Development and function of the thymus in teleosts

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Abstract

The thymus plays a pivotal role in the development of the adaptive immune system, an important factor that separates higher vertebrates from the rest of the animal phyla. The development of functional T-cells from thymocytes is a crucial step in the development of a functional vertebrate immune system and whilst recent advances in molecular and developmental biology have advanced our understanding of T-cell development, they have also provided potential model species across the vertebrate phyla including the zebrafish (Danio rerio). However, this species is one of more than 20,000 species of fish that could assist in elucidating the development of the vertebrate thymus and, consequently, the evolution of the vertebrate immune response. In this paper we review the knowledge of the teleost thymus through the organogenesis and development studies in teleosts together with advances in molecular and functional approaches. Where necessary we will combine this knowledge with that obtained in higher vertebrates.

Keywords: Thymus; Teleost; T-cell; Immune system; Immune function; Development

1. Introduction

The thymus is considered a key organ of the immune system in vertebrates. It is a unique vertebrate organ whose development can be traced through the evolutionary scale beginning in early fish species as a thickening in the epithelium of the pharyngeal area of the gastro-intestinal tract. The thymus is responsible for production of self-restricted and self-tolerant T-cells [1]. In this respect the thymus facilitates and regulates the interaction of lymphoid and non-lymphoid cells [2]. This process is required because of
two situations. The first is when self-tolerant lymphoid effector cells are generated through randomly compiled antigen receptors which do not have a pre-determined specificity. In this case, external cues are responsible for removing self-active clones. The second situation relates to the requirement for lymphoid organs to guide and control the adaptive immune response through the very precise interactions between antigen presenting cells and lymphocytes.

2. General morphology of the thymus

The thymus is a primary lymphoid organ, being the major site of T-cell development. In adult mammals the thymus is a single bi-lobed organ located in the mid-thorax close to the heart. The two lobes generally differ in size; they are occasionally united, so as to form a single mass; and sometimes separated by an intermediate lobe. The thymus is of a pinkish-grey colour, soft, and lobulated on its surfaces and is surrounded by mediastinal fat. Blood is supplied by the thymic artery and is drained through the thymic veins to the brachiocephalic vein. There is an age dependent involution such that in young animals the thymus is much larger than in adults and may extend upwards to the thyroid gland in the lower neck [3]. Despite the atrophy observed in older animals there is evidence that the human thymus remains active during aging [4].

3. Evolution of the thymus

The thymus first appears as an identifiable organ in the chondrichthyes and the osteichthyes (Fig. 1) [5]. This excludes the class cyclostomata, which includes modern lampreys and hagfish. Lampreys and hagfish retain notochord and a novel olfactory and hypophysial arrangement. Both these characteristics may be significant in thymus development as the thymus develops close to cartilage and is responsive to pituitary hormones [6]. Hagfish and lampreys are the first vertebrates to appear in the fossil record and although they possess morphologically identifiable lymphocytes in the peripheral blood and lymphohaemopoietic tissues in both the kidney and the intestinal lamina propria, they do not possess true lymphoid organs and, presumably, are involved in non-anticipatory immune responses. These types of response are more similar to the innate defence mechanisms of invertebrates than to the sophisticated adoptive immune responses of vertebrates [7]. However, it has been suggested that the typhlosole, a fold in the midgut found in

![Phylogenetic tree for teleost evolution. Timescale moves from left to right.](image-url)
invertebrates and agnathans, may have thymic activities in lampreys [8]. It has also been suggested that stem cells settle at different sites and proliferate to form lymphopoietic tissue under the influence of environmental factors [9].

The area of the mucosal surface is in the order of 400 times that of the surface area of the animal, with the mucosa being the site of major nutrient and gas exchange, as well as a major route for pathogen entry [10]. Within that context the mucosal-associated lymphoid tissue (MALT) has evolved as a defensive mechanism and the thymus evolved as a specialised area of that tissue. It has been suggested that an accumulation of phagocytes in the pharyngeal region may have given an ancestor an evolutionary advantage [11].

The thymus generally develops in the lamina propria of the gastro-intestinal tract in pouches located at the base of the gill arches. It then migrates to the underlying mesenchyme during ontogeny. In most teleosts the thymus is located near the gill cavity and is closely associated with the pharyngeal epithelium (Fig. 2) [7]. Although usually found as a paired organ in most vertebrates (Fig. 3), the thymus can appear as more than one pair of organs in teleosts. In the clingfish, Sicyases sanguineus, each gill chamber has a pair of thymus glands with one taking up a superficial position and the second located close to the gill epithelium [12].

Classification of cells and structures in fish thymus has largely been done on the basis of histological examination, with investigators varying the emphasis. Also a section examined may not be typical for the whole organ. In sections of young fish, the thymus can be more elusive [13,14], which may be a consequence...
of early age involution [15]. There can also be variations in reported morphology. An absence of cortico-medullary junction is often reported. One report examining a large sample of flounder (Paralichthys olivaceus) reported no medulla and total regression at 7 months [16]. Another reported being able to distinguish zones in the same species [17]. There appears to be similar problems with zebrafish [18]. However, it is important to recognise that differences do occur especially between species and within a species in an age-dependent manner. Zonation of the thymus has been observed in turbot (Scophthalmus maximus L.) and halibut (Hippoglossus hippoglossus L.) (Fig. 4), whilst in salmon (Salmo salar L.) none was observed [19,20]. Comparison of five-day-old and one-year-old rainbow trout indicated no observable zonation at either age [11]. Another group investigated apoptosis in sea bass thymus as an indication of T-cell selection, and recorded no apoptosis in early development whilst later apoptotic cells were visible in both cortex and medulla, concentrated at the indistinct border region [21]. In zebrafish there is clear zonation, with rag-1-positive cortex and rag-1-negative medulla [22]. Similar results were achieved in carp [23]. Vesicular spaces are characteristic and are thought to be sites of degeneration (Fig. 4) [24]. Differential cytochemical staining in channel catfish thymus showed acid phosphatase positive cells at the cortico-medullary junction which could be interdigitating cells [25].

Fig. 3. Thymus of 71-day-old Atlantic cod. The transverse section shows the two lobes of the thymus (T) in the branchial cavity on each side of the animal. Scale bar is 100 μm. Used with permission of S. Gudmundsdottir, Institute of Pathology, University of Iceland, Keldur, IS-112 Reykjavík, Iceland.
Serial sectioning, computer assisted measurement, the use of whole mounts, and specific markers yielded detailed information via 3D visualisations [18,23,26]. Classifications of the cells of the thymus are made using various criteria including site, morphology, size, keratin content, putative function, and dermal origin. It has been reported that cellular transformations are involved such that neither histological analysis of cells nor tissue morphology, nor gene expression/marker studies, are sufficient to determine lineage [27]. Cell labelling is the only way to determine origin. In one study seven subpopulations of keratin positive epithelial cells were identified [28]. Another group unified the terminology of carp with mammals and birds by classifying four cell types; limiting epithelial cells, reticular epithelial cells, nurse-like cells, and Hassall’s body-like structures [23]. Using flow cytometric analysis of surface phenotype, mouse thymic stromal cells have been classified as; dendritic cells (3 subsets), myeloid-derived cells (4 subsets), cortical epithelial cells (5 subsets), medullary epithelial cells (5 subsets), fibroblasts (4 subsets), and endothelial cells (5 subsets) [29].

The concept of developmental milestones is important in aiding the construction of the clinical model of human development, by clearly defining developmental stages. Developmental stages are used to monitor development in single species [30–32]. One report investigating immunological development suggested that species can be compared using age equivalence, with the appearance of small lymphocytes in the thymus as the first milestone [33]. However, another report marks the appearance of the alymphoid anlage as the first checkpoint [2]. In Harpagifer antarcticus, leucocytes are observed in the head kidney at 1-h post-hatch, whilst thymic infiltration was delayed until 4 weeks, this feature is interpreted as a cold-water adaptation [34].

There is a general agreement in the literature that the thymus is the first lymphoid organ to develop. In distinguishing between the development of a lymphoid organ and the first detection of lymphocytes it was...
noted that whilst the head kidney develops before the thymus, small lymphocytes have been observed in the kidney subsequent to those observed in the thymus [17].

In fish, the second pharyngeal pouch is often involved with thymic development. Tonsils develop in the second pharyngeal pouch of mammals (except rodents), having evolved 300 million years after the thymus. Tonsils include some structures similar to thymus but also germinal centres of B-cells, surrounding T-cell activity, and crypts that increase exposure to environmental antigens. In humans, thymus development starts in the first month whilst tonsil development is delayed until the fourth month of gestation [35,36]. It may be supposed that by the encapsulation and internalisation of the thymus, its original evolutionary function was lost. Tonsils then evolved in mammals where the thymus had evolved and develops in fish [35].

4. Development/ontogeny of the thymus

The classical model for thymus has been developed in mammals where the thymus develops in the third pharyngeal pouch. The structure is composed of a capsule, through which lymphocytes migrate into the cortex and in post-natal mice lymphocytes enter through vessels at the cortex-medullary junction [37]. In the cortex, further proliferation occurs in association with thymic nurse cells (TNC) and thymic epithelial cells (TEC) where positive selection takes place for lymphocytes with affinity for self-MHC and non-reacting self-MHC lymphocytes undergo apoptosis. Lymphocytes move to the thymic medulla through the cortico-medullary junction. This area is rich in macrophages that can phagocytose dying cells. Within the medulla, and in association with macrophages and dendritic cells, further selection is made against lymphocytes with high-affinity for self-MHC or affinity for self-antigen and these cells then undergo apoptosis. Finally, mature selected T-lymphocytes then migrate to the periphery.

The development of the thymus appears to require interaction of all three embryonic germ layers: ectoderm, mesoderm and endoderm [1,27,38]. In mammals, the thymus forms from an interaction between the endoderm of the third pharyngeal pouch and the neural crest mesenchyme in the third and fourth pharyngeal pouches [1]. Teleost fish develop seven pharyngeal arches. These form the mandible, hyoid and five sets of gill arches. Usually the second, third and fourth pharyngeal pouches are involved in fish thymus development. Neural crest cells, including cartilage precursors move into the area [39]. This generates primordia in contact with both the surface ectoderm and the pharyngeal endoderm, but surrounded by a mesenchymic capsule [27]. The mesenchymal cells are initially derived from the neural crest and pharyngeal mesoderm; they mediate epithelial cell proliferation and morphogenesis, and then establish an intra-thymic network of fibroblasts whilst contributing to the capsule and trabeculae [40].

There is evidence from chickens and mice that endoderm can generate both cortical and medullary epithelial cells [27,41]. An emerging model is that the endodermal primordia require a Hox3-Pax1/9-Eya1-Six1 transcription factor cascade acting in the pouch of the mesoderm. This patterns the area, with the primordia then encapsulated by the immigrant neural crest cells [27].

Neural crest cells form the capsule and perivascular tissue [1]. These primordia continue to develop, separate and migrate (medially, ventrally and caudally in both mouse and man) to their final positions at the midline above the heart. In birds thymic migration is linked with the neck elongation resulting in more thymic lobes along the neck region [42]. Pax9 is responsible for the invagination and internalisation of the thymus. Mice without Pax9 retain ectopic thymic tissue in the larynx. Pax1 deficiency would appear to block ventro-caudal movement [2]. Fish show internalisation of thymus, but it is not clear yet whether fish express these proteins.

In zebrafish, this development process has been shown to involve neural crest cells derived from the neuroectoderm of the sixth hindbrain rhombomere that migrate to the third and fourth pharyngeal pouches where they interact with the endoderm [38]. The thymus in zebrafish remains continuous with the pharyngeal endoderm, rather than migrating as in higher vertebrates. The angler fish, Lophius piscatorius,
has a thymus that is located behind the gill cavity [43,44]. This observation has led to the conjecture that this may represent the first step of the internalisation process that occurs during vertebrate evolution [45].

In older mammals, formation of vacuoles and enigmatic rings of flattened epithelial cells that are termed Hassall’s corpuscles accompany involution of the thymus. However, very little is known about the ageing of the teleost thymus. Whilst it appears that involution occurs in all vertebrates, the degree of involution seems to be variable in teleosts [46–48]. It has been suggested that the carp thymus may provide the environment for thymocyte maturation in adult life [23]. RAG1 and RAG2 have been found in the thymus of carp up to 6 years old, giving evidence of life time T-cell production [49]. Whilst no thymus was found in flounder after 7 months of age [16].

The mammalian thymic microenvironment has been compared with, firstly, the heart, and more recently, the brain [50]. This may be because capsular cells are derived from the neural crest, thymic hormones are similar to some neural peptides [51] and growth hormone is endogenously produced in sub-capsular epithelial cells [52]. It has been proposed that thymic nurse cells may have a neurohormonal function in the thymus [53]. Froehly and Deschaux [96] found hormonal factors, thyulin and thymosin in reticulo-endothelial cells of carp and sea bass [23,54].

In mammals the thymus is considered as an immunologically privileged site, however, in fish the close association with the branchial epithelium does not suggest this status. A study on the thymus of rainbow trout fry reported that antigens may gain access directly into the thymus from the gill cavity [11]. However, more recent evidence indicates that the thymus in teleosts is an immunologically privileged site with restricted access to antigens [55].

Early development of the thymus in fish has been studied in many diverse teleost species [15,17,18,30,34,56–62]. For instance, in Atlantic halibut with the expansion of thymocytes the thymus protrudes into the opercular cavity (Fig. 5a). However, in other species, such as carp and sea bass, its predominant growth is internal (Fig. 5b and c). The development timeframe can differ from species to species even when accounting for temperature effects on growth (Fig. 6). The relationship between growth and development can be dynamic and physiological age expressed as degree-days does not factor out all differences in temperature history [63]. Lower temperatures inhibit immune responses, manifest notably in channel catfish by the inhibition of the generation and activation of virgin T-cells [64].

It is believed that the source of lymphoblasts is the head kidney [62,65]. One reported sequence for the division of thymic development has four divisions; the appearance of thymic primordium (this must be subjective), it’s colonization by lymphocyte precursors, the expansion of thymocytes and histological regionalization [18,66]. It is possible that involution and regression should be added to this list.

Much developmental work has been achieved using histological analysis. However, advances in identifying protein and gene markers that could be linked to thymic function, have aided these studies [38,66]. It is now possible to confirm morphological studies by the use of immunohistochemical and in-situ hybridisation techniques using probes for genes and their products such as; recombination activation gene RAG1 [67,68], RAG2 [69], T-cell receptor (TCR) [70–72], major histocompatability complex (MHC) class I [73,74] and II [75] ika1 [76]. These genes are linked to the immune function of the thymus as is another gene of interest, ikaros, which is generally required for lymphoid cell development even before the appearance of the thymus [77]. More recent studies have started to identify other genes linked to thymic development such as Foxn1, which is a transcription factor expressed in thymic epithelial cells before the entry of lymphoid progenitors [78].

5. Molecular control of thymic development

The principal molecular steps in development of the thymus are reasonably well understood. Orchestration of several processes is necessary with neural crest cell development leading to potentiation.
of the pharyngeal endothelium, followed by neural crest migration to pharynx with the resulting epithelium—mesenchyme interactions and, finally, lymphocyte infiltration [2].

The pharyngeal endoderm becomes segmented before the neural crest cells arrive. It is thought this pre-patterning is determined by retinoic acid signalling [2]. Suzuki et al. [39] found retinoic acid depressed Hoxd-4 and shh causing malformations in cartilage. Retinoic acid patterning in the endoderm may determine where pre-determined neural crest cells entering the primordia condense to form cartilage. A mechanism like this would be needed or plates of cartilage rather than gills may result. In zebrafish, gcm is expressed in the epithelium of the third to seventh pharyngeal arches, and in macrophages following fgf3 expression. Gcm is thought to be a switch that acts on the neural crest cells to form cartilage. Hindbrain neural crest cells migrate mostly to the pharyngeal arches. Cells from rhombomere 6 migrate to pharyngeal arches 3 and 4, most commonly the site of thymus development. Myoblasts from somites follow the migratory trails of the neural crest cells. Hoxa3 is specifically involved in the formation of the third

Fig. 5. (a) The thymus of a 50-day-old Atlantic halibut. Dark staining lymphoid tissue is held within a capsule. Protrusion of the thymus into the pharyngeal cavity can clearly be seen. Th = thymus; OC = opercular cavity; Ot = otic capsule. Scale bars; for fry 1 mm; for micrograph 25 µm. The orientation is dorsal uppermost with anterior to the left. (b) Cross sections of carp 12 dpf, stained with haemalum eosin, showing the developing thymus (T) at the dorsal site of the branchial chamber, with gills (G) and operculum (O). (c) Sea bass thymus at 38 dph; the pharyngeal cavity is to the left. Used with permission of G. Scapigliati, Università della Tuscia, Viterbo, Italy.
branchial arch and the appearance of the thymic primordium [79]. The absence of Hoxa3 has effects on other pouch derivatives, so it is probably providing a positional signal [2]. Following this Foxn1 is required by the early thymus in order to attract thymocyte progenitors and has been detected in zebrafish [2].

Notch signalling is evolutionarily conserved and implicated in determination of binary cell fates. In mice B-cells develop by default in the thymus. Notch-1 is a large trans-membrane receptor that mediates cell–cell interactions, Notch signals are essential for T-cell development, both in thymus and GALT. Notch-1 signalling directly affects the T/B cell fate choice. Similarly in Drosophila, Notch inhibits the neuronal fate resulting in epidermal development [37,40,80]. Notch signalling is also implicated in the choice and ratio between αβ and γδ T-cell lineage [81].

Thymus is a specialised organ for T-cell production/selection. In many fish species, threads of cells connecting with the head kidney are reported [24]. This may be the route for lymphocyte migration from the thymus to the head kidney since it appears that the thymus is lymphoid before the head kidney. The first lymphocytes may migrate from an area near the liver [2,40].

6. Anatomical studies

The structures that characterise a thymus in fish are a capsule enclosing a cortex of lymphatoid tissue. In zebrafish, it has been shown that granulomas, which are aggregates of macrophages within a capsule, can be formed without the involvement of T-cells [82]. Basically, thymus can be considered as an encapsulated aggregation of macrophages that process the proliferation of T-cells. Differentiation of the structure of the thymus is highly variable within teleosts [12,83,84]. In many fish species there is no clear cortico-medullary differentiation as would normally be seen in higher vertebrates [16,84,85]. In mammals T-cell progenitors enter through the cortico-medullary blood vessels and can differentiate into NK cells, dendritic cells and T-cell lineages [86]. The parenchyma consists of lymphocytes, macrophages, dendritic/interdigitating cells and myoid cells [7]. Within the cortex are lymphocytes in a stroma of cells of epithelial morphology, and macrophages.
The capsule invaginates into the stroma producing trabeculae and giving passage to capillaries (in Atlantic halibut, Fig. 7). Trabeculae are very distinct in yellowtail [17]. This vascularisation by endothelial cells may provide the organising signals for formation of the medulla [27]. Interestingly, one report has emphasised that the medulla may be considered as a secondary lymphoid organ as it is accessible to exogenous antigens and lymphocytes [87]. The capsular cells are epithelial, and immediately beneath the capsule in the cortex is a layer of blast cells. These naïve thymocytes are in close contact with stromal cells. Within the medulla are large macrophages that phagocytose apoptotic lymphocytes. This must be accomplished effectively to avoid the risk of self-antigens stimulating autoimmunity [88] as in myasthenia gravis where the thymus is implicated in an autoimmunity to muscle [89]. Myoid cells have been shown in rats to secrete haemopoietic biglycan that is implicated in this condition. Soluble biglycan stimulates growth and differentiation in monocytes, including the microglia of the brain and they also produce a number of cytokines including IL-1alpha and monocyte growth factors [90,91]. Myoid cells have been reported in some species including Japanese flounder [16,17], rainbow trout [92], turbot [15,19], and zebrafish [18]. In Japanese flounder these myoid cells were about 25 μm in diameter [16,17].

The capsule encloses a 3D structure which develops from a 2D epithelium. The 2D arrangement persists in the GALT. The 3D is considered important for positive selection (affinity to MHC) to allow sufficient interaction with the MHC molecules of the reticular epithelium. Negative selection (self and excessive MHC) is more easily attained outside the thymus and dendritic cells may mediate this process [93].

With this development of specialisation the thymus loses the requirement to remain at its origin. With this freedom it is often internalised and migrates. The capsule confines the thymic microenvironment.

Fig. 7. Trabeculae invaginate into the cortex providing a route for vascularisation in a 100-day-old Atlantic halibut. Cap = capsule; T = trabeculae; S = sub-capsular layer; N = nurse-cell like aggregation; D = dendritic-like cell. Scale bar 5 μm.
Within the stroma is a matrix of epithelial cells. Classification of the epithelial cells varies in complexity between authors. For carp, as stated above, these have been classified into four types; limiting epithelial cells; reticular epithelial cells; nurse-like cells; Hassall’s body-like structures (as seen in Atlantic halibut, Fig. 8) [23]. Nurse cells are keratin containing epithelial cells that engulf thymocytes, and have been reported in fish. Reviewing work in chickens and mammals, it was reported that macrophages could be seen moving in and out of thymic nurse cells and the authors suggest this internalisation process represents clearance of non-functional apoptotic thymocytes [53]. These movements are probably in response to cytokines and chemokines [40,94].

In carp, more apoptotic cells were detected in the cortex than the medulla [23]. Inner and outer zones are reported in Yellowtail, red seabream and Japanese flounder [17]. Schneider reported the absence of zonation in young carp thymus but later on a complex intermingling of cortex into the medulla has been described for developing carp, zonation starts to become visible in the second week [23,49,95]. In carp, reticular cells are seen with large, long nuclei, loose chromatin, spongy-like nucleoli, and cytoplasm that is granular with processes between lymphocytes [95]. The size of lymphocytes can vary with species. A comparative study of three fish species revealed that lymphocytes are typically basophilic and 3–5 μm in diameter, whilst populations of darker staining small lymphocytes (2–2.5 μm) were observed in later development [17]. In rainbow trout, an outer and inner zone was differentiated with lymphocytes ranging from small 6–7 to large 10–11 μm diameter, and the nucleus was round, with a dent corresponding to an apparent chromatin adhesion [92,95]. Comparisons between species are not possible, however, as the size is not related to general cell size in the species.

Fig. 8. Hassall’s corpuscle-like object (H) in thymus of 100-day-old Atlantic halibut. Scale bar 5 μm.
7. Conclusion

The use of zebrafish, medaka and fugu as model species for the study of vertebrate development has advantages over mammal models as the embryos develop ex utero, are transparent and possess short lifecycles. An increasing range of genetic mutant strains of zebrafish is helping to unlock the complexities of vertebrate development. So studies on the fundamental mechanisms of vertebrate development, using zebrafish as a model, should advance our understanding of thymic development in teleosts as well as higher vertebrates.

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References