

Review

## Recognition strategies in the innate immune system of ancestral chordates

Konstantin Khalturin<sup>a,\*</sup>, Zeev Panzer<sup>b</sup>, Max D. Cooper<sup>b,1</sup>, Thomas C.G. Bosch<sup>a</sup>

<sup>a</sup> Zoological Institute, Christian-Albrechts-University, Olshausenstrasse 40, 24098 Kiel, Germany

<sup>b</sup> Howard Hughes Medical Institute, The University of Alabama at Birmingham, 378 Wallace Tumor Institute, 1824 Sixth Avenue South, Birmingham, AL 35294-3300, USA

### Abstract

Many components of the innate immune system in vertebrates can be reliably traced to urochordates and successful strategies for the detection and elimination of pathogens are present at that level of animal evolution, but the issue of where and how the adaptive immune system emerged is still obscure. There is a paucity of evidence for a gradual transition from the innate immune system of invertebrates to the recombinatorial immune system of higher vertebrates. None of the classical elements of MHC based transplantation immunity (MHC, TCR) or humoral immunity (Ig) have been found in urochordates or Agnathans. Nevertheless there is abundant evidence for adaptive immune responses in the agnathans. This remarkable paradox raises a number of questions. How do these ancestral chordates discriminate between the constituents of the external world and the constituents of “self”? Are these strategies universal within the animal kingdom and among chordates, or are different strategies used by representatives of the different taxonomic groups? The current state of our knowledge indicates that the immune system of lower chordates is very different from that of cartilaginous fishes. Pure homology hunting for vertebrate-specific immuno-relevant molecules in invertebrates is therefore of limited value. A more promising approach may involve unbiased functional screening methods. To understand better the evolution of adaptive immune systems, more comparative data from jawless vertebrates (lamprey or hagfish) and a representative of Acrania (e.g. *Amphioxus*) are clearly needed.

© 2004 Elsevier Ltd. All rights reserved.

**Keywords:** Urochordates; Agnatha; MHC; Complement; Innate immunity; Natural killer cells

### 1. Immune system in urochordates

Urochordates are dimorphic marine organisms. In their life cycle a nonfeeding, short living pelagic larva is followed by the sessile filter-feeding adult ascidian. While urochordates are invertebrates, they belong to chordates because of the presence of a notochord in the larval stage (Berrill, 1955). Currently existing urochordates and vertebrates diverged in evolution around 570 million years ago but their monophyletic origin suggests the presence of common mechanisms of immunity. Urochordates are particularly interesting because of their phylogenetic position at the root of vertebrate evolution and the availability of a large body of

molecular data. A large EST project (Satou et al., 2002) and the draft genome sequence (Dehal et al., 2002) of one urochordate—*Ciona intestinalis*: allow quick, extensive “in silico” searches for immunorelevant molecules. At the moment *Ciona* is the only animal of the deuterostome lineage outside of mammals whose genome has been sequenced. This makes *Ciona* and other urochordates attractive models in comparative and evolutionary immunology. Comparative studies of urochordate and vertebrate immune systems may shed light on the evolution of the innate immune system in deuterostomes, the emergence of adaptive immunity and the mechanisms of allorecognition.

#### 1.1. Innate immunity in urochordates

The search for predecessors of the innate immune system has revealed that urochordates share many components of innate immunity with vertebrates. Urochordates, at least

\* Corresponding author. Tel.: +49 431 880 4169; fax: +49 431 880 4747.  
E-mail addresses: [kkhalturin@zoologie.uni-kiel.de](mailto:kkhalturin@zoologie.uni-kiel.de) (K. Khalturin),  
[max.cooper@ccc.uab.edu](mailto:max.cooper@ccc.uab.edu) (M.D. Cooper), [tbosch@zoologie.uni-kiel.de](mailto:tbosch@zoologie.uni-kiel.de) (T.C.G. Bosch)

<sup>1</sup> Tel.: +1 205 975 7203; fax: +1 205 975 7218.

*Ciona intestinalis* (Azumi et al., 2003) and *Boltenia villosa* (Davidson and Swalla, 2002), possess the Toll-like receptors (TLRs) and corresponding signal transduction cascades. This fact is not surprising given the great degree of functional conservation of this cascade and receptors even between protozoans and vertebrates (see reviews Kimbrell and Beutler, 2001; Hoffmann, 2003). Moreover, two TLR-like molecules as well as MyD88 and perleppin-like protease have been identified recently in the basal metazoan *Hydra magnipapillata* (Zill and Bosch, personal communications). These findings support the view that the Toll/TLR receptors and the corresponding signal transduction cascades are ancient inventions that may be typical for all Eumetazoa.

Urochordates possess a complement system with both activation and lytic pathways. Many soluble factors and receptors of the complement system have been systematically cloned from urochordate species (see reviews Nonaka and Miyazawa, 2002; Fujita, 2002) or identified in the *Ciona* genome and ESTs (Azumi et al., 2003). The activation pathway is of the lectin type and consists of mannose-binding lectins (MBLs), mannose associated serine proteases (MASPs), C3 and corresponding CR3/CR4 receptors present on macrophage-like cells (Kenjo et al., 2001; Sekine et al., 2001; Marino et al., 2002; Endo et al., 2003; for review see Fujita, 2002). The lytic pathway consists of a large set of soluble proteins in the urochordate hemolymph, which have MAC/Perforin domains and an organisation similar to that of the terminal vertebrate complement components, C6/C7/C8/C9 (Azumi et al., 2003). Interestingly, the complement system in urochordates appears to be much more advanced than previously thought. The notion that “urochordates are like vertebrates, but only simpler” (Dehal et al., 2002) is certainly not applicable to the urochordate complement system. The number of genes encoding putative proteins of complement system are much larger in number in ascidians than in mammals (Azumi et al., 2003). For poorly understood reasons, this group of molecules has expanded considerably in the urochordate lineage.

## 2. The question of adaptive immunity in urochordates

Although many components of the innate immune system in vertebrates can be reliably traced to urochordates and successful strategies for the detection and elimination of pathogens are present at that level of animal evolution, the issue of where the adaptive immune system emerged is much more complicated. There is a paucity of evidence for a gradual transition from the innate immune system of invertebrates to the adaptive immune system of higher vertebrates. None of the elements of MHC based transplantation immunity (MHC, TCR) have been found in urochordates. Moreover, lacking the mechanisms of recombination for antigen recognition units, the urochordates are unable to produce antigen-specific antibodies. Accordingly, immunoglobulin genes have not been

found in the *Ciona* genome. This places the urochordates near the border which separates animals with both adaptive and innate immune systems from those with only innate immune system (see Fig. 1).

Despite their apparent “simplicity” and lack of obvious predecessors of an adaptive immune system, urochordates have several well documented forms of allorecognition. This remarkable paradox raises a number of questions. How do urochordates discriminate between the constituents of the external world and the constituents of “self”? Are these strategies universal within the animal kingdom and among chordates, or are different strategies used by representatives of the different taxonomic groups?

## 3. Allorecognition in urochordates

Colonial urochordates, in contrast to vertebrates, undergo transplantation reactions naturally. This led to the proposal that they contain the ancestral molecular machinery for vertebrate allorecognition (Burnet, 1971). Two types of allogeneic recognition are observed in urochordates. One is “colony specificity”, which has been observed in compound ascidians (see review of Saito et al., 1994; Magor et al., 1999; Hirose, 2003), and the other is “self-sterility” (block of self fertilization) which has been reported in colonial ascidians like *Botryllids* (see reviews by Saito et al., 1994), solitary ascidians such as *Halocynthia roretzi* (see review Sawada, 2002), and *Ciona intestinalis* (Murabe and Hoshi, 2002). The latter phenomenon has been studied extensively at the molecular level in *Halocynthia roretzi* and *Ciona intestinalis*.

## 4. Colony specificity

Colony specificity was first observed by Bancroft, who found that when two pieces of a single colony of *Botryllus schlosseri* come into contact with each other, they easily fuse to form a single colony (Bancroft, 1903). Two pieces of different origin, however, never fuse after grafting. Bancroft’s work did not attract much attention until Oka and Watanabe (1957) analyzed this phenomenon in the Japanese botryllid ascidian *Botryllus primigenus*. These investigators showed that this phenomenon was a type of self-nonsel self recognition that is under genetic control. Pairs of colonies that meet naturally or that are placed in contact under laboratory conditions either fuse their contacting peripheral ampullae to form a vascular parabiont (cytotoxic chimeric; Rinkevich and Weissman, 1987), or they develop cytotoxic lesions in the contact zone (see Fig. 2A; reviewed in Taneda et al., 1985; Weissman et al., 1990; Rinkevich, 1992). Within 2–5 h after the first contact various types of blood cells accumulate at the tips of interacting ampullae. Within 48 h after contact in case of two compatible colonies, the blood vessels will fuse and a chimeric organism will be formed. When the colonies are not compatible, a lesion area develops between them (Fig. 2A) that

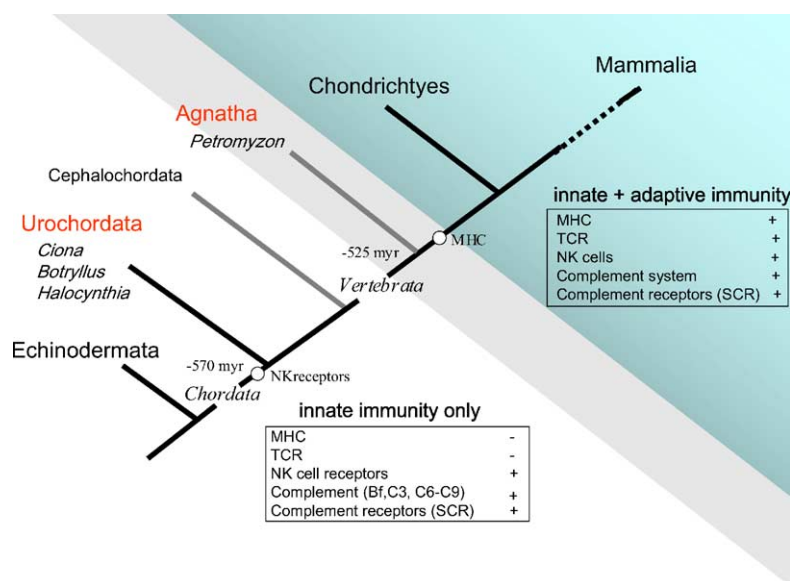


Fig. 1. Urochordates separated from the predecessors of a vertebrate lineage around 570 million years ago and share many features of the innate immune system with vertebrates. However, they do not possess the molecular machinery of the adaptive immune system found in jawed vertebrates (Gnathostomata). The emergence of a recombinatorial adaptive immune system featuring immunoglobulins, TCR and MHC took place sometime during the transition from Agnatha to Gnatostomata. A search for these cardinal elements of a recombinatorial immune systems in lymphocyte-like cells of the sea lamprey, *Petromyzon marinus*, has not been fruitful, hence the agnathans remain a grey zone in the evolution of adaptive immunity in vertebrates. Blue background, phylogenetic groups with both adaptive and innate immune system.

includes necrosis at all points of rejection due to the activation of a prophenoloxidase system by morula cells (Rinkevich et al., 1998). This leads to the physical isolation of the two colonies from each other through the selective elimination of cells within the contact zone.

Genetic experiments in *Botryllus primigenus*, in which large sets of rejecting colonies were crossed and their progeny tested for the ability to fuse or reject, indicated that allorecognition is genetically controlled by a single highly polymorphic gene locus containing multiple codominantly expressed alleles (Oka and Watanabe, 1960; reviewed by Saito et al., 1994). Publication of the hypothesis that the *Botryllus* fusibility/histocompatibility (FU/HC) locus may represent an ancestral form of vertebrate MHC (Scofield et al., 1982) attracted widespread attention, and *Botryllus* became one of the most widely studied invertebrate models for studies designed to unravel the evolutionary origin of MHC. Using a genetic approach based on arbitrary fragment length polymorphism (AFLP) analysis, the putative Fu/HC locus was first delineated to a 5 cM region and, more recently, to an area of around 800 kb in the *Botryllus* genome (De Tomaso et al., 1998; De Tomaso and Weissman, 2003). However, 20 years after the Scofield paper (1982), the molecular basis of *Botryllus* allorecognition is still largely unknown. No MHC related molecules and no good candidates for Fu/HC receptors or ligands are reported as yet. We also note that in order to avoid MHC based allograft rejection in vertebrates both haplotypes must be identical between the two individuals. In *Botryllus*, in contrast, the genetic constraints appear to be different: *Botryllus* colonies will fuse when they share one allele of the presumable FU/HC

locus out of many different co-expressed alleles. This, at least phenomenologically, resembles the mechanism for self pollination blockage in a Brassicacea (S-locus) more closely than MHC based transplantation immunity in vertebrates.

In addition to observations of natural transplantation there is a large body of experimental data on allotransplantation in urochordates. Transplantation experiments of allogeneic tissue in solitary ascidians, particularly *Styela plicata* (Raftos, 1991, 1990), indicate that individuals can recognize and reject allogeneic tunic grafts as nonself. In this allorecognition reaction, lymphocyte-like cells may detect nonself determinants on allogeneic cells (Raftos et al., 1987b). Although specific immune memory is proposed to exist in this species (Raftos et al., 1987a) there is as yet no convincing experimental data that support this idea. The analysis of histocompatibility in solitary ascidians has revealed a cell-mediated immune system with functional characteristics that are similar to those possessed by vertebrates (Kelly et al., 1993). Moreover, in another solitary ascidian, *Halocynthia roretzi*, an interesting phenomenon called “contact reaction” was reported (Fuke, 1980; Fuke and Numakunai, 1982). When a *Halocynthia* blood cell contacts an allogeneic blood cell, both of them undergo rapid lysis within 90 seconds after initial contact. Most of the blood cells are involved in this reaction for which humoral components play no role. The genetic background for this “contact reaction” is based on polymorphic alleles at one or two histocompatibility loci (Fuke and Nakamura, 1985). The phenomenon of “contact reaction” resembles the natural killer cell mediated lysis in jawed vertebrates. In several *Botryllid* species, injection of allogeneic blood plasma into the blood vessels of the colony causes rejection responses

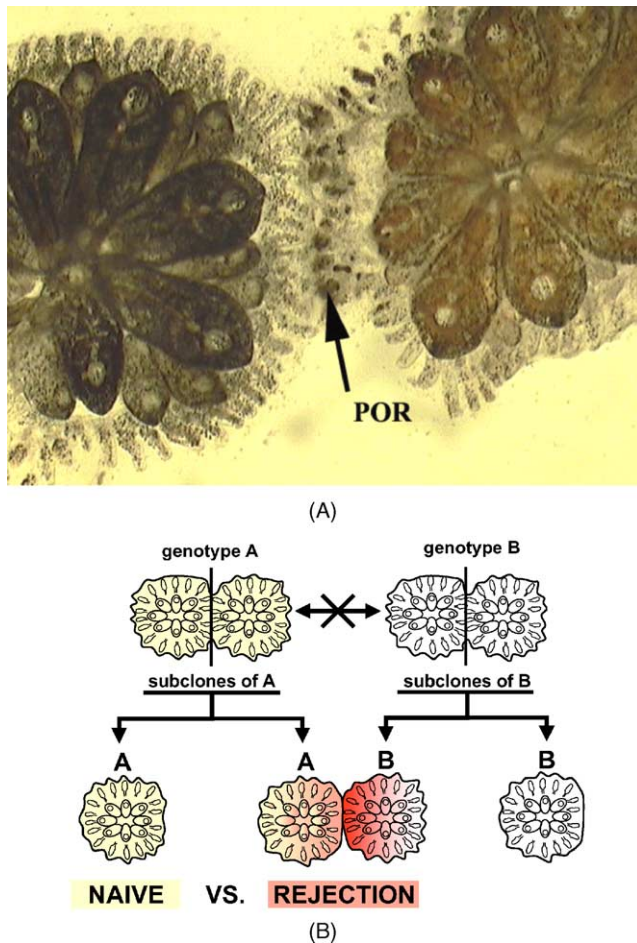


Fig. 2. Analysing allogeneic recognition in *Botryllus schlosseri* (A) two rejecting *Botryllus* colonies 48 h after the first contact. An area of necrosis is visible between the two colonies and the zooids of the right colony have retracted from the area of contact. POR—points of rejection. (B) The unbiased screening set-up for the detection of the genes which are differentially expressed in response to the allogeneic contacts in *Botryllus*. RNA was extracted from naïve and rejecting colonies and the sets of expressed genes were compared by differential display PCR (DD-PCR).

(Taneda and Watanabe, 1982). In addition, allospecific responses of hemocytes can be induced by incubating them in the blood plasma of an incompatible colony (Ballarin et al., 1995, 1998). Thus, soluble components of blood plasma in *Botryllus* are the determinants of self/nonself recognition. One may speculate that this phenomenon involves participation of the complement components, the genes for which are present in unexpectedly large number in urochordates (Azumi et al., 2003).

## 5. Self-incompatibility

Many species of solitary and colonial urochordates are hermaphrodites, and they release sperm and eggs simultaneously. They therefore have to evade self-fertilization and some species are strictly self-sterile. In *Halocynthia roretzi*, *Ciona intestinalis* and *Botryllus schlosseri*, study of the ge-

netic background of self-incompatibility has yielded several intriguing findings at the molecular level. There is increasing evidence indicating that self/nonself discrimination during natural or experimental transplantation in ascidians employs the same genetic machinery as that used to block self fertilization.

In deuterostomes, fertilization is a precisely controlled process wherein the sperm binds to the vitelline coat (*zona pellucida*) of the egg in a species-specific manner and undergoes an acrosome reaction. Lytic agents, called sperm lysins, are released during the acrosome reaction to digest the vitellin coat and allow sperm penetration and fertilization. The sperm lysins in deuterostomes are usually proteases. Three types of lysins, acrosin, spermosin, and chymotrypsin like proteases, have been found to be essential for fertilization in ascidians (reviewed by Sawada, 2002). However, it is not the lysins in *Halocynthia roretzi*, but rather the 26S-like proteasome, which is released from sperm during the acrosome reaction, that is responsible for lysis of the vitelline coat. The main lytic target in the vitelline coat is HrVC70, a 70 kDa type I transmembrane protein with 12 EGF-like repeats at the N-terminus and *zona pellucida* domain near the transmembrane region. Before proteasomal degradation, HrVC70 is polyubiquitinated by the ubiquitin-conjugating enzyme system of the sperm (Sakai et al., 2003). In ascidians which possess self incompatibility, only a heterologous sperm is able to bind to the vitelline coat and the acrosome reaction proceeds only if the sperm recognizes the vitelline coat as nonself. HrVC70 has been shown to be the receptor in the vitelline coat which binds sperm (Sawada et al., 2002). Therefore, it would be of a great interest to determine whether variability exists in the amino acid sequence of HrVC70 among *Halocynthia* individuals. Another interesting point is that in *H. roretzi* the same monoclonal antibodies that prevent lysis between hemocytes from different individuals (“contact reaction”) also prevent fertilization (Arai et al., 2001). The antigens recognized by these antibodies are present on the surface of hemocytes and the vitelline coat of the eggs. This is the first direct evidence that cell surface determinants responsible for allorecognition in urochordates may also be responsible for the block of self-fertilization (Arai et al., 2001). It has not yet been determined whether or not these antibodies recognize HrVC70. We also note that within the 800 Kb of the putative *Botryllus* FU/HC locus, there are genes coding for ubiquitin-conjugation enzymes as well as a gene with a similarity to Notch receptor-like protein containing multiple EGF domains (De Tomaso and Weissman, 2003). This could be pure coincidence, but it is also possible that molecules with multiple EGF domains may play a role in self/non-self recognition in ascidians.

## 6. Non-model organisms and unbiased screening approaches

The common ancestors of vertebrates and urochordates diverged around 570 million years ago. Therefore, molecules

with common ancestry may have evolved to the degree where their homology is no longer detectable. Alternatively, the two groups may have developed different strategies for immune responses and allorecognition (Klein, 1998). Even given the draft genome of *Ciona intestinalis*, our knowledge of the composition of the immune system in Urochordata is far from being complete and comprehensive. *Ciona* is a highly specialized and diverged member of this taxon, and its system of immunity may not represent the general situation within the class. However, the current state of our knowledge already indicates that the immune system of lower chordates is very different even from that of cartilaginous fishes (Fig. 1). Pure homology hunting for vertebrate-specific immuno-relevant molecules in invertebrates is therefore of limited value. A more promising approach is the use of unbiased (functional) screening methods (Khalturin et al., 2003) and a third comparator in the analysis, such as a jawless vertebrate (lamprey or hagfish, see Mayer et al., 2002a; Uinuk-Ool et al., 2002) or a representative of Acrania (e.g. *Amphioxus*).

## 7. The discovery of a CD94/NKR-P1 homolog in *Botryllus*

To gain insight into the molecular changes associated with allorecognition in *Botryllus*, we have developed a DD-PCR based screening strategy that makes use of the remarkable allojection process of *Botryllus schlosseri* and the “colony allorecognition assay” (Khalturin et al., 2003). The strategy is based on a comparative analysis of gene expression profiles between naive and rejecting colonies. This unbiased screening method does not require information about the system studied (Fig. 2). One of the *Botryllus* genes whose expression we found to be altered in response to allogeneic contacts is a homolog of CD94/NKR-P1 receptor termed bsCD94-1 (Khalturin et al., 2003). RT-PCR analysis indicated that this gene is expressed in the blood cells of *Botryllus*, and its transcription level is down-regulated after allogeneic contact. Immunostaining with a polyclonal antibody produced against the C-type lectin domain (CTLD) of bsCD94-1 indicates its presence on the surface of blood cells. Because the degree of structural conservation between the *Botryllus* bsCD94-1 protein and the vertebrate orthologs implies functional conservation, we propose that *Botryllus schlosseri* blood cells carrying this receptor are mediators of allorecognition. Since key molecules of vertebrate NK cells are present in a subpopulation of *Botryllus* blood cells, these cells may be considered as ancestral NK cells. NK cells thus may have evolved much earlier than previously thought.

The presence of a close homolog of bsCD94-1 in the *Ciona intestinalis* genome is supported by the identification of more than 100 corresponding ESTs (see *Ciona* EST database in Kyoto at <http://ghost.zool.kyoto-u.ac.jp/indexr1.html>; or the *Ciona* genome database at <http://genome.jgi-psf.org/ciona4/ciona4.home.html>). The consensus cDNA en-

codes a protein of 280 amino acids with a molecular mass of 31.2 kDa (GeneBank accession AY505423). This protein has 45% identity to its *Botryllus* homolog and has the characteristic features of a type II transmembrane protein (Fig. 3A). Interestingly, the *Ciona* protein has even higher homology to mammalian CD94 and NKR-P1 than does the *Botryllus* homolog. According to a BLASTP search at the NCBI database, the closest known relatives of ciCD94-1, other than *Botryllus* bsCD94-1, are CD94-B receptors from *Macaca mulatta* (AAF74528, E = 1e-04) and man (CAA03845, E = 1e-04) with 30 and 29% amino acid identity, respectively, within the C-type lectin domain (CTLD). The next closest group of receptors identified by BLASTP includes the mast cell function-associated antigens (MAFA, NP\_058666, E = 7e-04). These are inhibitory C-type lectin receptors that are expressed on NK cells and activated CD8<sup>+</sup> T cells. The draft genome sequence indicates that three assembled genomic regions of different size called “scaffolds” comprise the ciCD94-1 gene (see Fig. 3C). Scaffold 450 [59 kb] contains the whole ciCD94-1 gene (gene model ci0100135570), a portion of the 5' flanking region, and the downstream genomic region. Scaffold 196 [182 kb] contains the 203 bp of the first exon including 5' UTR and the adjacent upstream genomic region. Scaffold 6085 is small (1153 bp) and contains 117 bp of the first exon of ciCD94 and its 5' flanking region. According to the gene structure prediction in Scaffold 450, ciCD94 consists of six exons and resembles the exon/intron structure of the CD94 gene in mammals (Fig. 3B). At the moment it is difficult to conclude whether or not scaffolds 450 and 196 overlap and therefore represent one contiguous genomic region. Our preliminary data suggest that ciCD94 is present in at least two copies in the *Ciona* genome (Khalturin and Bosch, unpublished). Hence, there might be two non-overlapping scaffolds, each of which contains a ciCD94-1 gene copy. The genes for NK receptors with CTLDs are organized in a cluster spanning an area of around 4 Mbp on chromosome 12 in humans, chromosome 6 in mice, and chromosome 4 in rats. *Ciona* scaffolds 450 and 196 do not contain another gene with a C-type lectin domain other than ciCD94. However, since these scaffolds are relatively short (59 and 182 Kb), the issue of an ancestral NK cluster in *Ciona* and other urochordates will only be clarified when larger genome maps are available. Interestingly, seven of the 27 genes present in scaffolds 450 and 196 are expressed exclusively in gonads and/or blood cells (see Fig. 3C). Neither the ciCD94-1 protein function nor localization in *Ciona* is known. However, taking into consideration the high structural similarity to the family of NK receptors, one may speculate that ciCD94-1 participates in the NK cell-like cytotoxic activity described previously for *Ciona* blood cells (Parrinello, 1996).

The presence of a CD94-like protein in the *Ciona* genome in the absence of MHC genes raises the question about possible ligands. Recently, ligands other than MHC were found for the NKR-P1 family of NK receptors. Clrg and Clrb, which were previously referred to as orphan lectin-like NK cell

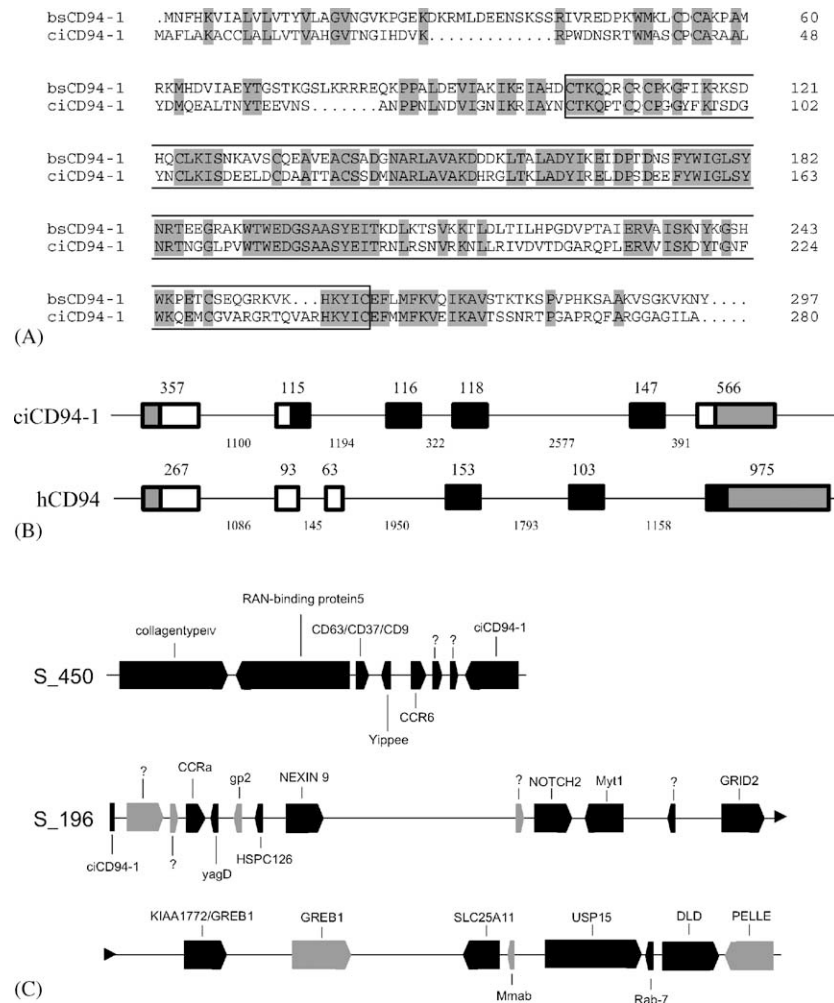


Fig. 3. A gene homologous to bsCD94-1 and to mammalian natural killer cell receptor CD94 is present in the *Ciona* genome. (A) Amino acid alignment of CD94-like proteins from *Botryllus schlosseri* and *Ciona intestinalis*. The C-type lectin domain (CTLD) is boxed and identical amino acids are shaded. (B) Comparative exon/intron structure of ciCD94-1 (GenBank accession AY505423) and human CD94. The exons coding for CTLD are in black. Sizes of exons and introns are shown above and under the scheme, respectively. (C) Regions of the *Ciona* genome (Scaffold\_450 and Scaffold\_196) which contain CD94-like gene. The genes within scaffolds were predicted by GeneScan program and the names are given according to the closest BLAST hits. ?—putative genes coding for the proteins without any considerable homology to known proteins; grey boxes—genes with the transcripts found only in blood cells and/or gonads.

receptors, are specific ligands for NKR-P1f and NKR-P1d (Iizuka et al., 2003). Interestingly, the genes for the NKR-P1 receptors and their ligands are colocalized in the adjacent areas in the genome within the NK cluster. Moreover, NKR-P1 and Clr are situated in a region with strong recombination suppression, in this respect being similar to the plant self-incompatibility loci. The finding of Iizuka et al., therefore, expands the “missing self” concept from involving exclusively MHC class I molecules to include lectin-like ligands. That opens a new perspective for the application of the NK cell based “missing self” concept to animals which lack MHC genes. The *Ciona intestinalis* genome contains many genes with similarity to C-type lectins (Azumi et al., 2003) and several homologs of mammalian NK receptors other than CD94/NKR-P1. Their potential role in the immune system of urochordates will be discussed elsewhere (Khalturin and Bosch, in preparation).

## 8. Conclusions about urochordate immunity

In mammals, the immune system distinguishes self from nonself by three mechanisms: (i) molecules or metabolites typical for certain groups of pathogens are distinguished by pattern recognition molecules (e.g., toll-like receptors for bacterial surface antigens), (ii) T-cell receptors (TCR) that recognize non-self determinants presented by MHC and (iii) CTLD receptors and KIRs of NK cells that are used to screen for the presence of “self” determinates. Cells lacking these “self” markers are eliminated. Two of these three mechanisms which play a role in mammalian transplantation immunity resemble the allorecognition reactions in urochordates, at least in a phenomenological way. First, in MHC mediated recognition by TCR, cells are eliminated if “nonself” determinants are present on their surface. Second, cells missing “self” determinants are eliminated due to the lytic activity of NK cells.

There are two current views concerning the mechanism of allorecognition in urochordates. One view holds that allorecognition functions as MHC recognition. This would demand the detection of “nonself” with the consequence of self tolerance maintenance via clonal elimination of self-reactive cells. The alternative view assumes that allorecognition is based on missing self. Observations of self-incompatibility in urochordates and a large body of data indicating that this self-incompatibility is based on the same mechanism as allorecognition supports the latter view. Therefore, allorecognition and self-incompatibility in urochordates probably is based on the detection of “missing self” markers. The urochordates thus appear to share mechanisms of innate immunity with the vertebrates, but they do not yet have the elaborate molecular machinery of adaptive immunity.

### 9. Search for the rudiments of adaptive immunity in jawless vertebrates

An adaptive immune system based on recombinatorial genetic elements has been shown to exist in all jawed vertebrates (Gnathostomes) including the cartilaginous fish (Chondrichthians), sharks, skates, and rays (see Fig. 1). The hallmarks of this type of adaptive immunity are lymphocytes and their ability to rearrange variable (*V*), diversity (*D*) and joining (*J*) gene segments to generate a vast repertoire of B cell and T cell receptors that can recognize virtually any potential antigen. The central molecular components of adaptive immunity in jawed vertebrates include the polymorphic major histocompatibility complex (MHC) class I and II receptors, T cell receptors (TCR), immunoglobulins (Igs), and the recombination activating genes (RAG1, RAG2) that initiate *V(D)J* recombination. A thymus, hematopoietic tissue-based lymphopoiesis, and compartmentalized secondary lymphoid tissues are also conserved features of the immune system in jawed vertebrates (Flajnik and Kasahara, 2001; Flajnik, 2002). In invertebrates convincing evidence for the presence of the pivotal genes involved in combinatorial adaptive immunity has not been forthcoming (Laird and De Tomaso, 2000; Dehal et al., 2002; Kaufman, 2002). The cardinal elements of this type of adaptive immune system therefore probably emerged over the 40–50 million years period during which jawless and jawed vertebrates diverged. This rather sudden appearance of adaptive immunity over a relatively short time period led to the idea of a ‘Big Bang’ origin of a combinatorial immune system (Schluter et al., 1999).

The earliest known vertebrates are the craniates. These jawless fish with a skull (Agnatha), were derived from a common Cephalochordate ancestor some 550 million years ago. Then about 500–510 million years ago the jawed cartilaginous fish diverged from the jawless ostracoderms. The armored shell and skin covered fish became extinct some 390 million years ago (Forey and Janvier, 1993; Kumar and Hedges, 1998) leaving the lampreys (Petromyzontiformes) and the hagfishes (Myxiniiformes) as the only remaining

species representing the early agnathan radiation. These jawless vertebrates are thus the closest living relatives of jawed vertebrates, and hence the best available candidates to search for the earliest recognizable elements of an adaptive immune system. Of the two remaining families of jawless vertebrates, the lamprey is considered the most recently evolved. Lampreys have also been characterized better than hagfish in their capacity to mount an immune response.

### 10. Lymphocyte-like cells in the lamprey

Blood cell types formed in hematopoietic tissues of larval and adult lampreys include erythrocytes, thrombocytes, granulocytes, neutrophils, monocytes, macrophages. Lampreys also have lymphoid cells similar to small and medium size lymphocytes, inflammatory lymphoblast cells and mature and immature plasma cells that are found in the jawed vertebrates (Finstad and Good, 1964; Finstad et al., 1964; Perey et al., 1968; Piavis and Hiatt, 1971; Kilarski and Plytycz, 1981; Zapata et al., 1981; Fujii, 1981, 1982; Fujii and Hayakawa, 1983; Ardavin and Zapata, 1987). The lymphoid cells in the sea lamprey are of special interest because they have been described as the cellular cornerstone of adaptive immune responses in these species (Good et al., 1989). They are indistinguishable from mammalian lymphocytes in their general morphology, ultrastructural features, cellular density and light scatter characteristics (Cooper, 1971; Mayer et al., 2002a). Moreover, lymphocyte-like cells in the lamprey have been shown to express PU.1/Spi-B, a transcriptional regulator of immune system gene. Spi-B is a member of a family of transcription factors that is involved in differentiation of jawed vertebrates lymphocytes (Shintani et al., 2000; Anderson et al., 2001). Ikaros-related transcription factors that are critical for the development of B and T lymphocytes, NK and dendritic lineages in jawed vertebrates, have also been identified in the lamprey (Haire et al., 2000; Mayer et al., 2002b). Lymphopoiesis and hematopoiesis both occur in the “primitive spleen” located in the typhlosolar invagination along the larval intestine, in the anterior kidney, and in the bone marrow-like fat tissue of the protovertebral arch in the adult lamprey (Finstad and Good, 1964; Finstad et al., 1964; Piavis and Hiatt, 1971; Kilarski and Plytycz, 1981; Fujii, 1982; Hagen et al., 1983; Ardavin and Zapata, 1987; Mayer et al., 2002a). Tiny foci of 4–20 lymphocytes have also been found in thymus-like epithelial structures in the lamprey pharyngeal gutters (Finstad and Good, 1964; Finstad et al., 1964), but doubt has been expressed about whether these lymphoid foci represent a proto-thymus (Potter et al., 1982).

### 11. Evidence for adaptive immune responses in the lamprey

Lamprey lymphocytes have been shown to undergo aggregation and proliferation in response to a variety of stimuli.

These include phytohemagglutinin, staphylococcal filtrate, hapten-conjugated serum, picrylated *Salmonella typhi*, and other antigenic stimuli (Finstad and Good, 1964; Cooper, 1971; Piavis and Hiatt, 1971). Skin allograft rejection in lampreys resembles that seen in fish and other vertebrates. Chronic rejection of first set allografts usually occurs within 21–50 days post transplantation in the lamprey. Second set skin allografts are rejected at an accelerated rate, usually within 18 days (Finstad et al., 1964; Perey et al., 1968). The graft rejection bed is infiltrated by polymorphonuclear leukocytes, eosinophilic granulocytes, small and medium sized lymphocytes, and lymphoblastoid cells (Perey et al., 1968; Fujii and Hayakawa, 1983). This pattern of allograft rejection clearly denotes a form of immunologic memory in these jawless fish.

There are many reports indicating that lampreys can also produce specific agglutinins in response to immunization (Boffa et al., 1967; Marchalonis and Edelman, 1968; Hildemann, 1970; Litman et al., 1970; Pollara et al., 1970; Fujii, 1981; Hagen et al., 1983; Raison and Hildemann, 1984; Hagen et al., 1985). Particulate antigens have been found to be especially potent for the elicitation of specific agglutinins against human type O red blood cells (RBC) and killed *Brucella abortus* in lamprey (Litman et al., 1970; Pollara et al., 1970; Fujii, 1981; Hagen et al., 1985). In addition, hemocyanin, a soluble antigen, was shown to be efficiently cleared from the lamprey circulation (Finstad and Good, 1964; Pollara et al., 1970). The lamprey agglutinins or “antibodies” are very labile proteins lacking intrachain disulfide bonds. In comparison with vertebrate immunoglobulins, the lamprey “antibodies” have a very different subunit structure, molecular weight heterogeneity, sedimentation properties, and electrophoretic mobility. These features are more reminiscent of the invertebrate agglutinins than of the immunoglobulin molecules made by the jawed vertebrates (Litman et al., 1970; Pollara et al., 1970).

## 12. A lymphocyte based search for lamprey immune system genes

A systematic analysis of genes expressed in lamprey lymphocyte-like cells has been conducted recently. This search led to the identification of a variety of lamprey lymphocyte transcripts for molecules that resemble important components of the vertebrate adaptive immune system. These include transcripts for genes involved in lymphocyte development, signal transduction, proliferation, differentiation, activation and chemotaxis. Transcripts for the CD45 transmembrane tyrosine kinase that regulates lymphocyte activation and proliferation, CD9/CD81, the homologs of which are involved in homing and cell adhesion, BCAP, the B cell adaptor for phosphoinositide 3-kinase, CAST, a CD3-associated signal transducer involved in transduction of activation signals in T lymphocytes; and CD98, an amino acid transporter of

activated lymphocytes (Mayer et al., 2002a; Uinuk-Ool et al., 2002), were identified. An ABCB transporter related to the mammalian ABCB9, TAP1 and TAP2 proteins that load peptides for presentation on MHC class I was also identified (Uinuk-Ool et al., 2003). In an extension of this analysis of the lamprey lymphocyte transcriptome, we have sequenced 8723 EST clones, and 1507 clones from a library enriched in messages of activated larval lymphocytes by a subtraction strategy (Z. Pancer et al., unpublished). A composite list of cell surface receptor homologs identified in this survey is presented in Table 1. We identified only two molecules that resemble central elements of the vertebrate adaptive immune system, prototypic TCR-like and CD4-like molecules, in this extensive database of lymphocyte genes. The lamprey TCR-like molecule has both *V* and *J*-like sequences in its IgV domain, an IgC domain, a transmembrane domain, and a cytoplasmic domain. However, this TCR-like chain is encoded by a single non-rearranging gene without close relatives. In fact, only seven of the 40 putative cell surface receptors identified in this search have Ig superfamily domains, and the only one of these having a canonical IgV is the TCR-like molecule described above. The ancestral TCR gene in the lamprey thus cannot explain the diverse adaptive immune responses reported for the agnathans. The most logical conclusion from this survey of 17,323 lymphocyte messages is that there are no identifiable agnathan homologs of the key molecules responsible for adaptive immunity in jawed vertebrates, namely the genes for MHC class I and II molecules and the rearrangeable *V*, *D* and *J* gene elements for TCRs and immunoglobulins. Our current studies favor the latter alternative.

## 13. Wherefore the genetic basis for adaptive immune responses in the lamprey?

The as yet unanswered question of the molecular basis for adaptive immune response in the lampreys is especially intriguing given that their immune responses resemble adaptive immunity in jawed vertebrates. First, the lamprey has circulatory lymphoid cells that morphologically and genetically resemble the lymphocytes in gnathostomes. Second, these lymphocyte-like cells differentiate and proliferate in hematopoietic tissues in a manner similar to that observed for lymphocytes in fish and other jawed vertebrates. Third, the agnathans exhibit specific immunologic memory of both cell mediated and humoral types. We suggest two possible explanations for this enigma. The agnathan adaptive immunity genes may have diverged beyond recognition after the split between the lamprey ancestral lineage and the gnathostomes. Alternatively, agnathans may use other lymphocyte gene products to mediate immune responses that resemble those of the jawed vertebrates. Hopefully, ongoing investigations will soon yield a definitive answer to this long-standing question.

Table 1

Cell surface receptor gene homologs expressed by lymphoid cells in the lamprey

TCR-like (Ig superfamily)	HSPC194 (CD34 <sup>+</sup> hematopoietic stem/progenitor cell markers)
CD4-like (Ig superfamily)	HSPC213 (CD34 <sup>+</sup> hematopoietic stem/progenitor cell markers)
CD9/CD81 (tetraspanin)	HSPC307 (CD34 <sup>+</sup> hematopoietic stem/progenitor cell markers)
CD29 integrin beta-1	TNFR2 (tumor necrosis factor receptor)
CD33 myeloid cell surface antigen (Ig superfamily)	TNFR14 (tumor necrosis factor receptor)
CD42A (platelet glycoprotein)	TNF-alpha induced protein
CD42B-alpha (platelet glycoprotein)	14 kDa interferon induced protein
CD42B-beta (platelet glycoprotein)	TM4S8 (tetraspanin)
CD45 (receptor protein tyrosine phosphatase)	TM4S9 (tetraspanin)
CD63 (tetraspanin)	Coagulation factor II receptor 1
CD98 (amino acid transporter)	DMBT1 (receptor for lung surfactant protein D)
CD111 poliovirus receptor (Ig superfamily)	G protein-coupled receptor 12A
CD130 interleukin receptor beta chain	GPI-anchored protein p137 (nutrient transporter)
High affinity IL-8 receptor A	Factor H
IL-17 receptor	Minor histocompatibility antigen
Chemokine receptor 4 alpha	Peripheral myelin protein 22
Opioid-binding CAM (Ig superfamily)	Receptor type protein tyrosine phosphatase A
Papilin (Ig superfamily)	Receptor tyrosine kinase tie-1
Plasma membrane protein 1B3 (Ig superfamily)	Saliva (RAG-1 induction)
Stromal cell derived factor receptor 1 (Ig superfamily)	VEGFR-2 (vascular endothelial growth factor receptor)

## References

- Anderson, M.K., Sun, X., Miracle, A.L., Litman, G.W., Rothenberg, E.V., 2001. Evolution of hematopoiesis: three members of the PU.1 transcription factor family in a cartilaginous fish, *Raja eglanteria*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 553–558.
- Arai, M., Suzuki-Koike, M., Ohtake, S., Ohba, H., Tanaka, K., Chiba, J., 2001. Common cell-surface antigens functioning in self-recognition reactions by both somatic cells and gametes in the solitary ascidian *Halocynthia roretzi*. *Microbiol. Immunol.* 45 (12), 857–866.
- Ardavin, C.F., Zapata, A., 1987. Ultrastructure and changes during metamorphosis of the lympho-hemopoietic tissue of the larval anadromous sea lamprey *Petromyzon marinus*. *Dev. Comp. Immunol.* 11, 79–93.
- Azumi, K., De Santis, R., De Tomaso, A., Rigoutsos, I., Yoshizaki, F., Pinto, M.R., Marino, R., Shida, K., Ikeda, M., Arai, M., Inoue, Y., Shimizu, T., Satoh, N., Rokhsar, D.S., Du Pasquier, L., Kasahara, M., Satake, M., Nonaka, M., 2003. Genomic analysis of immunity in a Urochordate and the emergence of the vertebrate immune system: “waiting for Godot”. *Immunogenetics* 55 (8), 570–581.
- Ballarin, L., Cima, F., Sabbadin, A., 1995. Morula cells and histocompatibility in the colonial ascidian *Botryllus*. *Zool. Sci.* 12, 757–764.
- Ballarin, L., Cima, F., Sabbadin, A., 1998. Phenoloxidase and cytotoxicity in the compound ascidian *Botryllus schlosseri*. *Dev. Comp. Immunol.* 22 (5–6), 479–492.
- Bancroft, F.W., 1903. Variation and fusion of colonies in compound ascidians. *Proc. Calif. Acad. Sci.* 3, 137–186.
- Berrill, N.J., 1955. *The Origin of Vertebrates*. Clarendon Press, Oxford.
- Boffa, G.A., Fine, J.M., Drilhon, A., Amouch, P., 1967. Immunoglobulins and transferrin in marine lamprey sera. *Nature* 214, 700–702.
- Burnet, F.M., 1971. Self-recognition in colonial marine forms and flowering plants in relation to the evolution of immunity. *Nature* 232, 230–235.
- Cooper, A.J., 1971. Ammocoete lymphoid cell populations in vitro. In: McIntyre 4th, O.R. (Ed.), *Leukocyte Culture Conference*. New York: Appleton Century-Crofts, pp. 137–147.
- Davidson, B., Swalla, B.J., 2002. A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. *Development* 129 (20), 4739–4751.
- Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., Harafuji, N., Hastings, K.E., Ho, I., Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I.A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H.G., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-Bow, S., DeSantis, R., Doyle, S., Francino, P., Keys, D.N., Haga, S., Hayashi, H., Hino, K., Imai, K.S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B.I., Makabe, K.W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Shin-i, T., Spagnuolo, A., Stainier, D., Suzuki, M.M., Tassy, O., Takatori, N., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P.D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N., Rokhsar, D.S., 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* 298, 2157–2167.
- De Tomaso, A.W., Saito, Y., Ishizuka, K.J., Palmeri, K.J., Weissman, I.L., 1998. Mapping the genome of a model protochordate. I. A low resolution genetic map encompassing the fusion/histocompatibility (Fu/HC) locus of *Botryllus schlosseri*. *Genetics* 149 (1), 277–287.
- De Tomaso, A.W., Weissman, I.L., 2003. Initial characterization of a protochordate histocompatibility locus. *Immunogenetics* 55 (7), 480–490.
- Endo, Y., Nonaka, M., Saiga, H., Kakinuma, Y., Matsushita, A., Takahashi, M., Matsushita, M., Fujita, T., 2003. Origin of mannose-binding lectin-associated serine protease (MASP)-1 and MASP-3 involved in the lectin complement pathway traced back to the invertebrate, amphioxus. *J. Immunol.* 170 (9), 4701–4707.
- Finstad, J., Good, R.A., 1964. The evolution of the immune response. III. Immunologic responses in the lamprey. *J. Exp. Med.* 120, 1151–1167.
- Finstad, J., Papermaster, B.W., Good, R.A., 1964. Evolution of the immune response. II. Morphologic studies of the thymus and organized lymphoid tissue. *Lab Invest.* 13, 490–512.
- Flajnik, M.F., Kasahara, M., 2001. Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity* 15, 351–362.
- Flajnik, M.F., 2002. Comparative analyses of immunoglobulin genes: surprises and portents. *Nat. Rev. Immunol.* 2, 688–698.
- Forey, P.L., Janvier, P., 1993. Agnathans and the origin of jawed vertebrates. *Nature* 361, 129–134.
- Fujii, T., 1981. Antibody-enhanced phagocytosis of lamprey polymorphonuclear leukocytes against sheep erythrocytes. *Cell Tissue Res.* 219, 41–51.
- Fujii, T., 1982. Electron microscopy of the leukocytes of the typhlosole in ammocoetes, with special attention to the antibody-producing cells. *J. Morphol.* 173, 87–100.

- Fujii, T., Hayakawa, I., 1983. A histological and electron-microscopic study of the cell types involved in rejection of skin allografts in ammocoetes. *Cell Tissue Res.* 231, 301–312.
- Fujita, T., 2002. Evolution of the lectin-complement pathway and its role in innate immunity. *Nat. Rev. Immunol.* 2 (5), 346–353.
- Fuke, M.T., 1980. Contact reaction between xenogenic and allogeneic coelomic cells of solitary ascidians. *Biol. Bull.* 158, 304–315.
- Fuke, M.T., Numakunai, T., 1982. Allogeneic cellular reactions between intra-specific types of a solitary ascidian, *Halocynthia roretzi*. *Dev. Comp. Immunol.* 6 (2), 253–261.
- Fuke, M.T., Nakamura, I., 1985. Pattern of cellular alloreactivity of the solitary ascidian, *Halocynthia roretzi*, in relation to genetic control. *Biol. Bull.* 169, 631–637.
- Good, R.A., Finstad, J., Litman, G.W., 1972. *The Biology of Lampreys II: Immunology*. Academic Press, London.
- Hagen, M., Filosa, M.F., Youson, J.H., 1983. Immunocytochemical localization of antibody-producing cells in adult lamprey. *Immunol. Lett.* 6, 87–92.
- Hagen, M., Filosa, M.F., Youson, J.H., 1985. The immune response in adult sea lamprey (*Petromyzon marinus* L.): the effect of temperature. *Comp. Biochem. Physiol.* 82, 207–210.
- Haire, R.N., Miracle, A.L., Rast, J.P., Litman, G.W., 2000. Members of the Ikaros gene family are present in early representative vertebrates. *J. Immunol.* 165, 306–312.
- Hildemann, W.H., 1970. Transplantation immunity in fishes: agnatha, chondrichthyes and osteichthyes. *Transplant. Proc.* 2, 253–359.
- Hirose, E., 2003. Colonial allorecognition, hemolytic rejection, and viviparity in botryllid ascidians. *Zool. Sci.* 20 (4), 387–394.
- Hoffmann, J.A., 2003. The immune response of drosophila. *Nature* 426, 33–37.
- Iizuka, K., Naidenko, O.V., Plougastel, B.F., Fremont, D.H., Yokoyama, W.M., 2003. Genetically linked C-type lectin-related ligands for the NKR1 family of natural killer cell receptors. *Nat. Immunol.* 4 (8), 801–807.
- Kaufman, J., 2002. The origins of the adaptive immune system: whatever next? *Nat. Immunol.* 3, 1124–1125.
- Kelly, K.L., Cooper, E.L., Raftos, D.A., 1993. A humoral opsonin from the solitary urochordate *Styela clava*. *Dev. Comp. Immunol.* 17 (1), 29–39.
- Kenjo, A., Takahashi, M., Matsushita, M., Endo, Y., Nakata, M., Mizuuchi, T., Fujita, T., 2001. Cloning and characterization of novel ficolins from the solitary ascidian, *Halocynthia roretzi*. *J. Biol. Chem.* 276 (23), 19959–19965.
- Khalturin, K., Becker, M., Rinkevich, B., Bosch, T.C., 2003. 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. U.S.A.* 100 (2), 622–627.
- Kilarski, W., Plytycz, B., 1981. The presence of plasma cells in the lamprey (agnatha). *Dev. Comp. Immunol.* 5, 361–366.
- Kimbrell, D.A., Beutler, B., 2001. The evolution and genetics of innate immunity. *Nat. Rev. Genet.* 2 (4), 256–267.
- Klein, J., 1998. In an immunological twilight zone. *Proc. Natl. Acad. Sci. U.S.A.* 95 (20), 11504–11505.
- Kumar, S., Hedges, S.B., 1998. A molecular timescale for vertebrate evolution. *Nature* 392, 917–920.
- Laird, D.J., De Tomaso, A.W., Cooper, M.D., Weissman, I.L., 2000. 50 million years of chordate evolution: seeking the origins of adaptive immunity. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6924–6926.
- Litman, G.W., Frommel, D., Finstad, F.J., Howell, J., Pollara, B.W., Good, R.A., 1970. The evolution of the immune response. VIII. Structural studies of the lamprey immunoglobulin. *J. Immunol.* 105, 1278–1285.
- Magor, B.G., De Tomaso, A., Rinkevich, B., Weissman, I.L., 1999. Allorecognition in colonial tunicates: protection against predatory cell lineages? *Immunol. Rev.* 167, 69–79.
- Marchalonis, J.J., Edelman, G.M., 1968. Phylogenetic origins of antibody structure. 3. Antibodies in the primary immune response of the sea lamprey, *Petromyzon marinus*. *J. Exp. Med.* 127, 891–914.
- Marino, R., Kimura, Y., De Santis, R., Lambris, J.D., Pinto, M.R., 2002. Complement in urochordates: cloning and characterization of two C3-like genes in the ascidian *Ciona intestinalis*. *Immunogenetics* 53 (12), 1055–1064.
- Mayer, W.E., Uinuk-Ool, T., Tichy, H., Gartland, L.A., Klein, J., Cooper, M.D., 2002a. Isolation and characterization of lymphocyte-like cells from a lamprey. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14350–14355.
- Mayer, W.E., O'Huigin, C., Tichy, H., Terzic, J., Saraga-Babic, M., 2002b. Identification of two Ikaros-like transcription factors in lamprey. *Scand. J. Immunol.* 55, 162–170.
- Murabe, N., Hoshi, M., 2002. Re-examination of sibling cross-sterility in the ascidian, *Ciona intestinalis*: genetic background of the self-sterility. *Zool. Sci.* 19 (5), 527–538.
- Nonaka M., Miyazawa S., 2002. Evolution of the initiating enzymes of the complement system. *Genome Biol.* 3 (1), 1001.
- Oka, H., Watanabe, H., 1957. Colony specificity in compound ascidians as tested by fusion experiments (a preliminary report). *Proc. Jpn. Acad.* 33, 657–659.
- Oka, K., Watanabe, H., 1960. Problems of colony specificity in compound ascidians. *Bull. Mar. Biol. Stn. Asamushi.* 10, 153–155.
- Parrinello, N., 1996. Cytotoxic activity of tunicate hemocytes. *Prog. Mol. Subcell Biol.* 15, 190–217.
- Perey, D.Y., Finstad, J., Pollara, B., Good, R.A., 1968. Evolution of the immune response. VI. First and second set skin homograft rejections in primitive fishes. *Lab. Invest.* 19, 591–597.
- Piavis, G.W., Hiatt, J.L., 1971. Blood cell lineage in the sea lamprey *Petromyzon marinus* (Pisces: Petromyzontidae). *Copeia* 4, 722–728.
- Pollara, B., Litman, G.W., Finstad, J., Howell, J., Good, R.A., 1970. The evolution of the immune response. VII. Antibody to human O cells and properties of the immunoglobulin in lamprey. *J. Immunol.* 105, 738–745.
- Potter, I.C., Percy, L.R., Barber, D.L., Macey, D.J., 1982. The morphology development and physiology of blood cells. In: Hardisty, M.W., Potter, I.C. (Eds.), *The Biology of Lampreys*, 4A. Academic Press, London, pp. 233–292.
- Raftos, D.A., Tait, N.N., Briscoe, D.A., 1987a. Allograft rejection and alloimmune memory in the solitary urochordate, *Styela plicata*. *Dev. Comp. Immunol.* 11, 343–351.
- Raftos, D.A., Tait, N.N., Briscoe, D.A., 1987b. Cellular basis of allograft rejection in the solitary urochordate, *Styela plicata*. *Dev. Comp. Immunol.* 11, 713–725.
- Raftos, D.A., 1990. The morphology of allograft rejection in *Styela plicata* (urochordata: ascideacea). *Cell Tissue Res.* 261, 389–396.
- Raftos, D.A., 1991. Cellular restriction of histocompatibility responses in the solitary urochordate, *Styela plicata*. *Dev. Comp. Immunol.* 15 (1), 93–98.
- Rinkevich, B., Weissman, I.L., 1987. Chimeras in colonial invertebrates: A synergistic symbiosis or somatic and germ-cell parasitism? *Symbiosis* 4, 117–134.
- Rinkevich, B., 1992. Aspects of the incompatibility nature in botryllid ascidians. *Anim. Biol.* 1, 17–28.
- Rinkevich, B., Tertakover, S., Gershon, H., 1998. The contribution of morula cells to allogeneic responses in the colonial urochordate, *Botryllus schlosseri*. *Mar. Biol.* 131, 227–236.
- Raison, R.L., Hildemann, W.H., 1984. Immunoglobulin-bearing blood leukocytes in the Pacific hagfish. *Dev. Comp. Immunol.* 8, 99–108.
- Saito, Y., Hirose, E., Watanabe, H., 1994. Allorecognition in compound ascidians. *Int. J. Dev. Biol.* 38 (2), 237–247.
- Sakai, N., Sawada, H., Yokosawa, H., 2003. Extracellular ubiquitin system implicated in fertilization of the ascidian, *Halocynthia roretzi*: isolation and characterization. *Dev. Biol.* 264 (1), 299–307.
- Satou, Y., Yamada, L., Mochizuki, Y., Takatori, N., Kawashima, T., Sasaki, A., Hamaguchi, M., Awazu, S., Yagi, K., Sasakura, Y., Nakayama, A., Ishikawa, H., Inaba, K., Satoh, N., 2002. A cDNA resource from the basal chordate *Ciona intestinalis*. *Genesis* 33 (4), 153–154.

- Sawada, H., Sakai, N., Abe, Y., Tanaka, E., Takahashi, Y., Fujino, J., Kodama, E., Takizawa, S., Yokosawa, H., 2002. Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. *Proc. Natl. Acad. Sci. U.S.A.* 99 (3), 1223–1228.
- Sawada, H., 2002. Ascidian sperm lysin system. *Zool. Sci.* 19 (2), 139–151.
- Schluter, S.F., Bernstein, R.M., Bernstein, H., Marchalonis, J.J., 1999. Big Bang emergence of the combinatorial immune system. *Dev. Comp. Immunol.* 23, 107–111.
- Scofield, V.L., Schlumpberger, J.M., West, L.A., Weissman, I.L., 1982. Protochordate allorecognition is controlled by a MHC-like gene system. *Nature* 295, 488–502.
- Sekine, H., Kenjo, A., Azumi, K., Ohi, G., Takahashi, M., Kasukawa, R., Ichikawa, N., Nakata, M., Mizuuchi, T., Matsushita, M., Endo, Y., Fujita, T., 2001. An ancient lectin-dependent complement system in an ascidian: novel lectin isolated from the plasma of the solitary ascidian, *Halocynthia roretzi*. *J. Immunol.* 167 (8), 4504–4510.
- Shintani, S., Terzic, J., Sato, A., Saraga-Babic, M., O'huigin, C., Tichy, H., Klein, J., 2000. Do lampreys have lymphocytes? the Spi evidence. *Proc. Natl. Acad. Sci. U.S.A.* 97, 7417–7422.
- Taneda, Y., Watanabe, H., 1982. Studies on colony specificity in the compound ascidian *Botryllus primigenus* II. In vivo bioassay for analysing the mechanism of nonfusion reaction. *Dev. Comp. Immunol.* 6, 243–252.
- Taneda, Y., Saito, Y., Watanabe, H., 1985. Self or non-self discrimination in ascidians. *Zool. Sci.* 2, 433–442.
- Uinuk-Ool, T., Mayer, W.E., Sato, A., Dongak, R., Cooper, M.D., Klein, J., 2002. Lamprey lymphocyte-like cells express homologs of genes involved in immunologically relevant activities of mammalian lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14356–14361.
- Uinuk-Ool, T.S., Mayer, W.E., Sato, A., Takezaki, N., Benyon, L., Cooper, M.D., Klein, J., 2003. Identification and characterization of a TAP-family gene in the lamprey. *Immunogenetics* 55, 38–48.
- Weissman, I.L., Saito, Y., Rinkevich, B., 1990. Allorecognition histocompatibility in a protochordate species: is the relationship to MHC semantic or structural? *Immun. Rev.* 113, 227–241.
- Zapata, A., Ardavin, C.F., Gomariz, R.P., Leceta, J., 1981. Plasma cells in the ammocoete of *Petromyzon marinus*. *Cell Tissue Res.* 221, 203–208.