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A cold-blooded view of adaptive immunity

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Abstract

The adaptive immune system arose 500 million years ago in ectothermic (cold-blooded) vertebrates. Classically, the adaptive immune system has been defined by the presence of lymphocytes expressing recombination-activating gene (RAG)-dependent antigen receptors and the MHC. These features are found in all jawed vertebrates, including cartilaginous and bony fish, amphibians and reptiles and are most likely also found in the oldest class of jawed vertebrates, the extinct placoderms. However, with the discovery of an adaptive immune system in jawless fish based on an entirely different set of antigen receptors — the variable lymphocyte receptors — the divergence of T and B cells, and perhaps innate-like lymphocytes, goes back to the origin of all vertebrates. This Review explores how recent developments in comparative immunology have furthered our understanding of the origins and function of the adaptive immune system.

Most studies that have defined the field of immunology were done in mammals, especially mice and humans, and most of our laws and paradigms are derived from these models. Yet, one of the fairly new laws developed by Janeway and Matzinger[1,2], which states that adaptive immunity is called to action not by foreignness but rather by external or internal danger via pattern recognition receptors (PRRs), has its origins in the study of *Drosophila melanogaster* Toll-like receptors[3]. Additionally, more than 50 years ago, the divergence of two major subsets of lymphocytes (namely, B cells and T cells) was partially revealed in studies of the bursa of Fabricius in birds[4]. In order to appreciate the origins of adaptive immunity, we must look to the cold-blooded (also referred to as ectothermic or poikilothermic) vertebrates, as well as to the immediate ancestors of the vertebrates, the so-called lower deuterostomes. Breakthrough discoveries over the past decade have ushered in a growing awareness of adaptive immune origins and alerted us to new possibilities.

A convenient way to appreciate the evolution of immunity is to compartmentalize the immune system into components that are conserved over time versus those that change rapidly, an idea originally put forward by Jan Klein[5]. Certain immune features, which will be described in some detail in this Review, such as the structure and function of

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immunoglobulin M (IgM) and the presence of a thymus, spleen, conventional αβ T cell receptors (TCRs) and MHC class II molecules, are highly conserved in almost all gnathostomes (jawed vertebrates). By contrast, other immunological components like IgD, the γδ TCR, natural killer (NK) receptors (NKRs) and nonclassical MHC molecules are plastic, always present but often differing in gene numbers, domain organization and function. Keeping this standard in mind provides a framework for understanding the foundation of the immune system: preserve the tried and true, but permit evolutionarily rapid changes with other complementary features to combat ever-changing pathogens.

When studying the evolution of any system, one makes the reasonable assumption that features shared between two divergent species were likely present in their common ancestor. Some similar characteristics, however, may be derived by convergent evolution; examples include the emergence of two different sets of antigen receptors in jawless and jawed vertebrates[6] and of single-domain immunoglobulin variable (V) regions in sharks and camels[7]. One must also recognize, and this is a common egregious error, that the shorthand manner of drawing representative species in figures (for example, see FIG. 1) is not meant to imply that the species derived from older taxa are ancestral to those derived from a more recent common ancestor. That is, the common ancestor of two distantly (or even closely) related organisms almost certainly was quite different from either descendant[8,9]. Finally, certain characteristics of a system can be lost in certain groups, which is the case for many of the bony fish that have been examined but is also true of all vertebrate classes. Keeping all these evolutionary attributes in mind, however, the salient adaptive immune features that we take for granted arose over a fairly short period of time in early gnathostomes, most likely in the extinct placoderms, in the so called evolutionary 'Big Bang'[10] (Fig. 2).

While the jawless fish have cells like T cells (both αβ and γδ T cells) and B cells (FIGS. 1,2), as well as a thymus equivalent, the MHC has proved elusive; like the variable lymphocyte receptors (VLRs), an MHC may have arisen in this group via convergent evolution[11,12]. Mucosal adaptive immunity is present and unique for each group, as studied in amphibians and bony fish (and mammals)[13–15]. Cartilaginous fish, bony fish and amphibians display features that distinguish the entire vertebrate phylum (FIG. 1) but also (in some cases) have distinctive features that are found in subtaxa. Prominent in the bony fish, and consistent with the general rapid evolution of this vertebrate class, the immune system has unique features in different taxa such as the loss of an MHC class II system and certain immunoglobulin isotypes[16,17]. Cartilaginous fish have a distinctive immunoglobulin organization that has permitted the emergence of antigen receptor genes with novel functions[18,19]. Although NK cells clearly exist in ectotherms, identification of NKRs has been exceedingly difficult owing to the rapid evolution of this system[20]; nevertheless, NKR genes, which are linked to the MHC, have been found in all vertebrates, demonstrating an early (likely primordial) association of MHC class I and NKRs[21,22]. Ancient lineages of genes encoding MHC class I molecules, immunoproteasome and transporter associated with antigen processing (TAP) molecules are found in the MHC of all fish and amphibians studied so far, but in mammals, this prototype has been superseded by a system that is less rigid in MHC class I peptide specificity[21,23–25]. A large leap forward in evolution is clear in amphibians, which show canonical antibody class switching and have an IgG class (known as IgY) that is involved in typical memory immune responses[26] (FIG. 2). The last major

advance in vertebrate evolution was the advent of lymph nodes and the formation of germinal centres in mammals[27–29]; the ectothermic vertebrates allow for the study of immunity that preceded the emergence of follicular dendritic cells (FDCs), the major cellular player in mammalian affinity maturation[28,30]. This Review will delve into all of these characteristics of adaptive immunity, focusing on basic questions that intrigue all immunologists, and explore what we can and should tackle over the next decade. It should be noted that endothermy is found in several taxa in the lower vertebrates, but by and large, their adaptive immunity has not been examined. Here, I will concentrate on the bulk of studies in fish, amphibians and reptiles.

Evolution of antigen receptors

Gnathostome antibodies and TCRs are members of the immunoglobulin superfamily (IgSF)[31] and are derived from an unknown precursor; a general model has been proposed and is discussed below[32–34]. By contrast, the jawless fish VLRs belong to the ancient leucine-rich repeat (LRR) receptor family, possibly originating from (and most related to) a cell surface receptor expressed on platelets[35]. Very high levels of diversity can be generated in both types of antigen receptor during lymphocyte development, but the gene rearrangement mechanisms used to generate diversity are entirely different.

Variable lym phocyte receptors.

Some old studies examining basic immunity, both humoral and cellular, suggested that agnathans had adaptive immunity, with specific responses to different foreign antigens and alloantigens[36]. However, extensive searches for antibodies, TCRs and MHC molecules over 30 years proved fruitless[37], and most comparative immunologists came to believe that, despite the earlier functional work, there was no adaptive immune system in agnathans[38]. This assumption was proved incorrect by Pancer and Cooper, who examined the transcriptomes of lymphocytes from immunized lampreys and discovered a large number of LRR-containing proteins with varying numbers of internal repeats[6] (FIG. 3). These LRR-containing receptors were shown to be clonally expressed and generated by rearrangement during lymphocyte ontogeny, and they were christened VLRs. The first of these genes to be studied was *VLRB*, which is expressed in a subset of lamprey lymphocytes. The VLRB protein is anchored by glycosyl phosphatidylinositol on the surface of naive lymphocytes in lampreys and then secreted (through an unknown mechanism) as a pentamer of dimers following antigenic stimulation[39] (FIG. 3). At first, it was thought that stimulation of the lamprey lymphocytes was similar to a T cell-independent immune response in mammals, that is, the crosslinking of antigen receptors on lymphocytes, likely in combination with another signal through a PRR, might activate B cells to induce VLRB secretion[40]. However, surprisingly, it was later found that a second rearranging VLR gene locus, *VLRA*, was expressed by another subset of lymphocytes, with a transcriptome similar to that of gnathostome T cells[11]. Further analysis revealed a third antigen receptor, VLRC, expressed by cells largely in epithelia and mucosa, and thus agnathan T cells seem to be split into two subsets, perhaps like gnathostome $\gamma\delta$ and $\alpha\beta$ cells[41].

IgM antibodies.

Multiple antibody isotypes are found in all gnathostomes (FIG. 3). IgM has been known for many years to be the primordial antibody class[26], although there are unique and intriguing features of IgM in each vertebrate class. In mammals, IgM is secreted as a pentamer associated with the joining (J) chain. IgM in cartilaginous fish is found in two forms: a multimeric form, as is present in all other vertebrates, and a monomeric form, which is most common in shark antigen-specific responses and is likely produced in a T cell-dependent manner[42–44] (note, while monomeric IgM is also found in all other vertebrates, only in cartilaginous fish has it been shown to be of major physiological relevance in the course of an immune response). The secreted form of bony fish IgM is a tetramer, and the J chain gene has been lost from IgM in this group, as it is present in the older cartilaginous fish[45–47]. Interestingly, the extent of disulfide bonding in secreted teleost IgM is modified over the course of an antigen-specific response in a type of affinity maturation that seems unique to this antibody class[48]. In addition, the transmembrane form of bony fish IgM is alternatively spliced so that it has only three constant (C) domains (as opposed to the usual four) for unknown functional reasons[49]. In summary, IgM is the major serum antibody found in bony fish and is produced by these animals in response to conventional antigens; cartilaginous fish use IgM as an innate antibody class as well as in adaptive responses but also use other antibody isotypes in adaptive immunity, and amphibian IgM (to our best knowledge) functions much like its mammalian orthologue.

IgD and IgW antibodies.

IgD, long believed to be a recent addition in vertebrates, is actually very old, also going back to gnathostome origins as an isotype found in cartilaginous fish called IgW[50,51] (FIG. 3). An immune-globulin resembling IgD was found first in bony fish, which was very surprising considering that in 1998, IgD had been found only in some mammals[52]. Subsequently, the IgD gene was uncovered in the genomic database of the amphibian genus *Xenopus* and shown to be expressed, like in mammals, via alternative splicing of IgM and IgD mRNA in naive B cells[50,53]. The cartilaginous fish homologue of IgD, IgW[54,55], was also found in lungfish[56], and in coelacanths, it seems to be the major isotype, with this famous species having lost IgM[57,58]. Recent data in bony fish and humans suggest that IgD is involved in inflammatory responses, binding to an unknown Fc receptor on basophils[59,60]. Compared with IgM, IgD is quite plastic in both structure and function; IgM is quite similar throughout evolution, whereas IgD is highly variable in terms of numbers of C domains (and even in the presence of IgD itself) and likely in terms of function as related to the transmembrane (largely unknown) and secretory (arming of innate cells) forms[51].

IgG and IgY antibodies.

Mammalian IgG emerged as an isotype called IgY[61] in amphibians. IgY arose concurrently with the first canonical class switch recombination (CSR) (FIGS. 2,3). Like IgM, the IgY heavy (H) chain has four C domains. Mammalian IgG and IgE are both related to an IgY common ancestor, with the IgE H chain maintaining four C domains and the IgG H chain losing its CH2 domain. In *Xenopus*, the switch to IgY, and hence its expression, is entirely T cell-dependent, and typical B cell memory generation as defined in mammals appeared in

this group. The cytoplasmic tail of both transmembrane IgY and IgG has a novel signalling motif that contributes to the proliferative burst of B cells in a memory response[62,63]. Besides its T cell-dependence, very little is known about the conditions for IgY class switching (see below) outside of mammals. An isotype related to IgY but lacking the two C-terminal domains, called IgF, was found in the *Xenopus tropicalis* genome; its function is unknown[53].

Other antibody isotypes and light chains.

There are other 'dead end' H chain immunoglobulin isotypes that arose in various vertebrate taxa, which have been recently reviewed in great detail[64–66], whose functions are unexplored. Mucosal isotypes, like the classical IgA in mammals, are described below.

Unlike what was thought for many years, the divergence of light (L) chains into κ and λ goes back to the origins of gnathostomes[67,68]. In most ectotherms, there is a third primordial L chain, σ, which was lost in reptiles[69]. There are L chain subgroups in some taxa, which are also dead ends like the H chains, especially in fish. In all the cartilaginous fish, the λ genes are 'germline-joined'[70], which allowed for an excellent analysis of somatic hypermutation (SHM) of all complementarity determining regions (CDRs) in sharks[71]. In all vertebrates, L chain preferences for certain H chain isotypes have been noted, which is one potential purpose for the multiple L chains[72,73]. Alternatively, it has been proposed that the different L chain isotypes might permit very different conformations of CDRs when paired with similar H chains[67,74]. Finally, multiple L chains allow for receptor editing in cells with a functioning H chain in cases where the L chain association provides a self-reactive receptor or the first L chain gene rearrangement is non-productive[75,76].

αβ T cell receptors.

Generally speaking, αβ TCRs are well conserved across evolution[77,78] (FIGS. 1,2). In cases where it is technically feasible, embryonic or neonatal thymectomy experiments have shown that adaptive immunity is dependent on T cells, including high-affinity, switched antibodies and cellular immunity to allografts and viral infection[79,80]. TCR diversity is quite high in all animals that have been studied, except in studies of cells resembling natural killer T (NKT) cells (see below). Next-generation sequencing in certain bony fish has shown a large diversity of αβ TCRs involved in their responses to antigens, such as viruses[80–82].

γδ T cell receptors.

During the years of TCR discovery, a new rearranging antigen receptor was discovered for which there was no known function; it was later named TCRγ[83]. After the discovery of its partner TCRδ a few years later[84], γδ T cells became the pariah of adaptive immunity, and only now are we beginning to grasp their recognition of antigens at the molecular level, at least for the γδ T cells involved in innate immunity[85]. In all ectotherms except bony fish (and in placental mammals), there are a large percentage of TCRδ genes that bear the immunoglobulin V region H chain (IgVH) as the recognition element (BOX 1). The immunoglobulin H chain (IgH) and TCRδ loci are linked in several vertebrates[86–88], suggesting that the loci were derived from a *cis* duplication[89,90], and it has been long recognized that TCRVδ and IgVH share attributes, especially a large size range of CDR3 sequences[91]. The relationship between IgH and TCRδ is both ancient, via the *cis*

duplication, and ongoing, via the transfer of IgVH into the TCRδ locus in several vertebrates. Additionally, *trans*-rearrangements of shark IgM and IgW V segments to TCRδ diversity (D) and J segments from nearby immunoglobulin clusters are found in high levels[78]. These data suggest that a large proportion of γδ T cells act in an adaptive fashion, which has been borne out in several studies in mammals[92,93]. Future work should be done to analyse γδ T cell adaptive responses in ectotherms, which is just beginning to be accomplished[93]. This is especially of interest in cartilaginous fish, where the TCRγ V genes have been definitively shown to hypermutate, similar to what has been detected in immunoglobulin genes[78,94].

Generating diversity: AID versus RAG.

Proteins encoded by recombination-activating genes (RAGs) are required for rearrangement of all antigen receptor genes in gnathostomes[95]. RAG1 does most of the work cleaving recombination signal sequences (RSSs) and is involved in RSS recognition, while RAG2 guides and cooperates with RAG1 in gene rearrangement[96]. Recently, a RAG transposon complete with RSSs (or at least terminal inverted repeats), insertion sites and genes encoding the RAG enzymes was uncovered in a lower deuterostome (predating the vertebrates) called amphioxus[97]. There are several of these transposons in the genome, and phylogenetic analyses suggest that they are still active in this and related species[98]. In addition, the catalytic core of *RAG1*, a transposon called Transib, is found encoded in several invertebrate species[99], showing that this transposon is quite old. Questions remain as to whether *RAG2* was part of the original transposon or whether it was an existing gene in the genome recruited to assist RAG1 in gene rearrangement[100,101]. Note that the RAGs are not used for VLR gene rearrangement in agnathans (see below), yet as mentioned, RAG1-like and RAG2-like genes have been found in the genomes of lower deuterostomes whose ancestors predated the vertebrates, such as sea urchins and amphioxi, where their functions, if any, are unknown[100,102].

Activation-induced cytidine deaminase (AID) was identified in mice and humans in 2000 as the enzyme required for both SHM and CSR in mammals[103,104]. Not long afterwards, AID was detected in all gnathostomes and shown in several species to be capable of mutation and expression in secondary lymphoid tissues[105–108]. The AID amino and carboxyl termini are required for SHM and CSR, respectively, thus it was surprising that AID from bony fish, a phylogenetic group clearly lacking CSR, was capable of inducing CSR in vitro[109,110]. One possibility is that the bony fish lost the CSR capacity because cartilaginous fish, formerly believed to be incapable of CSR because of the cluster-type organization of their genes (FIG. 1), indeed can undergo CSR among different IgM clusters, as well as between IgW and IgM clusters[111]. No typical switch boxes with either the repetitive elements or the targeting RGY (R=adenine or guanine, G=guanine, Y=Cytosine or Thymidine) motifs are found in the shark non-coding regions, so the switch mechanism is unknown as is the relationship to typical CSR. Nevertheless, this exciting finding in the oldest vertebrates with typical adaptive immunity heralds a new area for the study of AID and CSR.

AID is a member of the apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC) family of enzymes, which were first discovered as modifiers of mRNA

splicing[108]. Later, other APOBEC members were shown to be involved in viral defence and protection of the genome from retroviral invasion[112]. Two members of the APOBEC family are expressed in lamprey lymphocytes, one in developing T cells (cytidine deaminase 1 (CDA1)) and the other in B cells (CDA2), and these enzymes are likely required for the generation of VLR diversity[35,113]. The VLR genes are assembled via homology-based joins of the VLR cassettes in a process called 'copy choice'[114]. Apparently, the emergence of two enzymes, one specific for somatic rearrangement in T cells and the other for somatic rearrangement in B cells, has obviated the need for the complex regulation required for the one enzyme complex associated with RAG proteins working on gnathostome T cell and B cell antigen receptor genes. These CDA enzymes have been shown to be mutators in vitro, and CDA1 recently has been fused to a CRISPR cassette for in vivo mutagenesis and knockout experiments[115]. The discovery of AID and these related molecules has heralded an exciting foray into adaptive immunity, innate immunity and general cellular homeostasis. For example, the APOBEC family is also involved in scanning for retro-elements, that is, in general preservation of the genome[116]. This field is in its infancy, and a comparative approach to determine the role of APOBEC family proteins in non-typical immune processes holds much promise[112].

Evolution of the MHC

The MHC, complete with class I, class II and class III regions, was also first found in the cartilaginous fish (FIGS. 2,4). The levels of polymorphism of classical MHC class I and MHC class II molecules in most species are very high[117]. Furthermore, nonclassical MHC class I genes are also found in all gnathostomes tested, usually in regions distinct from the MHC locus itself (see below).

MHC class II molecules.

MHC class II α and β-chain genes are found in almost all gnathostomes. Generally, two or three isotypes are present, with high levels of polymorphism. Consistent with a slower evolution than MHC class I isotypes, mammalian MHC class II isotypes can be detected in ectotherms. The DO molecules, which in mammals are class II proteins that regulate the binding of peptides to MHC class II molecules through DM molecules, are not found in any ectotherm. In fact, the DM genes appear first in amphibians, are clearly lacking in bony fish[118] and, so far, have not been found in cartilaginous fish. It will be of interest to study how the lack of these catalysts impacts the association of peptides with MHC class II molecules; much work has been done on the biochemistry of MHC class II molecules in amphibians, but very little has been done in bony or cartilaginous fish[119]. However, the invariant chain (which is essential for the stable assembly of MHC class II molecules) is found in all ectotherms, with the attendant MHC class II-associated invariant chain peptide (CLIP) and the expected tissue distribution[120].

The Gadiformes order of teleost species has lost the MHC class II system, as first shown in the cod[16,121] and later in other species. It has been known for a long time that specific antibody responses cannot be generated in cod after immunization, essentially producing the same repertoire of IgM antibodies to each antigen[122]. How lifestyle has impacted this lack of

MHC class II molecules has been speculated on — these animals live in a cold-water environment, and perhaps a lack of pathogen pressure may have contributed to a 'use it or lose it' scenario. It has been hypothesized that a large number of nonclassical MHC class I molecules in cod might somehow compensate for the absence of MHC class II molecules (BOX 1), but other species bearing MHC class II molecules also have expanded MHC class I genes.

MHC class I molecules.

The classical and nonclassical MHC class I paradigm is clearly found in all cold-blooded gnathostomes. Classical class I molecules are recognized by their high levels of polymorphism, ubiquitous tissue expression and defined peptide-binding residues that lock in the amino and carboxyl termini of the bound peptides[123]. Unlike what is found in mammals, the antigen-processing TAP genes and immunoproteasome (especially proteasome subunit-β type 8 (PSMβ8)) genes are closely linked to MHC class Ia genes, generally in lineages (BOX 1). No functional studies have been carried out in ectotherms, but in birds, TAP and MHC class Ia alleles have been shown definitively to have coevolved in peptide transport and binding studies[124]. Again, biochemical work was done long ago in amphibians, in which MHC class I proteins were isolated using cross-reactive xenoantisera, alloantisera and monoclonal antibodies[119,125]. Now, it is time to step up with studies examining associations with peptides and the importance of linkage[24].

Nonclassical MHC or MHC class Ib genes are also present in all ectotherms. Amphibians have a large cluster of nonclassical genes downstream of the bona fide MHC gene (called *Xenopus* nonclassical or XNC) at the telomere[126]. Among *Xenopus* species, the number of genes found in the XNC varies greatly, as is the case for many large multigene families[127]. Early studies showed interesting tissue distributions of the XNC iso-types in the lung, intestine and spleen, with expression of all isotypes in the thymus, consistent with a positive selection of MHC class Ib-reactive T cells[128]. Recent work focusing on one of the isotypes, xnc10, has shown that NKT cells bearing an invariant TCR α-chain use this class Ib molecule as a restricting element, aiding in responses to tumour and viral antigens (see below for clarification; mammalian NKT cells use the nonclassical MHC class I molecules CD1 and MR1 as restricting elements[129]). Ancient nonclassical class I lineages in bony fish and cartilaginous fish are candidates for recognition by NKT-like cells or for other non-immune functions as shown in mammals[130,131]. For example, despite the general rapid evolution of the bony fish, there are deep lineages of MHC class I molecules, especially the so-called Z lineage in which the peptide-binding region is extremely conserved in all species[132].

Importance of MHC linkage.

In addition to the line-ages of antigen-processing and antigen-presenting genes found in ectotherms and the conservation of linkage groups, comparative analysis of the MHC has also permitted an understanding of the MHC before the emergence of adaptive immunity[117,133]. Linkage of MHC class I and MHC class II genes (as assessed by functional studies looking at genetic co-segregation of acute graft rejection and mixed leukocyte reaction) was shown in amphibians long ago, before cloning of the MHC genes[134]. In bony

fish, however, MHC class I and MHC class II genes were shown to segregate independently, suggesting that this configuration was the primordial state[135]. However, studies of shark families showed that, like all other vertebrates, MHC class I and MHC class II genes are linked, demonstrating that the teleost situation is derived, that is, it is specific to that taxon[136]. Furthermore, the gene encoding β2 microglobulin is also linked to the MHC in sharks and is encoded outside the MHC in all other vertebrates studied[137]. Preservation of this anticipated linkage in sharks suggests that other genes originally linked to the proto-MHC will also be uncovered in ongoing cartilaginous fish genome projects[88].

It has been known for many years that other genes encoding innate immune molecules are linked to the MHC in mammals, such as complement components C4, C2 and factor B, as well as tumour necrosis factor (TNF)[138]. It was originally speculated that these genes were MHC-linked by 'genetic accident'[5], but comparative analysis showed not only that several of these genes are also linked in cartilaginous fish but also that their linkage to framework MHC genes suggests that they were part of a pre-adaptive immune complex, that is, a 'proto-MHC'[117–139–140] (FIG. 4). Members of the B7 family, which are crucial co-stimulatory molecules involved in immunity, and several other immune molecules were also part of the original MHC[141,142]. NKR from both the C-type lectin superfamily and IgSF were also present in the MHC, strongly suggesting that the MHC, NK gene complex (NKC) and leukocyte immunoglobulin-like receptor complex (LRC) were syntenic ancestrally[117–143–144]. Additionally, IgSF members that have a specialized domain found in antigen receptor genes are also encoded by the MHC gene family of several ectotherms, suggesting that the immunoglobulin-TCR precursor was also encoded by the MHC gene family[117,145]. A working hypothesis is that the proto-MHC gene familywas a gene cluster already dedicated to immunity before the advent of adaptive immunity[146,147], and the major components of adaptive immunity, namely, immunoglobulins, TCRs, MHC class I and MHC class II molecules 'piggybacked' onto a region already programmed to respond with increased transcription upon infection (FIG. 4).

Is there an MHC in jawless fish?.

When it was revealed that lampreys had both B and T cells, one obvious question emerged: what are the restricting elements for VLRA recognition? Extensive database searches, as well as various molecular approaches, yielded no evidence for MHC class I or MHC class II molecules, TAP or an immunoproteasome, showing that either the genes are present but unrecognizable at the protein level or that they indeed are absent (perhaps lost?) from the lamprey genome[148]. If truly absent in the agnathans, there are several possibilities. There may be a convergent system to MHC class I and MHC class II molecules that presents peptides to agnathan T cells; a polymorphic agnathan cell surface antigen (called NICIR3/ALA) generates a strong alloantibody response in hagfish and may play such a convergent MHC role[149,150]. Alternatively, VLRA might recognize whole antigens associated with a cell surface molecule such as an Fc receptor or a complement receptor in order to initiate a T cell response. In one set of experiments, recombinant VLRA from expression libraries was capable of binding to an immunizing antigen (hen egg-white lysozyme (HEL))[151]; while TAP is not present in lampreys, a related molecule, TAP-like (TAPL), which is involved in cross presentation in mammals, is found in the lamprey genome[152] and also in

invertebrates[153]. It is possible that TAPL may have been co-opted for antigen presentation in the agnathans.

Lymphoid tissues: evolutionary insights

The thymus.

The thymus is found in all gnathostomes, usually with the typical cortical and medullary organization[78,154]. It can range from one lobule to a multilobed and even discontinuous structure, depending on the species or the developmental stage examined. Additionally, the proteasome subunit-β type 11 (PSMβ11; also known as B5T) and the autoimmune regulator (AIRE) arose early in the gnathostome lineage, suggesting that positive and negative selection occurs in a similar manner in mammals and in early jawed vertebrates[155,156] (FIGS. 1,2). Until recently, it was believed that the thymus was absent in agnathans[30], but with the discovery of VLRA-bearing T cells, this question was re-examined. In situ probes for Delta ligand (for the Notch receptor) and forkhead box protein N1 (FOXN1), a transcription factor shown to be essential in thymus development, defined a structure lining the pharynx of larval lampreys. Associated with these epithelia were lymphocytes expressing VLRA (and in later studies, VLRC) and the APOBEC family enzyme CDA1, mentioned above as the molecule believed to be important in VLRA and VLRC rearrangement[113,157]. This structure was named the thymoid, and it is believed to be the thymus equivalent in agnathans. Thus far, no PSMβ11 (no specialized proteasome components, actually) or AIRE genes have been found in agnathans, so further study may provide the original rationale for physically separating the development of T and B cells into different primary lymphoid tissues[158]. Structures in the gill region of lower deuterostomes like amphioxus also express FOXN1 and Delta ligand, so it will be of interest to study their roles (if any) in lymphocyte differentiation[154].

The spleen as the primordial secondary lymphoid organ.

Only warm-blooded vertebrates have lymph nodes, Peyer's patches and germinal centres, all of which are dependent on the cytokine lymphotoxin for their development[28,159]. Almost all gnathostomes, however, do have a spleen in which adaptive immune responses are generated, concentrating the antigens for interactions between antigen-specific T cells, B cells and antigen-presenting cells (APCs) (FIG. 1). The spleen can be partitioned into red pulp and white pulp in representative species to the level of cartilaginous fish, and B cells are also partitioned into segregated areas in many vertebrates (note that this segregated structure has been lost in several bony fish and amphibians.) During development, B cells are attracted to the chemokine CXC-chemokine ligand 13 (CXCL13), which is expressed by the splenic vasculature, and this forms the nascent white pulp. In mammals (and probably reptiles[160]) (FIG. 2), B cells are displaced into follicles, while T cells surround the vessel in the periarteriolar lymphocytic sheath. In amphibians and fish, B cells retain this embryonic characteristic[161] (BOX 1). It is interesting that, in the course of evolution, B cells formed segregated structures before the formation of T cell zones, as discussed extensively in a classic review article[161].

When did conventional and follicular dendritic cells emerge?

The dichotomy between lymphocytes and myeloid cells appears in the lower chordates, predating adaptive immunity. Cells with the morphology of dendritic cells (DCs) are found in all gnathostomes, and where studied, they have a high expression of MHC class II molecules[162–164]. These cells have been best described in bony fish, at least by morphology, peanut agglutinin staining and the ability to stimulate T cells (note, peanut agglutinin staining indicates a lack of terminal sialic acids on glycoproteins, which is believed to promote cellular interactions by eliminating the natural repulsive effects of negative charge). FDCs, by contrast, have not been reported in ectotherms[30]. Instead, it seems that typical DCs (or APCs) of the haematopoietic lineage present antigens to both T and B cells. In *Xenopus*, these cells, called XL cells[165,166], have high levels of MHC class II molecules for the presentation of antigens to T cells and bear immunoglobulins of all three isotypes on their surface, presumably in immune complexes (and coated with complement components) for the presentation of antigens to B cells (FIG. 5) (H. R. Neely, J. Guo, E. M. Flowers, M. F. Criscitiello and M.F.F., unpublished observations). It is proposed that XL cells provide a model for antigen presentation to both types of lymphocyte before the emergence of FDCs (BOX 1; FIG. 5).

Mucosal immunity

Dimeric IgA is the mucosal immunoglobulin in mammals and birds, and it has well-defined roles in mucosal immunity, such as in the coating of commensal organisms to prevent their intimate association with mucosal epithelia. In amphibians, there is a third antibody iso-type, called IgX (FIGS. 3,6), which forms pentamers (or hexamers) like IgM but is preferentially expressed in mucosae[167,168]. The first bioinformatics analysis showed IgX to be most similar to IgM in sequence, and thus it appeared to be one of the dead end isotypes mentioned above. As more immunoglobulins were cloned, however, it became clear that IgX is orthologous to IgA, re-estimating the emergence of this isotype to the origins of tetrapods[169]. IgX levels increase after immunization with mucosal antigens[170]. In addition, unlike IgY but similar to mammalian IgA, switching to IgX can occur in the absence of T cells. Studies have not been carried out to determine whether IgX, IgM or both (like in mammals) can coat commensal organisms in the gut lumen and elsewhere.

Bony fish have a unique mucosal antibody isotype as well, originally called IgZ and/or IgT and now IgT (for immunoglobulin teleost) (FIGS. 3,6). Like the mammalian TCR αδ locus, the D, J and C segments for this isotype are embedded in the IgM locus, so that the IgT gene is deleted upon IgM rearrangement[171,172]. Excellent work has shown that IgT is expressed primarily at mucosal or epithelial sites, that it can be induced specifically in response to mucosal pathogens and that both IgT and IgM coat commensal pathogens[15,173]. A molecule related to the poly-immunoglobulin receptor transports both isotypes despite the fact that the J chain has been lost in bony fish. As mentioned above, cartilaginous fish have an isotype called IgW, which is most related to IgD. It has several isoforms expressed in different tissues, but its high expression in the pancreas suggests that it is a mucosal isotype of cartilaginous fish[174] (FIG. 6).

While large numbers of lymphocytes are found in the lamina propria and epithelia of mucosae in ectotherms, as are specialized immunoglobulin isotypes that are enriched in these areas, there is no evidence of defined lymphoid tissues, such as Peyer's patches or mesenteric lymph nodes, in the ectotherm intestine. However, defined lymphoid tissue does exist in the olfactory epithelium, and this is an active area of research in lower vertebrates. It appears first in the lungfish and is also found in all amphibians studied to date (except the model species *Xenopus laevis*, where it was apparently lost)[13,175] (BOX 1).

T helper cell subsets

Mammalian T helper cell phenotypic categories have expanded over the past 10 years from the classical T helper 1 (T_H1) and T_H2 cell paradigms to include T_H17 cells, T follicular helper (T_{FH}) cells, regulatory T (T_{reg}) cells and several others T cell subsets[176]. While much work must be done to study T cell function in ectotherms, it is likely that such phenotypes will be found in all gnathostome classes (FIG. 2). A previous suggestion that cartilaginous fish lacked most of these phenotypes, as well as a functional CD4 molecules, was proved incorrect; several cytokines that were not detected in the genome project were uncovered with structurally based search programmes[88,177]; in addition, at least the expression levels of the shark CD4 molecule also appear to be conventional (Y. Ohta and M.F.F., unpublished results). To date, only jawless fish have been shown to have IL-17 (in T cells) and IL-17 R (in B cells), which are proposed to be involved in T-B cell collaboration[11], but these are early days in this line of research, and other cytokines will likely be uncovered.

Evolution of the T_{reg} cell lineage is of special interest. Studies in bony fish suggest that $FOXP3^+$ cells act as T_{reg} cells, perhaps as thymus-derived T_{reg} (tT_{reg}) cells (also referred to as natural T_{reg} cells)[178]. Studies carried out long ago in amphibians suggested that a population of suppressor cells was peripheralized at metamorphosis, where they were likely involved in regulating adaptive responses to newly arising adult-specific self-antigens[179]. Rudensky and colleagues have suggested that peripherally derived T_{reg} (pT_{reg}) cells (also referred to as adaptive or inducible T_{reg} cells) arose in evolution with the advent of placentation, consistent with a *FOXP3* genetic regulatory element (conserved non-coding sequence 1 (CNS1)) found in only placental mammals and no other vertebrates[180]. They propose that after the emergence of pT_{reg} cells for this purpose, such cells were co-opted to suppress adaptive immunity in a variety of situations. It will, of course, be of interest to determine whether this theory holds water, both in maternal-fetal interactions and generally in ectothermic vertebrates (and birds).

Innate-like lymphocytes

One of the most exciting current research areas in immunology is the study of innate-like lymphocytes, such as NKT cells, mucosal-associated invariant T cells (MAIT) cells, B1 cells, marginal zone B cells and innate lymphoid cells (ILCs), including NK cells. It is fitting that studies of their evolution should be a new priority, considering that it is tempting to propose that ILCs (especially) may have predated lymphocytes bearing antigen receptors[181,182].

Most immunologists believed that innate T cells (NKT cells and MAIT cells) were late evolutionary additions in mammals, similar to germinal centres (BOX 2). This notion was put to rest when Robert and colleagues detected NKT-like cells in the amphibian genus *Xenopus* that were specific for one of the non-classical MHC class I molecules in the XNC[129]. Like the mammalian NKT cells, the frog NKT cells bear an invariant TCR α chain and have an effector cell phenotype. Larval *Xenopus* express low levels of classical MHC class I molecules, and preliminary evidence suggests that the larval TCR repertoire is predominantly made up of clones bearing these invariant TCR α chains[183]. This work in amphibians, and the identification of CD1 molecules in reptiles and birds[184–186], has changed the paradigm for NKT emergence. Not only did NKT cells arise early in evolution but one could also propose that animals with moderately few lymphocytes predominantly use T cells that can fire rapidly, that is, that can proceed rapidly to effector function after antigen receptor stimulation, and that large TCR repertoires that favour clonal selection are not used in all cases. Robert's studies suggest that NKT cells will be found throughout the vertebrate subphylum, because as described above, lineages of nonclassical MHC class I genes are present in all vertebrate classes[187].

NK cell function has been detected in species of all vertebrate classes, but it has been difficult to identify their receptors (note that this was the case in studies in mammals as well in the 1990s). NKRs evolve at a very fast rate, which has been clear for many years, as primates and rodents do not even use the same gene family to encode their major NKRs[188]. This rapid evolutionary rate has made it difficult to detect NKRs in ectotherms[20,189]. Large gene families of NKR-like IgSF proteins have been detected in amphibians and fish, but for the most part, their role in NK cell recognition per se has not been determined, and many certainly have other functions. Conversely, clearly there are lymphocytes in fish and amphibians as shown in thymectomized animals, and in animals not bearing TCRs of any type that are still capable of directing cytolysis[190–192]. The challenge in the future will be to link such receptors with specific cellular functions.

As mentioned above, the MHC can harbour NKRs of both the IgSF and C-type lectin family (FIG. 4), depending on the taxon studied. Birds have two C-type lectin NKRs that are related (even orthologous) to mammalian NKRs in the MHC[144]. NK cell p30-related protein (NKp30; also known as NCR3), a specialized IgSF member that maps to the MHC in humans, is the most conserved NKR in gnathostomes and is also found in cartilaginous fish[24,142]. Amphibians have a direct homologue of NKp30 in the class III region of the MHC, called XMIV, while NKp30 has translocated outside the MHC and expanded on the telomere of another chromosome[21,142]. The ligand for NKp30, B7 homologue 6 (B7H6; also known as NCR3LG1)[193], is also found in cartilaginous fish; interestingly, in species in which NKp30 has been lost, B7H6 has been lost as well; conversely, when NKp30 genes are expanded, B7H6 genes are expanded as well. Generally, ILCs have not yet been examined in any detail in species other than humans and rodents. Lamprey lymphocytes have been detected that bear no antigen receptors, and these are candidates for NK cells and other ILCs[181].

Quo vadis?

Several major questions must be addressed in the field of comparative immunology (BOX 3), which have been touched on here. The discovery of the adaptive system in jawless fish has provided a goldmine of interesting questions including the primordial role of the thymus (selection or isolation?), the origins of lymphocytes (ILCs or antigen receptor-bearing cells?), the emergence of IgSF antigen receptors (present in which form?), and the (convergent?) role of the proto-MHC for presentation to VLRA-bearing (T cell-like) lymphocytes. Studies of fish and amphibians, with new reagents and powerful sequencing and knockout procedures, will provide more insight for the study of antigen presentation before the appearance of FDCs and germinal centres, the multifaceted nature of γδ T cells, NKR and their functions and the origins of mucosal immunity; especially for the latter, study of this system in animals lacking mucosal secondary lymphoid tissues may provide a useful framework for understanding the highly complex mammalian gut-associated lymphoid tissue. We have just scratched the surface in our understanding of the evolution of the APOBEC family. There is no doubt that future studies of immunity in cold-blooded vertebrates will surprise us and more importantly continue to alter our views of the adaptive immune system.

Note added in proof

Since the formal acceptance of this article, regulatory T cell-like cells have been described in zebrafish[194,195]. These cells express *foxp3a*, suppress tissue inflammation and can home to damaged organs to promote organ-specific regenerative responses.

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Box 1 |

Unique adaptive immune features in ectotherms

- The immunoglobulin heavy (H) and light (L) chain genes of cartilaginous fish are in the 'cluster organization', with variable (V), diversity (D), joining (J) and constant (C) elements found in each cluster[18] (FIG. 3). Despite this, each B cell shows antigen receptor exclusion, that is, the expression of only one H chain per cell[196,197]. This organization has allowed rapid evolution of distinct types of immunoglobulins, including a single-domain V region antigen receptor called immunoglobulin new antigen receptor (IgNAR)[198], intimate associations of immunoglobulin genes with T cell receptor (TCR) genes[86] and clusters that have been 'germ line-joined'[199,200] and are believed to serve unique functions early in ontogeny[201] and into adult life[70,71].
- A lineage of bony fish has lost MHC class II genes, the invariant chain and CD4 molecules, that is, apparently all the components necessary for T helper cell development[16,17]. Cod might compensate by expressing an inordinate number of nonclassical MHC class I genes, perhaps selecting for a system with many natural killer T (NKT)-like cells.
- Amphibians undergo metamorphosis, with extensive changes in adaptive immunity occurring at this transition[202]. Terminal deoxynucleotidyl transferase (TdT) is not expressed in larvae[203], so antigen receptor junctions in immunoglobulins and TCRs lack N-regions, and thus diversity is quite low. In addition, classical MHC class I gene expression is also low, and MHC class II gene expression is distinct in tadpoles (in B cells and antigen-presenting cells (APCs)) and adults (in all lymphocytes)[204]. After metamorphosis, a second wave of lymphocytes develops rapidly, now with a large amount of complementarity determining region 3 (CDR3) diversity. A working hypothesis is that in larvae, humoral immunity is regulated by $CD4^+$ cells, yet with low diversity of the immunoglobulin repertoire; cellular immunity might be the domain of NKT-like cells[187]. Suppressor cells may emerge at metamorphosis to prevent autoimmune reactions to newly arising adult-specific self-antigens[179].
- The genomic sequence of the famous coelacanth revealed a loss of immunoglobulin M (IgM), and it is the only vertebrate species to date with this feature[57]. IgW loci may have taken over the function of IgM. However, functional studies are difficult to perform, as these fish are rare and/or endangered species.
- The Antarctic fish immunoglobulins show clear evidence of selection, especially in their hinge regions, to allow for preservation of molecular flexibility in extremely cold temperatures[205–207].
- Unlike most mammals, ectotherms have lineages related to classical MHC class I, transporter associated with antigen processing (TAP) and

immunoproteasome genes[208,209]. These genes, again unlike mammals, are tightly linked with MHC class II genes21. Proteasome subunit-β type 8 (PSMβ8), but not PSMβ9 or PSMβ10, is included in these lineages, consistent with a critical function of PSMβ8 in the organization of immunoproteasomes. Note that both PSMβ8 and PSMβ11 (part of the thymic proteasome) are paralogues of the constitutive PSMβ5, again suggesting a crucial role for this member of the β-proteasome ring[210].

- The TCR δ chain in cartilaginous fish, amphibians and coelacanths (and archaic mammals and birds) utilizes V regions from the immunoglobulin H chain, and the genes are found at the TCR αδ locus. This phenomenon was first discovered in the cartilaginous fish version of IgNAR[86] and soon after in marsupials[211] and other species as a single V domain, or VH domain, associated with the TCR Cδ chain. Consistent with old theories of γδ TCR function[212], these findings suggest that γδ T cells in many vertebrates have adaptive functions[12].
- B cells, in all ectotherms tested, are capable of phagocytosis of particles and microorganisms[213]. This feature also applies to mammalian B1 cells, suggesting an ancient connection between ectotherm B cells and mammalian B1 cells, as well as between B cells and myeloid cells[214].
- Amphibians are in decline, and the best work has found a correlation with innate immune mechanisms and their suppression by pathogens[215,216]. Several studies, however, have also implicated adaptive immune mechanisms, specifically MHC polymorphisms, that correlate with susceptibility or resistance to the chytrid fungus[217,218].
- Many fish and amphibians are polyploid, and adaptive immune genes are under pressure to become diploidized over evolutionary time (especially MHC class I and MHC class II genes). This has been an active area of research for decades and has been reinvigorated in the era of genome sequencing[219–221].
- An immune hypothesis has been proposed for salmon migrating back to spawn in their original hatching grounds. During the stressful terminal migration, naive lymphocytes are depleted but plasma cells are spared, likely producing antibodies specific for pathogens where the fish first were exposed[222,223].

Box 2 |

Humoral immunity in the absence of germinal centres

Studies over the past 50 years have revealed that, by and large, specific antibody responses in cold-blooded vertebrates are of low affinity and do not mature over time to the high levels seen in mammals[26,224]. Before the molecular era, this was attributed to a potential lack of somatic hypermutation in non-mammals. However, mutations were discovered in cartilaginous fish[225] and amphibian immunoglobulin genes[29], and most conspicuously in the shark antigen receptor immunoglobulin new antigen receptor (IgNAR) genes[198], in the early 1990s. Despite this, affinity increases do not exceed 100-fold in any immunization study of ectotherms to date, although they show at least some level of selection[44]. As described in the text, at least at the taxonomic level of amphibians, follicular dendritic cells (FDCs) are not present, and recent work suggests that conventional, haem atopoietically derived antigen-presenting cells stimulate both T and B cells. Thus, the emergence of FDCs is believed to have provided the setting for the development of germinal centres and the selecting environment for the generation of high levels of affinity maturation[226]. As described in the previous review on the evolution of immunoglobulin genes and function, the discovery of activation-induced cytidine deaminase (AID) has provided some insight into the evolution of lymphoid tissues[105,107] but now should be used more extensively to study adaptive responses in lymphoid tissues over time. In addition, the jaw less fish apparently have no secondary lymphoid organs, so the locations in which cells come into contact with antigen and interact with each other are unknown40; the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APO BEC) family members implicated in the generation of diversity and likely mutations should be useful for such analyses.

Box 3 |

Key questions for evolutionary immunologists

The transcriptional network for lymphocyte development was set up before the emergence of adaptive immunity. Did this system rely on innate lymphoid cell (ILC)-like processes, or did classical lymphocytes emerge first and then lose their antigen receptors? Note that the adaptive cytokines, including IL-2, IL-4, IFNγ and many others, to this point have been found in only gnathostomes.

- Did antigen receptors containing immunoglobulin superfamily (IgSF) and leucine-rich repeat (LRR) domains coexist in a common ancestor? What drove the newer system to emerge? Was the original system for immunoglobulin and T cell receptor function actually first based on the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like(APO BEC) family of proteins (that is, mutation-based) and then superseded by the system based on the recombination-activating gene (RAG)-based (that is, rearrangement-based) system? And how do other APOBEC family members function throughout the ectotherm ic lineages?
- Was an IgSF family member encoded by the proto-MHC gene closely related to the precursor gene that was invaded by the RAG transposon to become a primordial antigen receptor? There are genes of this particular type of IgSF member found in antigen receptors (‘VJ type’) that are encoded by the MHC genes and are good candidates to be related to this ancestor.
- Somatic hypermutation, class switch recombination and T-B cell collaboration clearly existed before the emergence of germinal centres in ectotherms. How and why were follicular dendritic cells built into this system as a leap forward in selection for high-affinity antibodies? Are conventional dendritic cells truly ‘double-duty’ antigen-presenting cells presenting antigen to both T and B cells?
- B1 cells in mammals and conventional B cells in all ectotherms tested are capable of phagocytosis. Does this imply an ancient connection between myeloid and lymphoid lineages, and what is the utility of such a mechanism physiologically in cold-blooded and warm-blooded vertebrates?
- How do lamprey T cells recognize their antigen? Is there a convergent peptidic recognition of foreign antigen, or is there an entirely different mechanism?
- Why did the thymus evolve? If the agnathan thymoid is a model, perhaps the thymus emerged not for positive or negative selection in the gnathostome thymus but rather for sequestration of developing T cells away from the microenvironment where B cells develop (bone marrow equivalent). It (potentially) follows that, once a sequestered environment for T cell development was acquired, sophisticated and complex mechanisms for

positive and negative selection emerged, perhaps in conjunction with T cell recognition of peptide-MHC complexes.

- Translationally, the variable lymphocyte receptors and shark single-domain antibodies are novel platforms for diagnostic and/or therapeutic antibodies. The great phylogenetic distance between agnathans and/or cartilaginous fish and humans allows for the development of immune responses to evolutionarily conserved epitopes on human targets. How useful will such reagents prove in our pharmaceutical arsenal?
- Technologically, the advent of CRISPR technology for knockout and mutational studies will allow rapid progress in the basic immunology of model ectothermic organisms. Additionally, next-generation sequencing of whole genomes and transcriptomes, as well as rapid generation of proteomes, can, at least in some ways, obviate the requirement for model organisms in many studies.
- Will studies of nonmammalian vertebrates alert the general field of immunology to the potential for adaptive immunity of γδ T cells? How can we devise experiments to understand how native antigen is recognized by such cells in all vertebrates?
- Mucosal immunity in mice and humans can be quite dissimilar. How can studies of ectotherms further inform the entire field of the most basic, conserved elements of a mucosal immune system?
- Many years ago, suppressor cells were suggested to emerge at amphibian metamorphosis to suppress any responses to adult-specific antigens. Technologies exist now to re-examine this proposal with many new resources.
- Did peripherally derived regulatory T (T_{reg}) cells truly emerge as a subpopulation of cells that regulate paternal-specific immunity in placental mammals? That is, do ectothermic vertebrates (and birds) have only thymus-derived T_{reg} cells?
- The lifestyle of animals is clearly important to take into account when examining adaptive immunity. In addition to the cod and Antarctic fish, there are many other species of bony and cartilaginous fish living in diverse environments that should be studied.
- Which came first: MHC class I or MHC class II? For MHC class I, did classical or nonclassical molecules come first? The plasticity of MHC class I molecules suggests that they were primordial, while the thermodynamic stabilityof MHC class II molecules argues for their early emergence. Sim ilarly, CD4 and CD8 were not derived from a recent common ancestor, so their co-option for recognition of MHC molecules occurred independently. Which came first?

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Bursa of Fabricius

An organ derived from a cloacal outpocketing in birds in which B cells develop.

Deuterostomes

Embryonically, animals in which the blastopore becomes the anus, including all of the vertebrates. Lower deuterostomes such as tunicates, lancelets and echinoderms are descendants of ancestors before the emergence of adaptive immunity and the genome-wide duplications that occurred early in vertebrate history.

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Gnathostomes

Jawed vertebrates, including placoderms, cartilaginous fish, bony fish, amphibians, reptiles, birds and mammals.

Evolutionary 'Big Bang'

The rapid emergence of the majority of molecules, mechanisms and tissues that define human adaptive immunity, which most likely occurred in placoderms (see FIG. 2).

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Variable lymphocyte receptors

(VLRs). Antigen receptors (VLRA and VLRB) found in the agnathan jawless fish (lamprey and hagfish).

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Follicular dendritic cells

(FDCs). Cells found in only warm-blooded vertebrates that present native antigen to B cells in the follicles and germinal centres of secondary lymphoid organs.

Immunoglobulin superfamily

A family containing proteins with a specific immunoglobulin superfamily (IgSF) domain, including molecules such as immunoglobulins, T cell receptors and MHC class I and MHC class II molecules, in which there are unique members of the superfamily (VJ and C1 domains).

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Agnathans

The most ancient extant vertebrates (lamprey and hagfish), which lack jaws.

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Xenopus

A genus of aquatic amphibians that is a widespread model for basic science research, including in comparative immunology.

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Lungfish

Vertebrates that serve as a tractable model for the transition from fish to tetrapods and share features with both groups.

Coelacanths

Vertebrates with lobed fins, once thought to be extinct, that serve as a model for the transition from fish to tetrapods.

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'Dead end' H chain immunoglobulin isotypes

Immunoglobulin heavy (H) chain isotypes that arose in particular vertebrate taxa but are apparently not perpetuated throughout the vertebrate tree.

Somatic hypermutation

(SHM). A process that introduces activation-induced cytidine deaminase (AID)-initiating mutations into the immunoglobulin variable region genes of B cells during an adaptive immune response.

Complementarity determining regions

(CDRs). Loops on one face of variable immunoglobulin superfamily domains in regions of both immunoglobulins and T cell receptors that contact antigen (or antigenic peptide-MHC complexes) and that display the greatest variability.

CDR3

A complementarity determining region (CDR) that is generally considered to be the most diverse part of the immunoglobulin and T cell receptor binding site and is derived from recombination-activating gene (RAG)-mediated rearrangements during lymphocyte ontogeny.

RAG transposon

A hypothetical transposable element that contains insertion elements, recombination signal sequences and (at least) one gene encoding the V(D)J recombination-activating protein 1 (RAG1) catalytic core, which invaded an immunoglobulin superfamily gene, initiating the generation of diversity in antigen receptor genes.

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Switch boxes

Repetitive DNA elements upstream of every immunoglobulin H (IgH) isotype gene in mammals that are involved in class switch recombination.

APOBEC family

A specialized family of cytidine deaminases (CDAs) of which the best studied is activation-induced cytidine deaminase (AID), including its function in somatic hypermutation and class switch recombination. Members found in jawless fish (CDA1 and CDA2) are implicated in the rearrangement of the variable lymphocyte receptor genes, and other APOBEC family members in mammals are involved in viral defence and genome preservation.

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Copy choice

The gene conversion-like mechanism by which diversity is generated for the variable lymphocyte receptor genes in developing agnathan T and B cells.

CD1

The evolutionarily oldest nonclassical (MHC class Ib) molecule that presents lipid antigens to natural killer T cells and a subset of γ δ T cells.

NK gene complex

(NKC). A large family of C-type lectin genes in mammals involved primarily in natural killer (NK) cell recognition (for example, killer cell lectin-like receptor subfamily K member 1 (KLRK1) and CD94).

Leukocyte immunoglobulin-like receptor complex

(LRC). A large family of immunoglobulin superfamily genes in mammals (found on chromosome 19 in humans) involved in many immune reactions, including natural killer cell receptors.

Proteasome subunit-β type 11

(PSM β11). Also known as B5T; an immunoproteasome catalytic subunit expressed specifically by the thymic cortical epithelium in all gnathostomes that is vital for the production of peptides involved in the positive selection of $CD8^+$ T cells (cytotoxic T cells).

Autoimmune regulator

(AIRE). A protein that is expressed specifically by the thymic medullary epithelium in all gnathostomes and is vital for central tolerance of T cells via the expression of tissue-specific antigens.

Peanut agglutinin staining

A process that makes use of lectin, which recognizes desialylated glycoproteins, most conspicuously staining germinal centre B cells, double-positive thymocytes and dendritic cell subsets.

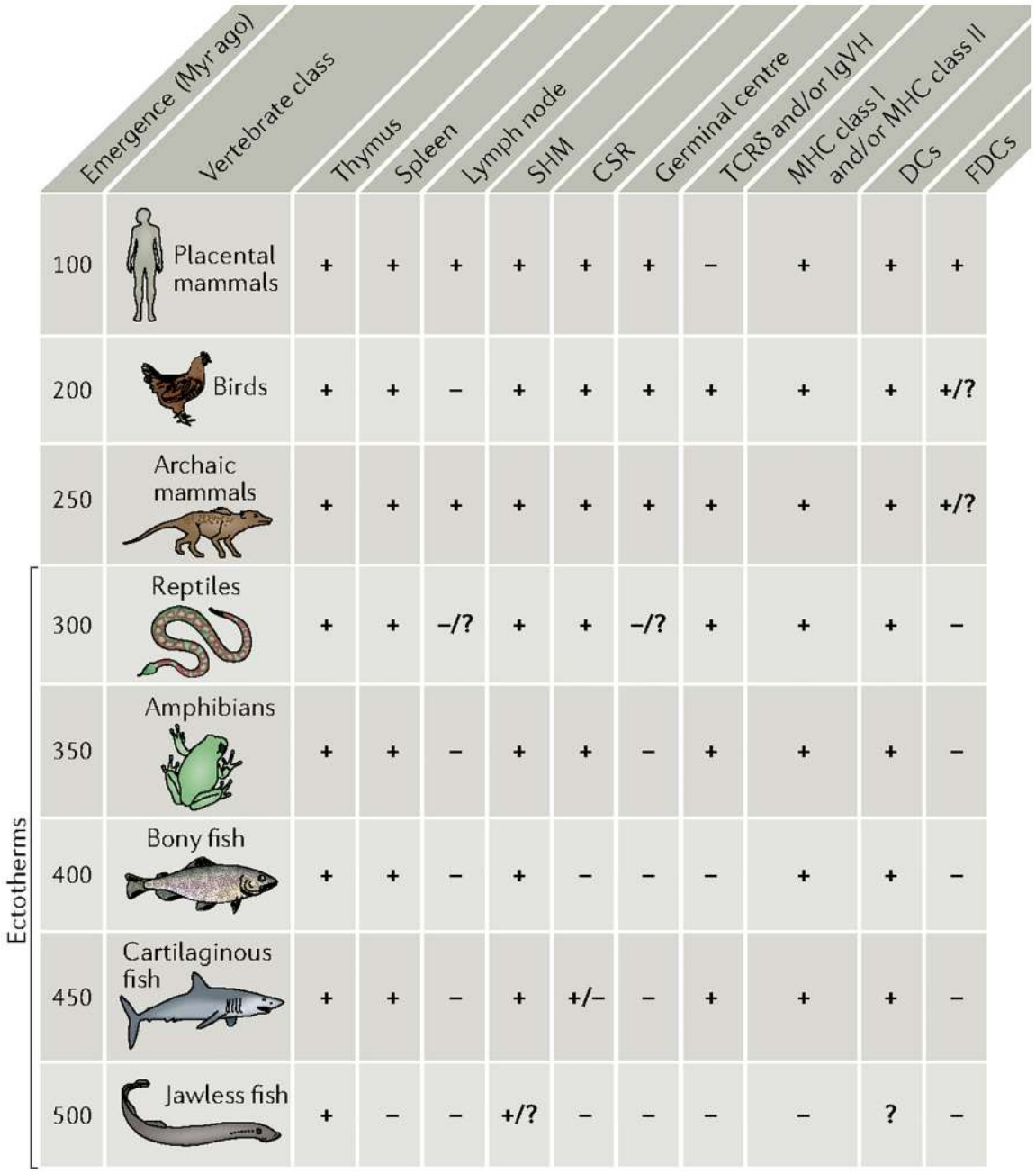


Fig. 1 |. General adaptive immune functions, mechanisms and molecules in the different vertebrate classes.

Numbers to the left indicate the approximate number of years (millions of years ago (Myr ago)) since the emergence of the ancestor of each vertebrate class. The term 'archaic mammals' refers to how different reptilian ancestors gave rise to birds and mammals, and the most ancient mammals most likely had these features. Note that while birds clearly have germinal centres, they have lost many features of adaptive immunity and are deserving of an entire review article of their own to explain their unique immune system. Animals discussed in this Review include amphibians (*Xenopus* and axolotl), bony fish (trout, salmon, medaka, zebrafish, cod, Antarctic fish, coelacanths and lungfish), cartilaginous fish (nurse shark,

skate and/or ray and elephant shark) and jawless fish (lamprey and hagfish). ?, unknown; CSR, class switch recombination; DCs, dendritic cells; FDCs, follicular dendritic cells; IgVH, immunoglobulin heavy chain variable region; SHM, somatic hypermutation; TCRδ, T cell receptor δ chain.

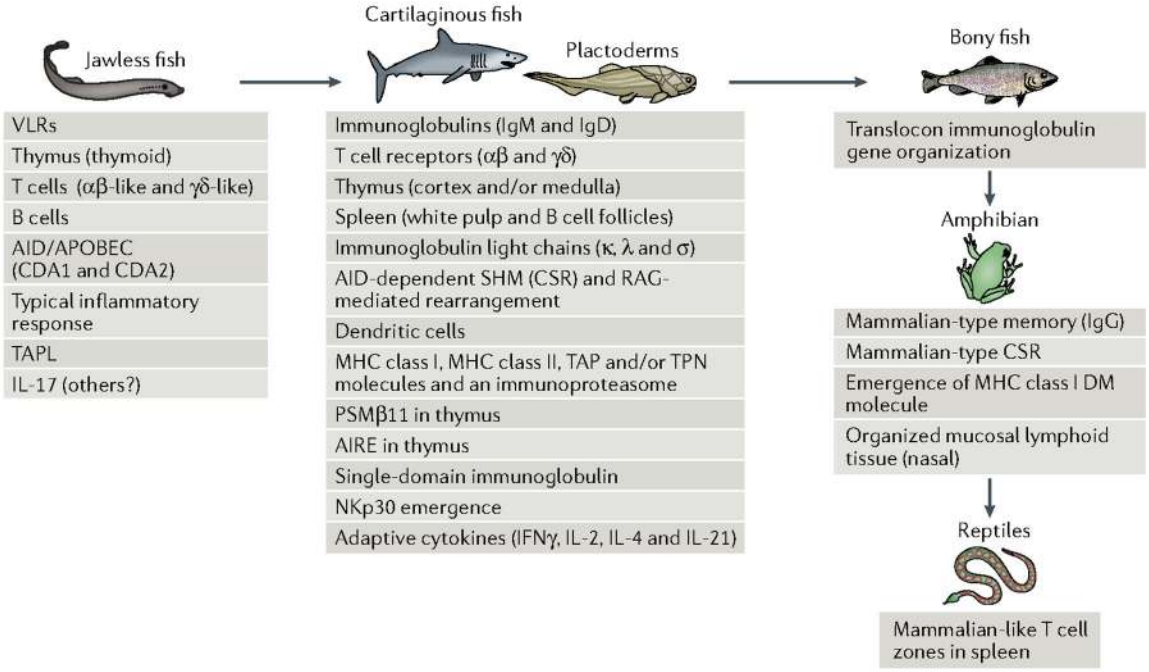


Fig. 2 |. The 'Big Bang' emergence of almost all features of human adaptive immunity early in gnathostome history.

Immune features of each ectothermic vertebrate class, both in terms of leaps forward in evolution and unique characteristics of each group, are shown below the representative animal. Note that there appears to have been two Big Bangs, one at the emergence of the jawless fish and another when the gnathostomes emerged. In this and other figures, it has been assumed that the extinct placoderms had all the immune features of the cartilaginous fish, but this is speculative. AID, activation-induced cytidine deaminase; APOBEC, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like; AIRE, autoimmune regulator; CDA, cytidine deaminase; CSR, class switch recombination; Ig, immunoglobulin; NKp30, natural killer cell p30-related protein; PSMβ11, proteasome subunit-β type 11; RAG, recombination-activating gene; SHM, somatic hypermutation; TAP, transporter associated with antigen processing; TAPL, TAP-like; TPN, tapasin; VLR, variable lymphocyte receptor.

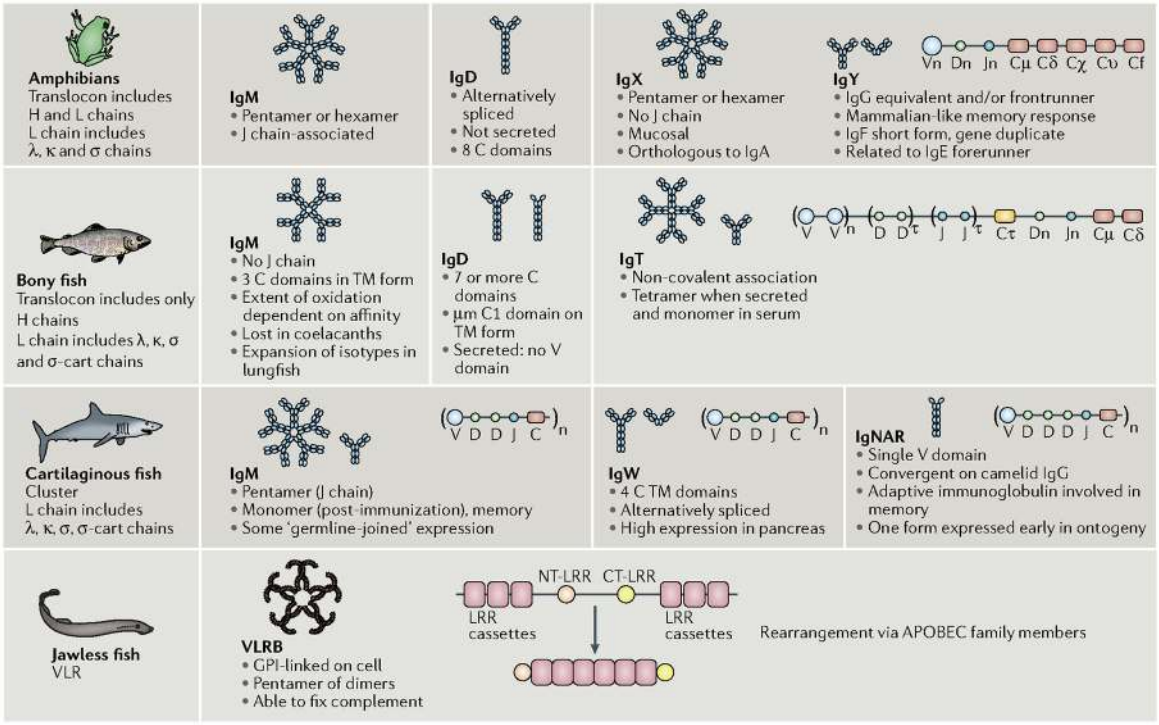


Fig. 3 |. Antibody and variable lymphocyte receptor proteins and genes throughout evolution.

The figure illustrates the main features of the antibodies found in amphibians, bony fish and cartilaginous fish and the variable lymphocyte receptors (VLRs) found in agnathans (jawless fish). Key differences in the variable (V), diversity (D), joining (J) and constant (C) domains of antibodies in each class are shown, as well as the structure of the leucine-rich repeat (LRR) cassettes found in agnathan VLRs. Note that naive B cells in the jawless fish also express cell surface VLRB in a monomeric form. The features noted below each molecule are described in the text and to some extent in FIG. 6. APOBEC, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like; CT-LRR, carboxy-terminal LRR; GPI, glycosyl phosphatidylinositol; H, heavy; Ig, immunoglobulin; L, light; NAR, new antigen receptor; NT-LRR, amino-terminal LRR; TM, transmembrane.

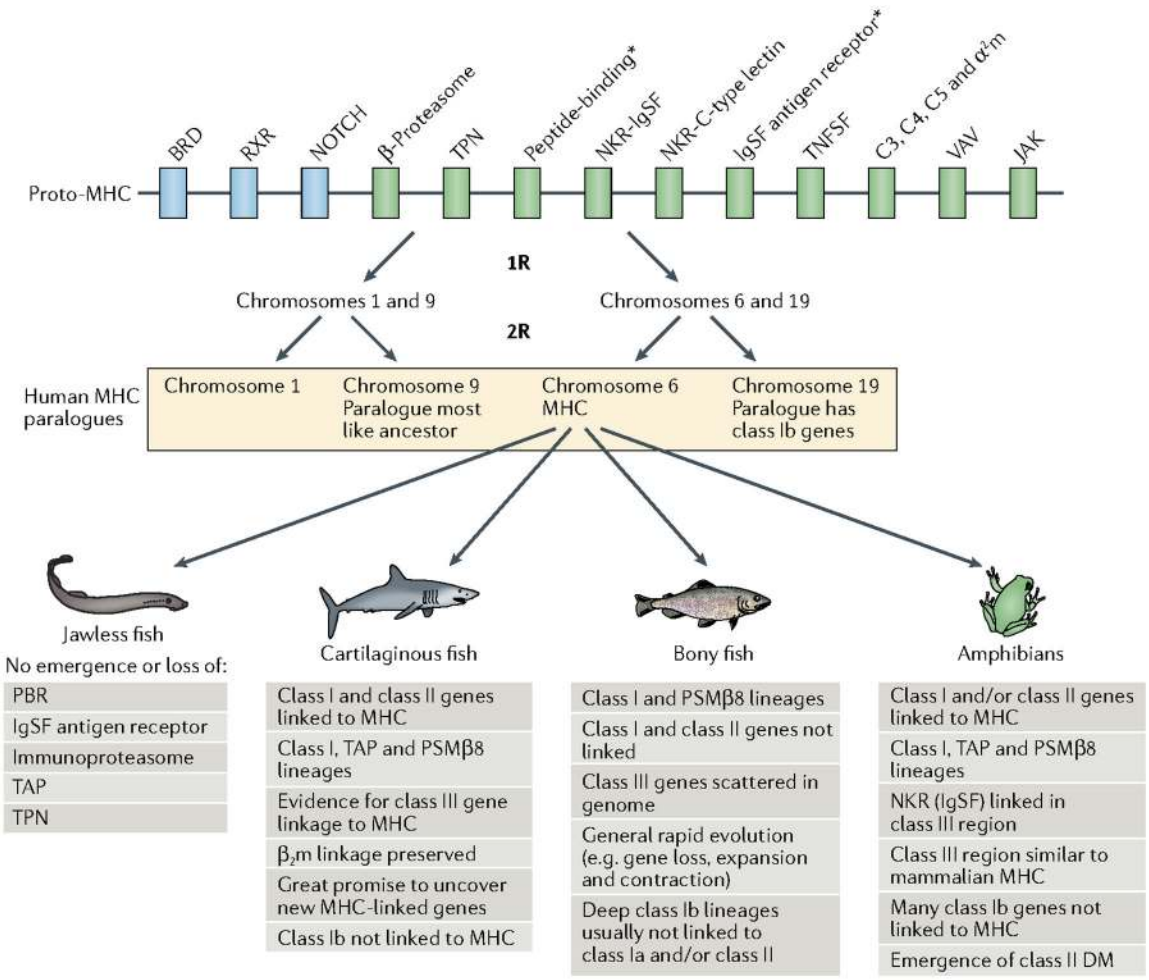


Fig. 4 |. MHC in the ectotherms, with an emphasis on what is unique in different classes.

The proto-MHC is shown at the top of the figure with several framework genes in blue found in all the MHC paralogous regions[141,227228]. The immune genes in blue are inferred to be part of the ancestral MHC, and genes with asterisks (*) are proposed to have existed. Chromosomes 1, 6, 9 and 19 are the MHC paralogous regions (numbering is based on human chromosomes, but all vertebrates have such paralogous regions) arising as a consequence of the two rounds (R) of genome-wide duplications (1 R and 2 R in the figure); these two rounds of genome duplications are shown, with short descriptions of what is special for each paralogous syntenic group. Features of the MHC of each vertebrate class are shown beneath the representative animal from each class. α2m, α2 macroglobulin; β2m, β2 microglobulin; BRD, bromodomain protein; C3, complement component C3; IgSF, immunoglobulin superfamily; JAK, Janus kinase; NKR, natural killer receptor; NKR-C-type lectin, natural killer receptor of the C-type lectin family; NKR-IgSF, natural killer receptor of the IgSF; NOTCH, neurogenic locus Notch homologue protein; PBR, peptide-binding region; PSMβ8, proteasome subunit-β type 8; RXR, retinoid X receptor; TAP, transporter associated with antigen processing; TNFSF, tumour necrosis factor superfamily; TPN, tapasin.

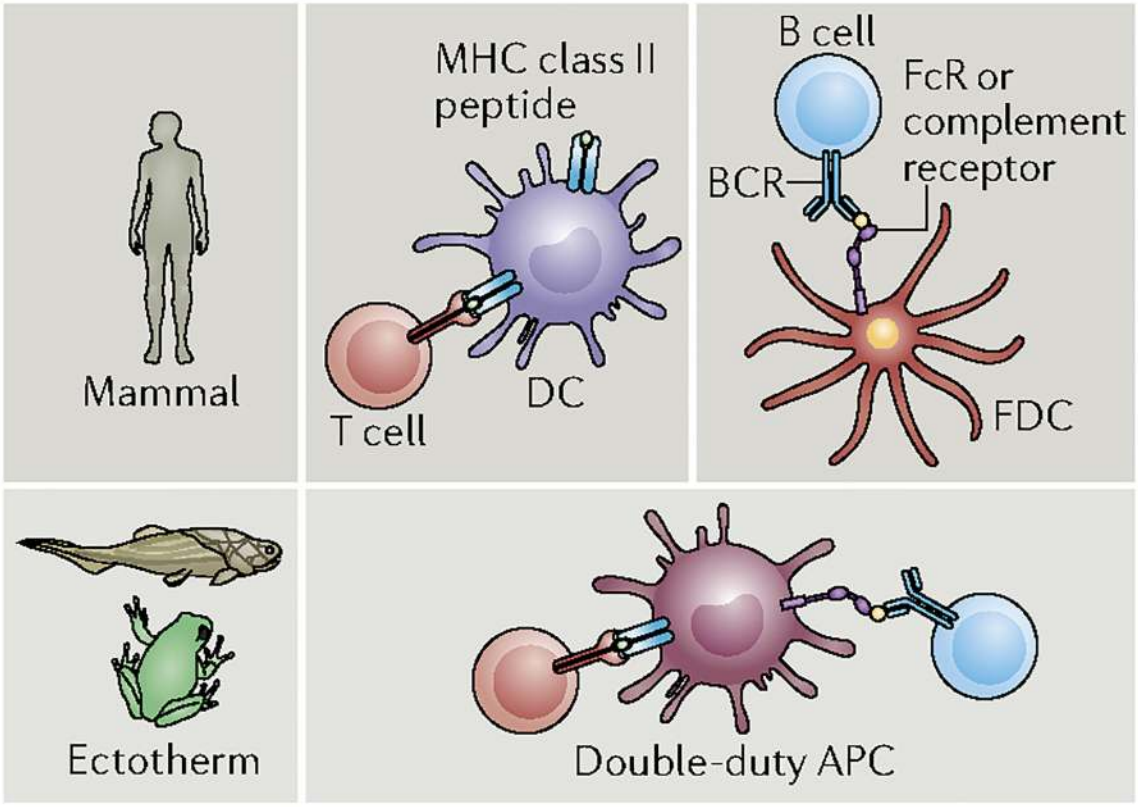
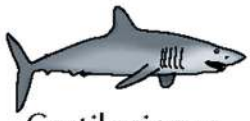


Fig. 5 |. 'Double-duty' APCs may have presented antigen to T cells and B cells before the emergence of FDCs.

It is proposed that a single population of conventional, haematopoietically derived dendritic cells (DCs) presents antigen to both T and B cells in ectotherms. This was likely the ancestral state before the appearance of follicular dendritic cells (FDCs) in mammals. Double-duty antigenpresenting cells (APCs) express MHC class II molecules, Fc receptors (FcRs) and complement receptors. BCR, B cell receptor.



	Mammals	Amphibians	Bony fish	Cartilaginous fish
Mucosal immunoglobulin	 IgA (IgM)	 IgX (IgM)	 IgT (IgM)	IgW? (IgM)
Monomers covalently associated	+	+	–	?
J chain: Presence	+	+	– (lost)	+
J chain: Association with immunoglobulin	+	–	–	?
Secretory piece	+	?	+	?
T cell dependence	+/–	+/–	?	?
Induced by mucosal immunization	+	+	+	?
Generated by CSR	+	+	–	?
Organized gut lymphoid tissue	+	+[a]	–[b] +[b]	–
Mucosal immunoglobulin to coat commensals	+	?	+	?
Poly-immunoglobulin receptor	5 domain	4 domain	2 domain	1 domain?

Fig. 6 |. Mucosal adaptive immunity throughout ectotherm evolution.

The figure illustrates the key features of adaptive mucosal immunity in mammals, amphibians and fish. The major mucosal immunoglobulin is indicated for each group, but IgM can also be transported across mucosal epithelia, although its role is less important, where studied.?, unknown; CSR, class switch recombination; J chain, joining chain. [a]Nasal organized lymphoid tissue is found in all amphibians examined except *Xenopus*[175]. [b]Organized nasal tissue is lacking in the majority of bony fish (actinopterygians) but present in the lungfish (sarcopterygians).