NK) receptors that bind proteins in a Ca^{2+}-independent manner, rather than sugars.

Hemocyte *Ci*CD94 gene expression modulated by LPS is involved in phagocytosis

C. intestinalis CD94 (*Ci*CD94-1) protein containing *Ci*CTLD is a homolog of the *Botryllus schlosseri* (50/66 % identity/similarity) *Bs*CD94/NKR-P-1 molecule (Khalturin *et al.*, 2003), and human (30/46 % I/S) CD94. *Ci*CD94-1 has been cloned and sequenced, and hemocytes have been stimulated *in vitro* with LPS (Zucchetti *et al.*, 2008). Even though sequence homology situates the *Ci*CD94-1 molecule close to CD94, several features do not suggest that they are complete orthologs. *Ci*CD94-1 could be considered a C-type lectin which lacks Ca^{2+}-binding property and its carbohydrate-recognition (mannose/galactose and related sugars) capacity, and it could be located along the evolutionary line leading to the NK receptors functionally related to the human CD94 which recognizes peptides in the groove of MHC class I molecules. On the other hand, the lack of MHC genes in *C. intestinalis* genome should indicate that *Ci*CD94 is functionally similar to the mice NK cells MHC-independent CD94 (Iizuka *et al.*, 2003; McNerney *et al.*, 2005). However the involvement of *Ci*CD94 in cytotoxic mechanism has not been shown, whereas it appears to be involved in phagocytosis of polystyrene latex beads by granular amebocytes inhibited in the presence of anti-*Ci*CD94-1 specific antibodies (Zucchetti *et al.*, 2008).

No assays have been reported to disclose a *Ci*CD94-1 dependent cytotoxic activity by unilocular refractile granulocytes which are known to be cytotoxic (Parrinello, 1996; Parrinello *et al.*, 1996). The *Ci*CD94-1 as receptor on phagocytes can be up-regulated by LPS, presumably part of a

mechanism of self-nonself recognition (Zucchetti *et al.,* 2008). Interestingly, granular amebocytes, together with compartment cells, are engaged in the production of *Ci*C3-1 and express *Ci*C3a-R following inoculation of LPS.

The presence in the genome of a CD94 homolog and molecules with inhibition (*Ci*ITIM) and activation (*Ci*ITAM) motifs reasonably sustain the activity of precursors of NK cells in *Ciona*. The *Ci*CD94-1 protein has been found in about 20 % of the granular amebocytes of naïve ascidians suggesting the existence of a cell population that constitutively express *Ci*CD94-1 presumably acting as a self-nonself sentinel.

Altogether these results indicate that hemocytes are provided with a complex array of surface receptors and effector molecules, enabling them to be active in several immune responses. Alternatively, distinct hemocyte populations originated from a same hemocyte type (lymphocyte-like cells) could express distinct receptors and exert different activities following an inflammatory challenge.

Ca^{2+}-dependent hemocyte cytotoxicity seems to be *Ci*CD94-independent

Apparently, a *Ci*CD94-independent a Ca^{2+}-dependent cytotoxic activity of *C. intestinalis* hemocytes has been shown. The hemocyte type named unilocular refractile granulocytes (URG, the unique large granule occupies the cytoplasm), constitutively display cytotoxic activity and lyse rabbit erythrocytes and K562 tumour cell line. Zucchetti and colleagues (2008) did not report any URG that express *Ci*CD94-1, furthermore immunocytochemical staining of Percoll gradient separated hemocytes shows that, after a short incubation with LPS, about 80 % granular amebocytes express the *Ci*CD94-1 protein. No signs of the receptor in hyaline amebocytes, that have been reported to be phagocytes (Rowely, 1981), have been found.

The receptor involved in hemocyte cytotoxic activity remain unknown. A plaque forming cell assay with rabbit erythrocytes showed that the cell-killing mechanism requires effector-target cells contacts for challenging the release *in vitro* of soluble cytolysins. The cytolysin is $Ca2^{+}$-dependent and is inhibited by sphingomyelin and carbohydrates (unpublished). The unique granule displays phenoloxidase activity, and URGs are components of the inflammatory reaction and densely populate the tunic matrix after LPS inoculation (Parrinello, 1996; Parrinello *et al.*, 1996; Cammarata *et al.,* 2008).

Although, any evidence exists on the involvement of *Ci*CD94-1-NKR in URG mediated cytotoxicity, in line with the observation that some C-type lectins bind carbohydrates in a Ca^{2+}-independent manner (Brown and Gordon, 2001), carbohydrates could be still be potential ligands for *Ci*CD94-1, and the possibility exists that a coreceptor may be involved.

Since lectins have also been claimed as recognition molecules (Quesenberry *et al*., 2003), presumably different self-nonself recognition molecules characterize ascidian hemocytes and tissues.

***Ci*FACIT-collagen expression as a component of the inflammatory reaction**

Inflammation plays an important role in many processes, protecting organisms against pathogens, and also potentially promoting damage progression (Henson, 2005). Collagens are major structural components of extracellular matrix in tissues of vertebrates and invertebrates, involved in defence and reparative processes (Singer and Clark, 1999).

In the mammalian acute inflammatory reaction, collagen fibres bundles are organized for tissue repair during the remodelling phase (Nwomeh *et al.*, 1998), moreover, the total collagens present in normal tissue, is increased from 2- to 9-fold in the chronically inflamed tissue (Narayanan *et al.*, 1983). In this respect, activation of the innate immune response leads to the production of proinflammatory cytokines that can promote collagenolysis. Collagen degradation, and collagen fragments modulate inflammation either augmenting or suppressing interleukine production from peripheral-blood cells (Thomas *et al.*, 2007).

A family of non-fibrillar collagens, including type IX (FACIT) collagen, contain short triple helical domains, composed of three genetically distinct polypeptide-chains, interrupted by short non-helical domains (Ricard-Blum *et al.*, 2005). This collagen type does not form fibrils, and interacts with fibrillar collagen (fibril-associated collagen of cartilage extracellular matrix) and with other extracellular matrix partners (Eyre and Wu, 2005). Since type-IX collagen interacts with the cellular receptor integrins it may have an important function as mediator of cell adhesion to collagen fibrils (Käpylä *et al.,* 2004).

A *C. intestinalis* type IX-like collagen cDNA (*Ci*-typeIX-Col 1α chain), with features of fibril associated collagens formed of interrupted triple helices (FACIT) has been cloned and sequenced (Vizzini *et al.*, 2002). The involvement of this collagen in the inflammatory response has been shown by real-time PCR analysis, ISH assay and immunohistochemical methods. In addition flow cytometry with anti-*Ci*-typeIX-Col 1α chain specific antibodies, showed a prompt (1-4h) and enhanced collagen expression in the circulating hemocytes treated *in vitro* with LPS, and in epidermis cells after *in vivo* LPS inoculation (Vizzini *et al.*, 2008). Morula cells with large granules (morular feature) express this collagen revealing a fibroblast-like role.

Conclusions

The barrel-shaped sea squirt *C. intestinalis* (non-colonial ascidian, Tunicata) has its life cycle in shallow sea and ocean waters around the world. One day after an egg is fertilized, it develops into a swimming small tadpole that settles down and metamorphoses into an immobile adult. The settled adult feeds by siphoning seawater using a basket-like filter to capture particulate food and oxygen. Despite the adult humble appearance, the tadpole larva, comprised of about 2,500 cells, is provided of notochord and dorsal neural tube revealing kinship

to vertebrates. The importance of tunicates as models for the vertebrate ancestor was recognized by Kowalevsky, who first identified them as chordates, and they have played an important role in various evolution scenarios (see Gee, 1996). Due to the very simple ascidian body plan and an apparently increased body complexity of cephalochordates, ascidians were previously thought to be living form of the earliest chordate lineage.

In the December 13, 2002, an issue of the journal Science, an international consortium of researchers reported on the draft sequencing, assembly, and analysis of the *C. intestinalis* genome (Dehal et al., 2002a). From then on, sequence comparison, cDNAs, EST and gene modulation and function studies provided new insights about the evolution of key vertebrate systems including immune system and development. Recently, taking advantage of the genomes sequencing, Delsuc and colleagues (2006) proposed that tunicates and not cephalochordates are the closest living relatives of vertebrates stimulating further research on Chordates evolution. Simplified form of vertebrate gene families were typically found in *Ciona,* whereas the lancelet lineage diverged before the tunicates and vertebrates. Genome sequences show that these relationships, reflected at the molecular level, indicates how similar systems and gene sets evolved in different ways from a common ancestor. The close relationship to vertebrates along with its compact genome (about 160 million base pairs, 1/20 the size of the human), makes this sea squirt an ideal model organism for studying chordate evolution.

Sequence analysis revealed that *C. intestinalis* genome contains about 16,000 genes, about 80 % of which are also present in humans and other vertebrates. However, the total number of *Ciona* genes is only about half the number in vertebrates, presumably due to the fact that it has single copies of a large number of genes whereas they are present in multiple copies in vertebrates (Francino, 2005). Anyway, it can be retained that comparative analysis of genes and knowledge of the immunity genes evolution models are consistent with Darwin's 1871 suggestion that ascidians and vertebrates diverged from a common ancestor. Increase in the extent of genome resources as well as understanding of transcription at both transcriptosome and spliceosome levels may unveil conserved features on the coding and non coding DNA that sustain genetic stability or promote changes (Litman and Cooper, 2007) either they mutate away and disappear, or they evolve to perform other functions and advance in complexity.

Altogether adaptive and innate immune system, form a tremendous complex system to recognize non-self and provide protection from a wide variety of pathogens. The innate immune system is the most ancient of the two systems, and the adaptive immune system appeared more recently developing a high degree of complexity and interconnectivity. Many components of the innate immune system in vertebrates can be reliably traced to urochordates. For example, genome analysis reveals a number of innate immunity vertebrate-like genes, including Toll-like and virus receptor genes, complement pathways components and receptors, CD94/NK-receptor-like, lectins, TNF, IL1-R, collagens. However, pure homology seeking for vertebrate-specific immunorelevant molecules in invertebrates is of limited value, and functional screening methods may be a more promising approach. Accordingly, the expression analysis of humoral and receptorial molecules in *Ciona*'s tissues and hemocytes following a challenge indicate their involvement in *Ciona*'s inflammatory response.

There is no evidence of MHC orthologs, TCR, Igs in urochordates and agnathans, and no evidence have been reported on the hundreds of key genes involved in vertebrate adaptive immunity. There is a lack of evidence for a gradual transition from the invertebrate innate immune system to the recombinatorial immune system of higher vertebrates (Khalturin *et al.,* 2004**)** and how the adaptive immune system emerged is still obscure. In the genome of *C. intestinalis*, genes that encode molecules with membrane receptor features have been found among many members of the Ig superfamily. They contain the V, and C1-like domains typical of vertebrate antigen receptors and MHC class I and II. The human homologs of these genes segregate in a single unit of four paralogous segments on chromosomes 1q, 3q, 11p, and 21q. In these regions there are several genes involved in the adaptive immune system, and MHC paralogs with some related members. Presumably, an ancestral receptor emerged before the RAG-mediated rearrangement originated the ancestor of Ig and TCR provided with a V domain, in which V and J regions were encoded by a single exon. It has been hypothesized that the simplest ancestral receptor could be a single chain made up of V and C domain, every one linked to a transmembrane segment and a short cytoplasmic tail for signaling (Azumi *et al.*, 2003; Du Pasquier, 2004; Kasahara *et al.*, 2004). The *status* of the urochordate genes reflects perhaps a primitive pre-duplication/pre-recombination-activating gene (RAG) stage that foreshadow the pathway leading to the genesis of the T-cell receptor (TCR) and antibodies (Du Pasquier *et al.*, 2004).

The absence of antigen-presenting molecules of the MHC-linked class I and II types, raise questions on the ancestral chordates self-nonself self and allorecognition constituents. Recently novel perspectives have been proposed upon a *C. intestinalis* molecular code for individuality in the absence of MHC (Khalturin *et al.,* 2005) as well as on the involvement of complement components. Kurn and colleagues (2007) proposed that early during chordate phylogenesis the components of the complement system in addition to their role in pathogen elimination may be involved in allorecognition. So far the phylogenetic analysis of complement components indicates that gene expansion was generated by duplication events that occurred independently in the ascidian and vertebrate lineages.

Furthermore, since diverse lectin-CRD repertoires in tunicates mediate broad recognition (Quesenberry *et al.,* 2003), molecular diversity in

non self recognition could be due to the glycome code. In addition, mounting evidence indicates that invertebrate immune-type receptors may undergo somatic diversification through elaborate RNA processing mechanism (Zhang *et al.*, 2004; Kalturin *et al.*, 2005; Watson *et al.*, 2005; Sadd and Schimid-Hemplel, 2006).

Nowadays, much is known about the evolution of the immune system, but the details of its origin and ancestral genes functions remain to be elucidated (Loker *et al.,* 2004). Obviously, we are expecting insight that will come by recognizing gene product functions and identifying the function of non-coding sequences of DNA lying between the genes, which could regulate gene expression.

Finally, understanding the complex orchestration of *Ciona* gene networks is crucial to much of biomedical research, while the comparative genomic studies are helping to determine the function and modulation of the genes and other DNA regions in the human genome.

Acknowledgements

This work was supported by the MIUR PRIN-COFIN 2006-2008 and the University of Palermo research grant to NP.

References

Arika S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL6 and related molecules (IL1 and TNF) FASEB J. 4: 2860-2867, 1990.

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.

Ballarin L. Immunobiology of compound ascidians, with particular reference to *Botryllus schlosseri*: state of art. Inv. Surv. J. 5: 54-74, 2008.

Beck G. Macrokines: invertebrate cytokine-like molecules. Front. Biosci. 3: 559-569, 1998.

Ben-Shlomo R. The molecular basis of allorecognition in ascidians. BioEssay 30: 11-12, 2008.

Beschin A, Bilej M, Torreele E, De Baetselier P. On the existence of cytokines in invertebrates. Cell. Mol. Life Sci. 58: 801-814, 2001.

Beschin A, Bilej M, Lucas R, De Baetselier P. Functional convergence of invertebrate and vertebrate cytokine-like molecules based on similar lectin-like activity. In: Beshin A (ed), Progress in molecular and subcellular Biology. Invertebrate cytokines, Springer, Berlin, Heidelberg, New York, pp 145-163, 2004.

Biassoni R, Cantoni C, Marras D, Giron-Michel J, Falco M, Moretta L, *et al*. Human Natural Killer cell receptors: insights into their molecular function and structure J. Cell. Mol. Med. 7: 376-387, 2007.

Bodmer WF. Evolutionary significance of the HLA system. Nature 237: 139-145 1972

Bonura A, Vizzini A, Salerno G, Parrinello N, Longo V, Colombo P. The expression of a cloned MBL-like collectin is enhanced in the inflamed body wall of the ascidian *Ciona intestinalis* as an effect of LPS injection. Mol. Immunol. [submitted].

Boyington JC, Riaz AN, Patamawenu A, Coligan JE, Brooks AG, Sun PD. Structure of CD94 reveals a novel C-type lectin fold: implications for NK cell-associated CD94/NKG2 receptors. Immunity 10: 75-82, 1999.

Bransteitter R, Pham P, Scharff MD, Goodman MF. Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. Proc. Nat. Acad. Sci. USA 100: 4102-4107, 2003.

Brown GD, Gordon S. Immune recognition. A new receptor for beta-glucans. Nature 413: 36-37, 2001.

Buss L. The evolution of individuality. Princeton University Press, Princeton, NJ, 1987.

Cadavid LF, Powel AE, Nicotra ML, Moreno M, Buss LW. An invertebrate histocompatibility complex. Genetics 167: 357-365, 2004.

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.

Chadwick-Furman N, Rinkevich B. A complex allorecognition system in a reef-building coral: delayed responses, reversal and nontransitive hierarchies. Coral Reef 13: 57-63, 1994.

Cebo C, Dambrouck T, Maes E, Laden C, Strecker G, Michalski JC, *et al*. Recombinant human interleukins IL1 alfa, IL1 beta, IL4, IL6, and IL7 show different and specific calcium-independent carbohydrate-binding properties. J. Biol. Chem. 276: 5685-5691, 2001.

Cebo C, Vergoten G, Zanetta JP. Review. Lectin activities of cytokines: functions and putative carbohydrate-recognition domains. Bioch. Biophys. Acta 1572: 422-434, 2002.

Darwin C. The descent of man, and selection in relation to sex. Hurst, New York, 1874.

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

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, 2002a.

Dehal P, Satou Y, Azumi K, Branno M, Campbell B, Degnan B, *et. al.* The complete genome sequence of the ascidian, *Ciona intestinalis*: insights into the evolutionary origins of chordates and vertebrates. Science 298: 2157-2167, 2002b.

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

De Tomaso AW, Weissman IL. Evolution of protochordate allorecognition locus. Science 303: 977, 2004.

De Tomaso AW, Nyholm SV, Palmeri KJ, Ishizuka KJ, Ludington WB, Mitchel K, *et al.* Isolation and characterization of a protochordate

histocompatibility locus. Nature 438: 454-459, 2005.
Du Pasquier L. Innate immunity in early chordates and the appearance of adaptive immunity. C.R. Biol. 327: 591-601, 2004.
Du Pasquier L, Zucchetti I, De Santis R. Immunoglobulin superfamily receptors in protochordates: before RAG time. Immunol. Rev. 198: 233-248, 2004.
Eyre DR, Wu J-J. Collagen cross-links. Top. Curr. Chem. 247: 207-229, 2005.
Francino MP. An adaptive radiation model for the origin of new gene functions. Nat. Genet. 37: 573-578, 2005.
Fumiko Y, Yoshizaki FY, Ikawa S, Satake M, Satoh N, Nonaka M. Structure and the evolutionary implication of the triplicated complement factor B genes of a urochordate ascidian, *Ciona intestinalis*. Immunogenetics 56: 930-942, 2005.
Fujita T. Evolution of the lectin-complement pathway and its role in innate immunity. Nature Rev. Immunol. 2: 346-353, 2002.
Fujita T, Endo Y, Nonaka M. Primitive complement system-recognition and activation Mol. Immunol. 41: 103-111, 2004.
Gasparini F, Franchi N, Spolaore B, Ballarin L. Novel rhamnose-binding lectins from the colonial ascidian *Botryllus schlosseri*. Dev. Comp. Immunol. 32: 1177-1191, 2008.
Gee H. Before the backbone: views on the origin of vertebrates. Chapman & Hall (eds), London, 1996.
Henson PM. Dampening inflammation. Nat. Immunol. 6: 1179-1181, 2005.
Imler JL. Hoffmann JA. Toll receptors in innate immunity. Trends Cell Biol. 11: 304-311, 2001.
Iizuka K, Naidenko OV, Plougastel BF, Fremont DH, Yokoyama WM. Genetially linked C-type lectin-related ligands for NKRP1 family of natural killer receptor. Nat. Immunol. 4: 801-807, 2003.
Käpylä J, Jäälinoja J, Tulla M, Ylöstalo J, Nissinen L, Viitasalo T, *et al.* The fibril-associated collagen IX provides a novel mechanism for cell adhesion to cartilaginous matrix. J. Biol. Chem. 279: 51677-51687, 2004.
Kaisho T, Akira S. Critical roles of Toll-like receptors in host defense. Crit. Rev. Immunol. 20: 393-405, 2000.
Kasahara M. Genome paralogy: a new perspective on the organization and origin of the major histocompatibility complex. Curr. Top. Microbiol. Immunol. 248: 53-66, 2000.
Kasahara M, Suzuki T, Du Pasquier L. On the origin of the adaptive immune system: novel insights from invertebrates and cold-blooded vertebrates. Trends Immunol. 25: 105-111, 2004.
Khalturin K, Becker M, Rinkevich B, Bosch TCG. Urochordates and the origin of natural killer cells: Identification of a CD94/NKR-P1-related receptor in blood cells of *Botryllus*. Proc. Natl. Acad. Sci. USA 100: 622–627, 2003.
Khalturin K, Kurn U, Pinnow N, Bosch TCG. Towards a molecular code for individuality in the absence of MHC: screening for individuality variable genes in the urochordate *Ciona intestinalis*. Dev. Comp. Immunol. 29: 759-773, 2005.
Khalturin K, Pancer Z, Cooper MD, Bosch TCG. Recognition strategies in the innate immune system of ancestral chordates. Mol. Immunol. 41: 1077-1087, 2004.
Klein J. The grapes of incompatibility. Dev. Cell 10: 2-4, 2006
Kürn U, Sommer F, Bosch TC, Khalturin K. In the urochordate *Ciona intestinalis* zona pellucida domain proteins vary among individuals. Dev. Comp. Immunol. 31: 1242-1254, 2007.
Lambris JD. The third component of complement - chemistry and biology. Springer, Berlin, Heidelberg, New York, 1990.
Lanier LL. Turning On Natural Killer Cells. J. Exp. Med. 191: 1259-1262, 2000.
Litman G, Cooper MD. Why study the evolution of immunity? Nat. Immunobiol. 8: 329-331, 2007.
Loker ES, Adema CM, Zhang S-M, Kepler TB. Invertebrate immune systems - not homogeneous, not simple, not well understud. Immunol. Rev. 198: 10-24, 2004.
Manis JP, Tian M, Frederick W. Mechanism and control of class-switch recombination Trends Immunol. 23: 31-39, 2002.
McNerney ME, Guzior D, Kumar V2B4 (CD244)-CD48 interactions provide a novel MHC class I-independent system for NK-cell self-tolerance in mice. Blood 106: 1337-1340, 2005.
Medzhitov R, Janeway CA. Innate immune recognition: mechanisms and pathways Immunol. Rev. 173: 89-97, 2000.
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.
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.
Murabe N, Hoshi M. Re-examination of sibling cross-sterility in the ascidian, *Ciona intestinalis*: genetic background of the self-sterility. Zool. Sci. 19: 527-538, 2002.
Narayanan AS, Engel LD, Page RC. The effect of chronic inflammation on the composition of collagen types in human connective tissue. Coll. Relat. Res. 3: 323-334, 1983.
Nonaka M, Yoshizaki F. Evolution of the complement system Mol. Immunol. 40: 897-902, 2004a.
Nonaka M, Yoshizaki F. Primitive complement system of invertebrates. Immunol. Rev. 198: 203-215, 2004b.
Nonaka M, Miyazawa S. Evolution of the iniziating enzymes of the complement system. Genome Biol. 3, REVIEWS1001, 2002.
Nwomeh BC, Yager DR, Cohen IK. Physiology of the chronic wound. Clin. Plast. Surg. 25: 341-356, 1998.
Ottaviani E, Malagoli D, Franchini A. Invertebrate humoral factors: cytokines as mediators of cell survival. In: Beschin A, Muller WEG. (eds),

Invertebrate Cytokines and the Phylogeny of Immunity: Facts and paradoxes. Progr. Mol. Subcell. Biol., Springer-Verlag, Berlin, Heidelberg, Vol. 34, pp 1-25, 2004.

Pancer Z, Amemya CT, Ehrhardt GR, Ceitlin J, Gartland GL, Cooper MD. Somatic diversification of variable lymphocyte receptors in agnathan sea lamprey. Nature 430: 174-180, 2004.

Parrinello N. The reaction of *Ciona intestinalis* L. (Tunicata) to subcuticular erythrocyte and protein injection. Dev. Comp. Immunol. 5 (Sup.1): 105-110, 1981.

Parrinello N. Cytotoxic activity of tunicate hemocytes. in Rinkevich B, Müller WEG (eds), Invertebrate immunology, Springer-Verlag, Berlin. pp 190-217, 1996.

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, 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, 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, Arizza V, Cammarata M, Giaramita FT, Pergolizzi M, Vazzana M, *et al.* Inducible lectins with galectin properties and human IL1α epitopes opsonize yeast during the inflammatory response of the ascidian *Ciona intestinalis*. Cell Tissue Res. 329: 379-390, 2007.

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 (CiTNFα) gene during the LPS-induced inflammatory response Cell and Tissue Res. 334: 305-317, 2008.

Pinto MR, De Santis R, Marino R, Usui N. Specific induction of self discrimination by follicle cells in *Ciona intestinalis* oocytes. Dev. Growth Differ. 37: 287-291, 1995.

Pinto MR, Chinnici C, Kimura Y, Melillo D, Marino R, Spruce LA, et al. CiC3-1a-Mediated chemotaxis in the deuterostome invertebrate *Ciona intestinalis* (Urochordata). J. Immunol. 171: 5521-5528, 2003.

Quesenberry MS, Ahmed H, Elola MT, O'Leary N, Vasta GR. Diverse lectin repertoires in tunicates mediate broad recognition and effector innate immune responses. Integr. Comp. Biol. 43: 323-330, 2003.

Ricard-Blum S, Ruggiero F. The collagen superfamily: from the extracellular matrix to the cell membrane. Pathol. Biol. 53: 430-442, 2005.

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.

Rogers S, Wong SYC. C-type lectin and lectin-like receptors in the immune system. In Wong SYC, Arsequell G. (eds), Immunobiology of Carbohydrates, Landes Bioscience/ Eurekah.com, Georgetown, Texas, USA and Kluwer Academic/Plenum Publishers, New York, USA, pp 101-118, 2003.

Rosati F, De Santis R. Studies on fertilization in the Ascidians. I. Self-sterility and specific recognition between gametes of *Ciona intestinalis*. Exp. Cell Res. 112: 111-119, 1978.

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

Sadd BM, Schmid-Hempel P. Insect immunity shows specificity in protection upon secondary pathogen exposure. Curr. Biol. 16: 1206-1210, 2006.

Satoh N. The ascidian tadpole larva: comparative molecular development and genomics. Nat. 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.

Seya T, Hirano A, Matsumoto M, Nomura M, Ueda S. Human membrane cofactor protein (MCP, CD46): multiple isoforms and functions. Int J. Biochem. Cell Biol. 31: 1255-1260, 1999.

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. Comm. 302: 207-218, 2003.

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

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

Terai I, Kobayashi K, Matsushita M, Fujita T, Matsuno K. α2-Macroglobulin binds to and inhibits mannose-binding protein-associated serine protease. Int. Immunol. 7: 1579-1584, 1995.

Terajima D, Yamada S, Uchino R, Ikawa S, Ikeda M, Shida K., Arai Y., Wang H.G. *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.

Thomas AH, Edelman ER, Stultz CM. Collagen Fragments Modulate Innate Immunity Exp. Biol. Med. 232: 406-411, 2007.

Tong L. Toll/interleukin-1 receptors and innate immunity. In: Waksman G, Caparon MG, Hultgren S (eds), Structural basis of bacterial pathogenesis, ASM Press, Washington, DC, pp 241-263, 2005.

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

Vivier E, Tomasello E, Paul P. Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition? Curr. Opin. Immunol. 14: 306-311, 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.

Wakoh T, Ikeda M, Uchino R, Azumi K, Nonaka M, Kohara Y, *et al.* Identification of transcripts expressed preferentially in hemocyte of *Ciona intestinalis* that can be used as molecular markers. DNA Res. 11: 345-352, 2004

Watson FL, Püttmann-Holgado R, Thomas F, Lamar DL, Hughes M, Kondo M, *et al.* Extensive diversity of ig-superfamily proteins in the immune system of insects. Science 309: 1874-1878, 2005.

Yokobori Y, Watanabe S, Oshima T. Mitochondrial genome of *Ciona savignyi* (Urochordata, Ascidiacea, Enterogona): comparison of gene arrangement and tRNA genes with Halocynthia roretzi mitochondrial genome, J. Mol. Evol. 57: 574-587, 2003.

Yoshizaki FY, Ikawa S, Satake M, Satoh N, Nonaka M. Structure and the evolutionary implication of the triplicated complement factor B genes of a urochordate ascidian, *Ciona intestinalis*. Immunogenetics 56: 930-942, 2005.

Yu C, Dong M, Wu X, Li S, Huang S, Su J, *et al*. Genese "waiting" for recruitment by the adaptive immune system: the insights from *Amphioxus*. J. Immunol. 174: 3493-3500, 2005.

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

Zhang SM, Adema CM, Kepler TB, Loker ES. Diversification of Ig superfamily genes in an invertebrate. Science 305: 251-254, 2004.

Zhong J, Kyriakis JM. Dissection of a signaling pathway by which pathogen-associated molecular patterns recruit the jnk and p38 mapks and trigger cytokine release. J. Biol. Chem. 282: 24246-24254, 2007.

Zucchetti I, Marino R, Pinto MR, Lambris JD, Du Pasquier, L, De Santis R. CiCD94-1, an ascidian multipurpose C-type lectin-like receptor expressed in *Ciona intestinalis* hemocytes and larval neural structures Differentiation 76: 267-282, 2008.