



Comparative study of cartilaginous fish divulges insights into the early evolution of primary, secondary and mucosal lymphoid tissue architecture[☆]

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ABSTRACT

Cartilaginous fish are located at a pivotal point in phylogeny where the adaptive immune system begins to resemble that of other, more-derived jawed vertebrates, including mammals. For this reason, sharks and other cartilaginous fish are ideal models for studying the natural history of immunity. Insights from such studies may include distinguishing the (evolutionarily conserved) fundamental aspects of adaptive immunity from the (more recent) accessory. Some lymphoid tissues of sharks, including the thymus and spleen, resemble those of mammals in both appearance and function. The cartilaginous skeleton of sharks has no bone marrow, which is also absent in bony fish despite calcified bone, but cartilaginous fish have other Leydig's and epigonal organs that function to provide hematopoiesis analogous to mammalian bone marrow. Conserved across all vertebrate phylogeny in some form is gut-associated lymphoid tissues, or GALT, which is seen from agnathans to mammals. Though it takes many forms, from typhlosole in lamprey to Peyer's patches in mammals, the GALT serves as a site of antigen concentration and exposure to lymphocytes in the digestive tract. Though more complex lymphoid organs are not present in agnathans, they have several primitive tissues, such as the thymoid and supraneural body, that appear to serve their variable lymphocyte receptor-based adaptive immune system. There are several similarities between the adaptive immune structures in cartilaginous and bony fish, such as the thymus and spleen, but there are mechanisms employed in bony fish that in some instances bridge their adaptive immune systems to that of tetrapods. This review summarizes what we know of lymphoid tissues in cartilaginous fishes and uses these data to compare primary and secondary tissues in jawless, cartilaginous, and bony fishes to contextualize the early natural history of vertebrate mucosal immune tissues.

1. Introduction – sharks and the evolution of immunity

1.1. Adaptive immunity

An adaptive immune response is initiated by one or both of two signals: the innate immune system being overpowered by the accumulation of antigen and lymphocyte antigen receptor signaling pathways. There are some pathogens that can be conquered by the innate immune system, but many pathogens require the intervention of the adaptive immune system in order to be cleared. Adaptive immune responses are activated in peripheral lymphoid tissues, such as spleen, lymph node, and GALT where naïve lymphocytes first encounter antigen and clonally proliferate. The mitotic lymphocyte proliferation maintains *specificity*

for antigen and creates memory cells against that antigen, providing the two hallmark characteristics of adaptive immunity.

Adaptive immunity has two major arms, cell-mediated immunity and humoral immunity. Cell-mediated immunity begins with naïve T cells, lymphocytes that have not been exposed to the antigen that is specific to their antigen receptor. These naïve T cells will circulate through the lymphatics, blood, and peripheral lymphoid tissues in search of antigen until the specific antigen is found. If the naïve lymphocyte does not find antigen, it will undergo apoptosis or programmed cell death. Once the naïve T cell has found specific peptide antigen presented by major histocompatibility complex (MHC) on a dendritic cell, or another activated antigen presenting cell (APC) expressing costimulatory signals, it no longer circulates through the vasculature and lymphatics and sustains a

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long proliferative phase, called clonal expansion, which includes differentiation into effector and memory T cells. These clonal descendants have the same antigen specificity as the original naïve T cell. The effector T cells enter the blood stream and migrate to the site of infection to perform their effector functions. There are two major classes of adaptive (generally $\alpha\beta$ T cell receptor-bearing) T cells: cytotoxic T cells, which don the coreceptor CD8, and helper T cells, which have the CD4 coreceptor. Cytotoxic T cells target and kill cells infected by intracellular pathogens such as viruses or intracellular bacteria. These target cells present peptide antigen on MHC class I on the cell surface to cytotoxic T cells which are MHC class I restricted. Helper, or CD4, T cells have several further subsets that carry out different functions via cytokines and the CD154 ligand for CD40. T_H1 cells help to control bacteria that persist in macrophages by evading traditional killing mechanisms of the phagocyte. The helper T cells activate infected macrophage microbicidal functions by recognizing peptide bacterial antigen being presented by MHC class II. T_H2 cells operate to control infections by parasites by activating and promoting eosinophil responses and instructing B cells to class-switch to the IgE isotype of antibody. T_H17 cells function to stimulate neutrophil responses and inflammation to protect against extracellular pathogens such as bacteria and fungi and can stimulate maintenance of epithelial barriers in the gut. Antigen presented to helper T cells generally must be presented by professional APCs (macrophages, dendritic cells, and B cells) using class II MHC, because CD4 T cells are MHC class II restricted and class II is not ubiquitously expressed, but MHC class I is.

1.2. Lymphoid tissues in mammals

Adaptive immunity requires lymphocytes to have interactions with antigen presenting cells and other lymphocytes in order to perform with regulated specificity and memory. These interactions occur in specialized sites in the body termed lymphoid tissues. Lymphoid tissues are broadly defined as any tissue in the body where an immune reaction or lymphocyte maturation takes place, largely based on studies in mammals. Lymphoid organs, first seen in jawed vertebrates, are thought to have evolved for the purpose of facilitating antigen-receptor gene assembly [1]. They are split into two main types, central (primary, or generative) and peripheral (secondary) lymphoid tissues.

Primary lymphoid tissues facilitate maturation of lymphocytes, bone marrow for B lymphocytes and thymus for T lymphocytes in mouse and man, as well as generating and shaping the repertoire of receptors for each cell type. All lymphocytes originate from hematopoietic stem cells in the adult mammalian bone marrow. Early thymocyte progenitors then migrate to the thymus and B lymphocyte precursors stay in the bone marrow for maturation. For both B and T cells, tests for autoreactivity occur before migration out of the primary lymphoid tissue and again in secondary tissues as mature naïve cells post-migration. B cells that do not react to self-antigen in the bone marrow survive and are said to have passed central tolerance. Upon leaving the bone marrow and journeying to the secondary tissues, such as the spleen, transitional B cells, which are not fully mature but have intact receptors, are exposed to self-antigen again. If the cells have too strong or too weak receptor signaling upon second exposure to antigen, they have not established peripheral tolerance and will not fully mature. Should the cells fail, they will either die or enter clonal anergy [2].

Mammalian T cell progenitors travel to the thymus from the bone marrow to mature. The thymus contains two anatomically distinct areas, the densely packed outer cortex and the exiguous inner medulla. This organization is conserved throughout jawed vertebrate evolution. The thymus contains many cell types from thymocytes (developing T cells) to stromal cells (epithelial cells of the thymus) to intrathymic dendritic cells and macrophages. The cortex contains immature thymocytes and sparse macrophages, whereas the medulla has more mature thymocytes and larger amounts of macrophages and dendritic cells. Thymocyte maturation mirrors that of B cells in sequentially testing the antigen

receptor for functional chains, signal transduction capacity and self-tolerance. In the T cell maturation process, positive selection additionally selects $\alpha\beta$ lymphocytes that recognize self-allelic forms of MHC, instructing cytotoxic or helper subset differentiation based on class I or class II recognition, respectively. If the cell reacts weakly to presented self-antigen in the context of self MHC, it is positively selected to survive in the cortex. Lymphocytes that react strongly to self-antigen are negatively selected and eliminated by programmed cell death (apoptosis) in the medulla. Only about 2% of T cells are successful in the maturation process and go on to differentiate into mature naïve subsets.

Once the lymphocytes have completed maturation and passed selection, they migrate to the secondary lymphoid tissues such as the spleen, lymph nodes and mucosal-associated lymphoid tissues (MALT). In these tissues, lymphocytes become activated upon exposure to antigen specific for their antigen receptor paratope.

Peripheral lymphoid tissues have distinct areas of T and B cell residency as well as besprinkled macrophages, dendritic cells, and stromal cells. The spleen contains specific lymphoid areas called white pulp that contain areas concentrated with T cells (periarteriolar lymphoid sheaths) and B cells (follicles). Germinal centers can develop in these B cell follicles, where follicular dendritic cells present native antigen complexes to B cells. Marginal zones of the spleen, locations between red and white pulp, also contain APCs such as dendritic cells and macrophages. Germinal centers are the location of genetic alterations such as class-switch recombination (CSR) and affinity maturation by somatic hypermutation (SHM). If the IgM made by the B cell is not the most effective functional class for a particular threat, CSR replaces the heavy chain constant region of that Ig to another Ig isotype, so the receptor may go from IgM to IgG, IgA or IgE [3]. Affinity maturation via SHM occurs when the rearranged V-region genes of the heavy and light chains of an Ig are bombarded with point mutations to produce a higher affinity antibody. This is not always the result; SHM can give rise to a higher, lower, or identical affinity [4], but competition for antigen and survival signals selects for the higher affinity in the iterative germinal center reaction. Lymph nodes, only present in birds and mammals, are small, lumpy peripheral lymphoid tissues for antigen concentration. They have multiple lobes and are enclosed by a tissue capsule. The body has a network of lymphatic vessels that carry lymph, a liquid containing antigenic material as well as immune cells, to the lymph nodes from all areas of the body. Lymph nodes are packed with lymphocytes, macrophages, and other APCs [5]. It is here where the APCs will present antigen to naïve antigen specific lymphocytes which will clonally expand to increase numbers of functional lymphocytes [6].

Mucosal associated lymphoid tissues (MALTs), such as respiratory epithelium, tonsils, and Peyer's patches of the intestine, provide physical barriers as well as lymphoid functions to combat pathogens. MALTs are arranged in a lymph node like structure with B cell follicles and T cell zones. Peyer's patches have specialized epithelial tissue, called follicle-associated epithelium (FAE), that lines the domes of the patch and houses T lymphocytes near the microfold (M) cells which transport antigen across the epithelial barrier [7]. A specific antibody, IgA, is restricted to mucosal immune tissues and plays a major role in mucosal immunity. IgA can be transported across the mucosal epithelium and secreted into the lumen in the form of secretory-IgA (sIgA) where it, then, binds antigen in the lumen or on the luminal surface of the mucosal tissue.

1.3. Sharks

"Cartilaginous fish" is an umbrella term for the Chondrichthyes, that encompasses sharks, rays, skates, sawfish, and chimeras. All these vertebrates have common characteristics of jaws, paired fins, gills, and a skeleton made of cartilage instead of bone.

Cartilaginous fish are the oldest group of living jawed vertebrates that have adaptive immune characteristics and lymphocyte antigen receptors similar to mammals, and, therefore, lie at a pivotal point in the

evolution of the immune system [8]. They are, also, the oldest group to have a polymorphic, polygenic MHC [9]. Cartilaginous fish are also the first group, phylogenetically, to have a true thymus whose structure was maintained, for the most part, throughout vertebrate evolution [1]. All of these indicate that cartilaginous fish mark the emergence of adaptive immunity as it is recognized throughout jawed vertebrate evolution, making their immune characteristics potential representatives of the ancestral building blocks of the system.

There are some peculiar immune characteristics seen in cartilaginous fish. For example, the genes for immunoglobulin (Ig) heavy and light chains are in many multicluster loci with single V, D, J, and C segments [10] which is seen in bony fish light chain loci as well [11], as opposed to the single large translocons containing many V(D)J segments in recent vertebrates. Without the multicluster organization of cartilaginous fish Ig loci the evolution of unique new antigen receptors (IgNAR and NARTCR) [12–14] as well as interactions between immunoglobulin genes with T cell receptor (TCR) genes, potentially, could not have occurred [15]. Classical MHC class I genes (UAA) are observed in many cartilaginous fish as well as some unique class I MHC genes such as UBA in nurse shark and horn shark [16–18] and UCA in dogfish [16,19]. Recent studies in nurse shark and horned shark show another nonclassical MHC class I gene, UDA present, to a lesser extent than the other nonclassical MHCs, in most organs [20].

Cartilaginous fish do not have bones or bone marrow but have other organs responsible for the development of blood cells and the maturation of B cells. The head kidney (anterior kidney, pronephros) is responsible for red blood cell production in addition to the spleen, which is also a site for antigen concentration [21]. The organs responsible for B cell maturation are the epigonal organ attached to the gonads [21] and Leydig's organ in the mid-dorsal area of the shark's body, associated with the esophagus [21]. Similar to other taxa of vertebrates, the T cells of sharks and rays mature in the thymus. The thymus originates from the pharyngeal pouches above the gills and, unique to fish, stays near each gill for the entirety of the fish's life. In humans, the thymus originates from the pharyngeal pouches but migrates to the anterior superior mediastinum and forms a single organ directly behind the sternum and in front of the heart.

Sharks are the most phylogenetically distant group from mammals to have immunoglobulins, long-term antibody-driven immunological memory [22], activation-induced cytidine deaminase (AID)-mediated somatic hypermutation (SHM), and recombination-activating gene (RAG) mediated somatic recombination. Recombination via RAG is the mechanism used in primary lymphoid tissues to form lymphocyte antigen receptors (e.g., TCRs and immunoglobulins) and this process is first seen in the cartilaginous fish. AID catalyzes somatic hypermutation for affinity maturation and related APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family members diversify variable lymphocyte receptors (VLR) in lamprey [23]. The AID gene and its immunogenetic activity is first seen in sharks, indicating its origin in cartilaginous fish.

2. Cartilaginous fish lymphoid tissues

2.1. Primary tissues in shark (and skate/ray)

The primary lymphoid organs seen first, in a primitive form, in jawless fish [24], gained complexity in cartilaginous fish and continued throughout the radiation of vertebrate evolution. As the name implies, cartilaginous fish have a cartilage frame as opposed to all more derived vertebrates which have bones. Several tissues contribute to B lymphopoiesis in cartilaginous fish, and different species employ them to varying degrees.

2.1.1. B cells in cartilaginous fish

Shark B cells are similar in appearance and antibody production to those of more evolutionarily recent vertebrates such as fish and

mammals. In fact, sharks are the oldest group, phylogenetically, to have these B cells [25].

Sharks possess a variety of Igs smaller than that of recent vertebrates, some of which are the same as or similar to those of mammals. IgM is an isotype seen in sharks as well as mammals, but the other two, IgW and IgNAR, are not like the immunoglobulins in mammals, although they may serve similar functions [25,26]. Primitive vertebrates, cartilaginous fish and lungfish, have IgW which resembles IgD in recent vertebrates with similarity in constant domain sequence and transmembrane portion suggesting that these heavy chain isotypes originate from the same ancestor [27]. IgNAR is an isotype that has two disulfide bonded heavy chains not associated with light chains, forming a homodimer that is exclusive to cartilaginous fish [12,28,29]. Though IgNAR is unique to cartilaginous fish and has no simple orthology to mammalian isotypes, IgNAR is functionally analogous to IgG, due to its late appearance in serum during development and affinity maturation [30–33]. Immunoglobulin isotypes are defined by the heavy chain constant region domains they employ, but sharks also use four different light chains: κ , λ , σ , and σ -2 [34].

The epigonal organ is attached to the gonads of the shark and is composed of sinuses similar to those observed in bone marrow; it is considered the closest to a bone marrow equivalent in sharks [31,35]. Essential steps in B lymphocyte maturation such as V(D)J rearrangement of immunoglobulins occurs in the epigonal organ [32,36]. The epigonal organ contains antibody secreting cells such as plasma cells and B1-a cells, similar to bone marrow in mammals [31,37,38]. Studies in clearnose skates (*Raja eglanteria*) show that expression of PU.1, an essential transcription factor in the development of lymphocytes, is restricted to the epigonal and Leydig's organs [39–41]. The Leydig's organ, the second of the specialized replacement lymphopoiesis organs in shark, is characterized as white, spongy masses on the dorsal and ventral sides of the esophagus beneath the epithelium, containing sinuses similar to those seen in bone marrow and the epigonal organs [42]. Not all cartilaginous fish have Leydig's organ. Some species, such as the nurse shark (*Ginglymostoma cirratum*), have only an epigonal organ [43]. Presence of a Leydig's organ will often result in the epigonal organ being dwarfed, suggesting they may serve similar functions and that the Leydig organ is a form of compensation of an overworked epigonal organ [44–46]. As in mammalian bone marrow, the epigonal and Leydig's organ both contain sporadic granulocytes [41], consistent as granulocytes mature in bone marrow and these organs appear to be the hematopoietic equivalent in sharks. Finally, there is some evidence that hematopoiesis occurs in the epigonal and Leydig's organ, as well as kidney and gut, in cartilaginous fish [47].

2.1.2. T cells in cartilaginous fish- thymus

The thymus is said to be the most ancient of lymphoid organs [48], as it is the first primary lymphoid organ present in phylogeny that has remained consistent throughout jawed vertebrate evolution. The thymus is a spongy organ located in the pharynx region of recent vertebrates, as it originates from one or more pharyngeal arches in development. Since sharks are fish and have gills for means of respiration one would find the thymus located above the gills bilaterally [49,50]. In sharks, some but not all pharyngeal arches in each gill basket provide the basis of development of the thymus.

Shark thymus can be single or multilobed depending upon species and stage of development [51–53]. For example, nurse shark thymus develops complexity as it develops, but this is not necessarily the case in all species of cartilaginous fish [54]. The thymus is covered in a connective tissue capsule, with extensions of the capsule, called trabeculae, dividing the lobes [55].

Sharks have several different T cell receptor (TCR) chains created using V(D)J somatic gene recombination thought to heterodimerize to give a fully functional TCR. There is TCR α predicted to dimerize with TCR β , TCR γ predicted to dimerize with TCR δ , and NARTCR. NARTCR is unique in that it is produced by doubly-rearranging V domain encoding

exons to make a TCR δ chain with a free variable without a pairing domain on TCR γ [13,56]. This protruding variable domain has high identity with IgNAR V, which also lacks a heterodimerization partner. *In situ* hybridization experiments in 2010 on nurse shark thymus localized the mRNA of these different TCR chains showing that TCR α and TCR β are located in the central cortex together with weak signal in the medulla, TCR γ is concentrated in the subcapsular region of the cortex as well as the central cortex, and TCR δ expression is highest in the subcapsular region of the cortex but has the most signal in the medulla of all TCRs [51]. These same experiments showed that MHC class I has a higher concentration in the medulla than the cortex, which is consistent with the idea of selection still occurring as the lymphocytes complete maturation [51].

Somatic hypermutation (SHM) is a process primarily used in B cell affinity maturation that nurse sharks also employ to diversify their T cell repertoire which is not seen in any other vertebrates, with the exception of camelids [57] thus far [51,58]. Ott et al. demonstrated SHM occurring in TCR γ , TCR α and TCR δ V segments with analysis of mutation data and following rearrangement lineages of nontemplate (N) and palindromic (P) nucleotides in sequences of nurse shark TCRs. Alongside this data, they confirmed SHM in the thymus by colorimetric *in situ* hybridization data showing expression of AID in the cortex. Fluorescent *in situ* hybridization on nurse shark thymus tissue for activation-induced cytidine deaminase (AID), the catalyst of SHM, showing sporadic signal throughout the cortex and increasing near strong foci of TCR α signal at the cortico-medullary junction provided further evidence of SHM via AID acting on TCRs in the thymic cortex [59].

2.2. Secondary tissues in shark (and skate/ray)

Secondary lymphoid tissues function as sites of antigen encounter allowing lymphocytes to interact with antigen, APCs, and one another to mount an immune response. The secondary lymphoid tissues of sharks are spleen and mucosal associated lymphoid tissues (MALT), including those associated with the gut, but lymph nodes are absent [60]. These organs each have characteristics unique to them that will be discussed in the sections to come.

2.2.1. Spleen

The spleen is considered the first peripheral lymphoid organ to be maintained throughout evolution, and sharks are the primordial living group to have a spleen [61,62]. Though there are other organs that provide a secondary function as lymphoid tissue, the spleen is the main hematopoietic organ and only bona fide secondary lymphoid organ in sharks [62]. It is suspected that the shark spleen could be the site of red blood cell formation as well as a potential site of plasma cell differentiation [31].

The lymphocyte zones, termed white pulp, are the areas composed of lymphocytes as well as developing plasma cells, active antibody secreting B lymphocytes [32]. Older nurse shark immunohistochemistry (IHC) studies show that the white pulp of shark spleen is divided into B and presumptive T cell zones with no defined border, but a concentration of B cell markers in the outer ring of the white pulp and T cell markers in the inner core [21,32]. More recent research in the model suggests that the white pulp is strictly B lymphocytes with no T cell zones [31]. Expression of RAG in the spleen of nurse sharks and clear-nose skates (*Raja eglanteria*) indicates it may have a role in B lymphocyte development as seen in Leydig and epigonal organs [63,64]. In the Aleutian skate (*Bathyraja aleutica*), secretory immunoglobulins are first seen in the spleen hinting it may be the primary site of B lymphocyte generation, but this has not been confirmed [65,66].

A characteristic of spleen seen in recent vertebrates is the germinal center, which appears post antigen exposure and is the location of affinity maturation of lymphocytes, proliferation of B lymphocytes into differentiated plasma cells, and the generation of memory B lymphocytes [67,68]. Periaarteriolar lymphoid (PALS) sheaths are collections of

T cells in the white pulp of spleen surrounding the central arterioles, also commonly seen in recent vertebrates. There is no evidence of germinal centers or PALS in sharks suggesting that these structures evolved later, perhaps in endotherms as frogs also appear to lack germinal centers [21, 69,70].

2.2.2. Mucosal associated lymphoid tissues (MALT)

Mucosal associated lymphoid tissues, also known as MALTs, are any secondary immune tissue serving the mucosal sites, either in the digestive tract, respiratory tract, reproductive tract or even skin in some species. MALTs, predominantly in digestive organs, are present in all vertebrates from lamprey to human [61]. The most common MALT, called GALT or gut-associated lymphoid tissue, can be found in the digestive tract, specifically the lower intestine. GALT can take many forms over many species; poikilothermic vertebrates lack organized MALT, whereas birds and mammals have organized MALT such as Peyer's patches and lymph nodes [71,72]. Studies in the Iberian ribbed newt (*Pleurodeles waltlii*) demonstrate that amphibians appear to have lymphoid aggregates in the gastrointestinal tract, but no organized tissue was observed [60].

As reviewed by Hart 1988, several species of cartilaginous fish, stingray (*Dasyatis skajei*), horned shark (*Heterodontus francisci*), and eagle ray (*Aetobatus narinari*), share common observations of GALT in the mid to low intestine [73] as well as dogfish (*Scyliorhinus canicula*) [74] which was not reviewed. Sharks do not have Peyer's patches, but they do have GALT located in the spiral valve, a region of the intestine whose function is to increase the surface area of the intestinal wall with extra folds and twists [64,71]. The spiral valve contains lymphoid aggregates beneath the epithelial surface but is not considered a lymphoid organ as the lymphoid aggregates are not encapsulated similar to the Peyer's patches in mammals; though it is thought that the GALT in spiral valve could be a primitive ancestor of the architecture in Peyer's patches [60,66]. In these lymphoid aggregates, one can expect to see a variety of cellular components including macrophages, lymphocytes, and granular leukocytes such as neutrophils, all of which are characteristic of sites of antigen encounter [74,75].

There is some evidence of B lymphopoiesis in spiral valve indicated by the expression of RAG, seen in skate as well as AID, seen in nurse shark [47,59,64]. Microscopy studies in the small-spotted catshark, *Scyliorhinus canicular* [75], and dogfish, *Scyliorhinus canicula* [74], showed a presence of lymphocytes and macrophages in the spiral valve.

Along the theme of GALT as a secondary lymphoid structure, studies in cartilaginous fish, *Scyliorhinus canicula* L., show that development of primary immune organs, such as the thymus, occurs before that of secondary immune organs, such as spleen and GALT [75]. Dogfish from different stages in development were collected and GALT tissue was embedded and imaged using light microscopy. Each specimen was dissected and inspected for the presence or absence of several lymphoid organs, for example the thymus, kidney, spleen, GALT, epigonal and Leydig's organs [75].

The gut is not the sole location of mucosal immune tissues in sharks. There is evidence of some immune aggregates in shark skin as well as gills in the presence of lymphocytes and granular cells [76,77]. Meyer et al. showed transmission electron microscopy (TEM) images of *Scyliorhinus canicular* skin as well as light microscopy images of toluidine blue stained nurse shark skin both with embedded granular cells in the epidermis [76]. Little else has been said on the potential for SALT, skin associated lymphoid tissue, in cartilaginous fish. Another potential site of shark MALT is the respiratory tract which is filled with mucus and a prime site of antigen entry. Sequence analysis and quantitative-PCR studies in the White-spotted bamboo shark, *Chiloscyllium plagiosum*, reveal MHC class II signal in the gill, suggesting it is a site of antigen presentation by professional APCs, and, therefore, a potential secondary lymphoid tissue [78]. Results from northern blot observing the expression of lymphocyte receptors in various tissues of nurse shark showed high expression of TCR γ and δ , in the gill [51] which indicates the T cell

defense present at mucosal sites, such as the gill, could have an early emergence in evolution.

3. Comparison of lymphoid tissue architecture in early vertebrate lineages

3.1. Agnathan to gnathostomes

Agnatha is the first group to demonstrate primitive adaptive immune characteristics, although they are much simpler than the characteristics of mammals or even sharks [62]. Lamprey have an elementary body plan which does carry over to their immune organs. Thymus, spleen, bone marrow, and other lymphoid tissues are lacking in lamprey (Fig. 1), but they do have analogous structures seeming to be the starting point for the immune structures we are familiar with in recent vertebrates (Fig. 2). Lymphoid-like cells do aggregate in certain areas of the lamprey such as the kidney, gill basket, and gut [24]. Where the definition of primary and secondary lymphoid organs is more rigid in recent vertebrates, primary is the site of maturation and differentiation and secondary is antigen exposure, those of jawless fish are far more fluid. Primary lymphoid organs in hagfish and lamprey can serve a multitude of functions as well as a site of lymphocyte-like cell maturation, a hematopoietic site, or even a site of antigen exposure [79]. Though some organs, such as the thymoid and typhlosole, are more restricted in their functions as primary lymphoid organs, several others, such as the kidney, gill and supraneural body, are lymphoid tissue with other purposes.

Hagfish and lamprey have different lineages of lymphocyte-like cells with receptors called VLRs that are analogous to receptors seen in adaptive immune cells of recent vertebrates. VLRA is an $\alpha\beta$ T lymphocyte analogue whereas VLRB is B cell and VLRC resembles $\gamma\delta$ T cells in location and putative function, based largely on transcriptional profiles

[80–82]. VLRB bearing cells are much more numerous than their counterparts in every tissue except the gill region where VLRA are dominant, but both are detectable in primary lymphoid organs such as blood and kidney [83].

The thymoid is a thymus-like structure in lamprey located in the gill basket and is considered the primary lymphoid organ of agnathans (Fig. 3). The thymoid is packed with lymphocyte-like cells, predominantly VLRAs, and epithelial cells [84]. The thymoid, much like the thymus, originates from the pharyngeal arches of the lamprey, but, unlike the thymus which develops from the third arch in tetrapods [85], it is not restricted to a particular pharyngeal arch from which to develop [84]. Upon antigen exposure, lymphoid cells of secondary organs proliferate whereas cells of primary lymphoid organs do not. In lamprey, the kidney and typhlosole lymphocyte-like cells proliferate, but those of the thymoid do not indicating that the thymoid is the primary lymphoid tissue [84]. The thymoid is also home to the assembly of VLRA and C which is also where the highest expression of these (potentially TCR analogous) genes is seen [84].

Hematopoietic activity in lamprey is seen concentrated in the intestine, kidney, gill region, and typhlosole (Fig. 3) [86]. The typhlosole is located in the gut of agnathans and is packed with lymphocyte-like cells and stromal cells; it is the main lymphopoietic and hematopoietic organ in developing lamprey larvae [24,83,87]. The typhlosole has higher expression of VLRB cells as opposed to VLRAs, and contain the lamprey equivalent to plasma cells, activated VLRB cells [83]. The kidney is considered another VLRB lymphopoietic organ in agnathans and is full of lymphocyte-like cells, which is similar to teleost fish who show hematopoietic activity in the anterior kidney [61,83,84]. These suggest physiology analogous to primary and secondary B lymphopoiesis in agnathan kidney and typhlosole, respectively, if VLRB and VLRB-expressing lymphocytes are indeed the humoral arm of agnathan








	Primary				Secondary		Mucosal	
	Thymus	Bone Marrow	Levdig's organ	Epigonal organ	Spleen	Lymph Node	GALT	Spiral Valve
 Jawless Fish	✓★						✓	
 Cartilaginous Fish	✓		✓	✓	✓		✓	✓
 Bony Fish	✓				✓		✓	
 Amphibians	✓	✓			✓		✓	
 Reptiles	✓	✓			✓		✓	
 Birds	✓	✓			✓	✓	✓	
 Mammals	✓	✓			✓	✓	✓	

Fig. 1. A table comparing classes of vertebrates and the lymphoid tissues present in each where checks indicate the presence of a tissue. Each tissue has its point of emergence, like the thymus in cartilaginous fish, the lymph node in birds, bone marrow in amphibians, and GALT as far back as jawless fish. The star indicates that lamprey have a thymoid region potentially with similar function to thymus, but it is not a true thymus.

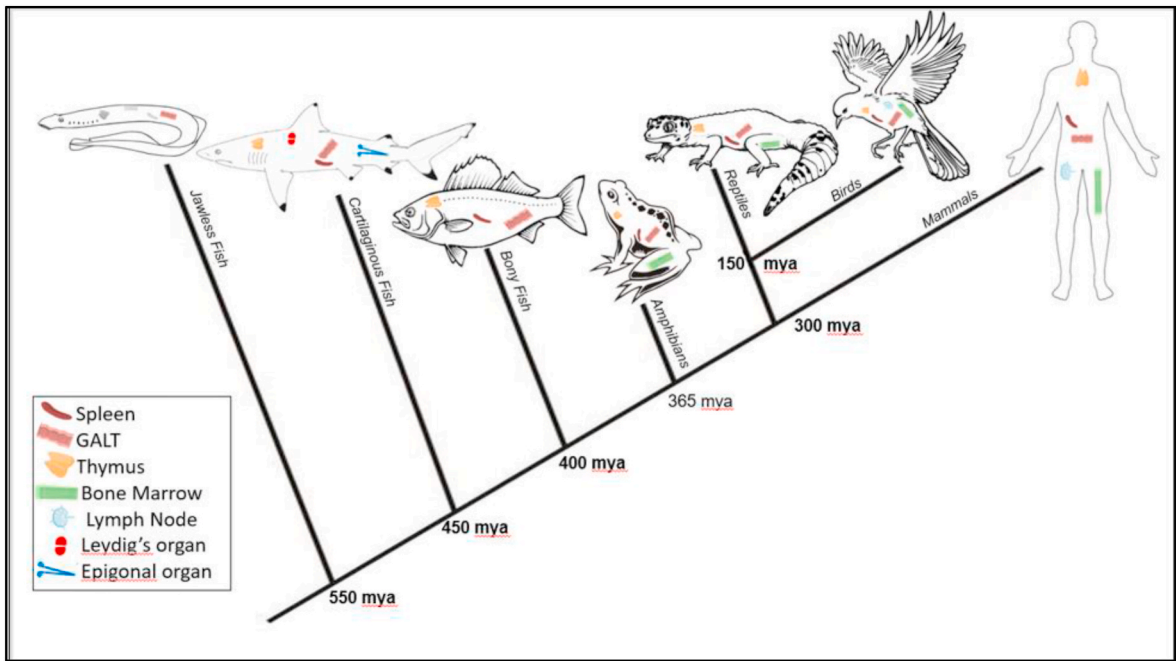


Fig. 2. Phylogenetic tree portraying the emergence of these key organs and tissues. As stated in Fig. 1, bone marrow is not present until amphibians, lymph nodes do not appear until birds, true thymus tissue is first seen in cartilaginous fish, and GALT is ubiquitous across all groups. The gray colored tissues in the jawless fish indicate the analogous primary lymphoid function of thymus and bone marrow and secondary function of spleen are present, but in other tissue unique to agnathans.

Jawless Fish			Cartilaginous Fish			Bony Fish		
Primary*	Peripheral*	Mucosal*	Primary	Peripheral	Mucosal	Primary	Peripheral	Mucosal
Thymoid A 	Kidney B 	Typhlosole (GALT) C 	Thymus D 	Spleen E 	Spiral Valve F 	Thymus G 	Spleen H 	GALT I
Supraneural body J 		Gill K 	Epigonal Organ L 	Leydig's Organ** M 	Gill N 	Head Kidney O 	Pancreas** P 	Gill Q

*not limited to these roles. Can have crossover roles of primary, secondary and mucosal immune function.
** A primary organ that is not present or immunological in every species.

Fig. 3. A table of diagrams illustrating the structure and microarchitecture of each lymphoid organ or tissue present in agnathans, cartilaginous fish, and bony fish. Beneath the diagram are photomicrographs of the organ or tissue represented in the diagram. (A,B,C and K) [76] (D and E) Personal images (F) [67] (G) [100] (H,O, and Q) [90] (I) [104] (J) [107] (L) [108] (M) [109] (N) [110] (P) [92] Copyright (2002) National Academy of Sciences, U.S.A.

adaptive immunity.

The supraneural body (SB), also called fat body, is placed dorsal to the agnathan vertebra and has been compared to bone marrow in recent vertebrates, because it is packed with a variety of blood cells in all stages of development as well as blood cell precursors [24,83,88]. Though there is some VLR rearrangement occurring in the SB, it is considered a secondary lymphoid structure (Fig. 3), because the lymphocytes present in the SB proliferate upon antigen exposure, a key characteristic of

secondary lymphoid tissues [24,83]. In adult sea lamprey, the SB is the most important blood forming organ, but hematopoietic activity begins before metamorphosis from larvae to adult lamprey replacing the functions of the typhlosole and kidney [24,88]. As the sea lamprey develops from larvae to adult, the blood cell forming activity of the typhlosole, nephric fold, and pharyngeal region decreases until it diminishes entirely just before metamorphosis where SB activity increases starting at metamorphosis through adulthood [24].

The expression of cytosine deaminase (CDA) 1 and 2 directly correlates with the presence of certain VLRs. As AID is to TCR and BCRs in recent vertebrates, CDA catalyzed receptor editing of VLRs. CDA1 expression is associated with VLRA where CDA2 is with VLRB; the expression of CDA can be used to assume locations of VLRs in tissues [84]. The expression of CDA1 fills the entire gill basket, which contains the thymoid, and suggests the thymoid has no true point of origin within the pharyngeal arches, as well as the thymoid is, most likely, the primary lymphopoietic tissue for VLRA lymphocyte-like cells [84].

3.2. *Gnathostomes to bony fishes*

The branch after cartilaginous fish in vertebrate phylogeny contains the bony fish, also known as Osteichthyes. This superclass is split into two classes: Sarcopterygii (lobe-finned fish) and Actinopterygii (ray-finned fish). Teleost fish, a group within bony fish, are the most studied group of Osteichthyes in comparative immunology. Even within this group, there is great variation between species regarding which organs they use for hematopoiesis and lymphocyte differentiation.

The term bony fish may be confusing from an immunological standpoint, because, though they do have bones, Osteichthyes do not have bone marrow as seen in recent vertebrates such as birds and mammals (Figs 1 & 2) [1,25,60]. Instead they have analogous structures in the head kidney, also called the anterior kidney or pronephros, and spleen that are responsible for hematopoiesis and B cell differentiation [47,72,89–91]. Expression of RAG [92,93] TdT [94], and Ikaros, a transcription factor essential to lymphocyte maturation and lineage commitment for both T and B lymphocytes [47,94], in Atlantic cod (*Gadus morhua*) and rainbow trout, *Oncorhynchus mykiss*, indicate the head kidney must be a site of immune cell production and differentiation. Fish express a AID-homologue, for somatic hypermutation, but fish do not perform class-switch recombination [12,28,95,96]. The AID enzyme characterized in catfish (*Ictalurus punctatus*) [97] and zebrafish (*Danio rerio*) [95] show a longer cytidine deaminase motif than that of mammals and chickens as well as ample substitutions in the carboxy-terminal region, both of which are required for CSR.

Salmonid studies in Atlantic salmon (*Salmo salar*) and rainbow trout reveal mature B lymphocytes in the head kidney that travel to the spleen for activation [98]. Studies in zebrafish show RAG1 expression in kidney demonstrating there is gene recombination occurring in the kidney, indicating it is a site of lymphocyte differentiation (Fig. 3) [99]. The pancreas of zebrafish, as well as the head kidney, serve as sites for B cell development [100], which is interesting due to the primary function of the pancreas being endocrine in the secretion of hormones, such as insulin and glucagon, and exocrine in the secretion of digestive enzymes, such as proteases and lipases.

Similar to those that came before them, bony fish use the thymus as a site of T lymphocyte differentiation and maturation. The thymus of bony fish is located dorsal to each set of gills and is said to have originated from the third pharyngeal pouch in development [60,101]. Studies in zebrafish and medaka (*Oryzias latipes*) show the teleost thymus only has one thymic lobule, but there is a range of single lobed to multilobed thymus structures in other teleost fish [25,102]. Histological findings in zebrafish [103], turbot (*Scophthalmus maximus* L.) [104], rainbow trout [105], salmon [106], carp (*Cyprinus carpio* L.) [89], sea bass (*Dicentrarchus labrax* L.) [107] and atlantic halibut (*Hippoglossus hippoglossus* L.) [108], indicate there is distinction between the cortex and the medulla of thymus, even if there is no clear corticomedullary junction

[109]. Genomic studies in zebrafish show expression of RAG in the thymus at exponentially higher concentrations than in kidney, implying it is the most productive primary lymphoid organ in zebrafish [99].

Teleost fish have similar secondary lymphoid organs, spleen and GALT, to the sharks that predate them as well as not yet evolving lymph nodes seen in mammals. Teleost spleen does resemble that of sharks in that it lacks germinal centers but still contains white and red pulp. Studies in trout and medaka indicate there is distinction between B and T cell zones in the white pulp of spleen, but in zebrafish there is no clear resolution between the two [62]. Teleost GALT is more dispersed than we see in earlier phylogeny as studies suggest the second portion of the gut is an immunogenic area high in cell-mediated response [110,111]. The GALT of teleost fish lacks defined organization such as a Peyer's patch, but they do have cells called intestinal epithelial lymphocytes (IEL) which function as epithelial cells with immunogenetic qualities similar to those seen in recent vertebrates [60,72,112].

Teleost fish have other secondary lymphoid tissue that is not as commonly seen in their cartilaginous counterparts, such as non-gut associated MALTs. The gills of fish are highly immunogenic due to the opportunity for pathogen entry [113,114] and are known as GALT or ILT, intrabronchial lymphoid tissue [98,115]. The skin also contains diffuse lymphoid tissue, referred to as SALT, that contain T cells, B cells expressing IgT (a mucosal antibody), and microbiota [115]. Also associated with the respiratory system of teleost fish, diffuse lymphoid aggregates in the nasopharynx, NALT, is located in the olfactory organ and has a very high percentage of B cells and T cell markers but no definite evidence of T cells [115]. NALT is not considered a secondary lymphoid organ, but a lymphoid aggregate [115,116].

4. Conclusions

Not enough studies look beyond anatomic, histologic and genetic characterization into function of these proteins, cells and tissues. More mechanistic studies of peripheral lymphoid tissues (including MALT) in animals immunized via distinct routes and infected with distinct classes of pathogen will be important for understanding the role of the cartilaginous fish peripheral immune tissues, and that early step in the evolution of our own. Understanding the journey of our immune system through evolutionary time is essential in fully understanding the physiology of the system. Sharks are at a pivotal point in evolution where they display so many of the characteristics of mammalian immunity, but often in its most basic form. More studies should be conducted in primitive vertebrates to better connect the dots in early adaptive immune evolution.

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