# Genome Duplication and T Cell Immunity

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The adaptive immune system (AIS) mediated by T cells and B cells arose ~ 450 million years ago in a common ancestor of jawed vertebrates. This system was so successful that, once established, it has been maintained in all classes of jawed vertebrates with only minor modifications. One event thought to have contributed to the emergence of this form of AIS is two rounds of whole-genome duplication. This event enabled jawed vertebrate ancestors to acquire many paralogous genes, known as ohnologs, with essential roles in T cell and B cell immunity. Ohnologs encode the key components of the antigen presentation machinery and signal transduction pathway for lymphocyte activation as well as numerous transcription factors important for lymphocyte development. Recently, it has been discovered that jawless vertebrates have developed an AIS employing antigen receptors unrelated to T/B cell receptors, but with marked overall similarities to the AIS of jawed vertebrates. Emerging evidence suggests that a common ancestor of all vertebrates was equipped with T-lymphoid and B-lymphoid lineages.



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# I. Introduction

When and how T cell immunity emerged is an important issue in understanding the origin and evolution of the adaptive immune system (AIS). Thanks to the decades-long efforts of immunologists and the advances of genome projects, we now know that the key components of T cell immunity, such as T cell receptors (TCRs) and major histocompatibility complex (MHC) molecules, are present in all classes of jawed vertebrates (gnathostomes) ranging from mammals to the cartilaginous fish, but absent in jawless vertebrates (agnathans) and invertebrates[1–7] (Fig. 1).

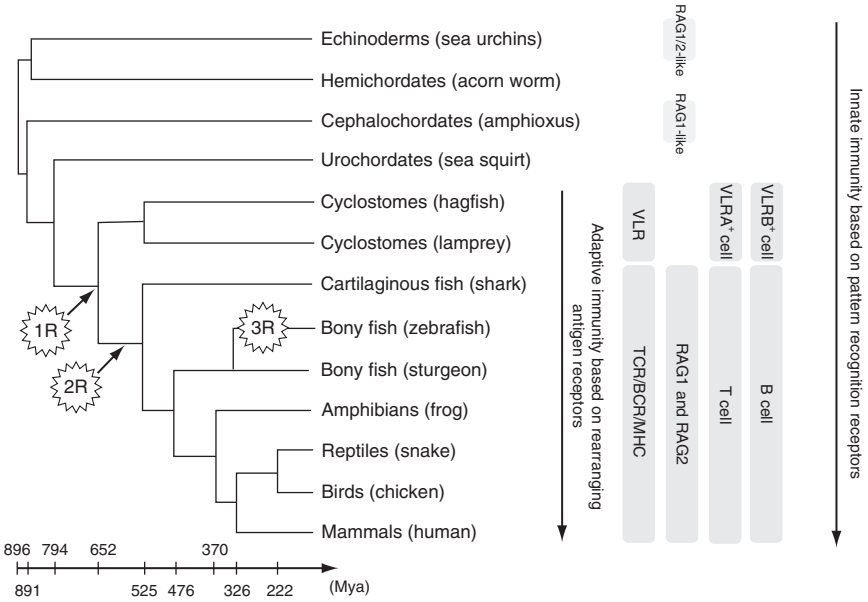


FIG. 1. Evolution of the AIS in deuterostomes. The figure shows schematically at which stage in phylogeny major immune molecules and cells emerged. RAG1-like genes are derived from a transposon; recently, they have been identified also in the genomes of sea urchins[8] and amphioxus.[9] "1R" and "2R" indicate the first and second rounds of WGD. The timing of WGD relative to the emergence of jawless vertebrates is controversial. For detailed discussions, see Section IV and Fig. 4. "3R" stands for a fish-specific WGD. Cephalochordates and urochordates are invertebrate chordates. Cyclostomes, represented by hagfish and lamprey, are jawless vertebrates. Cartilaginous fish, bony fish, amphibians, reptiles, birds, and mammals are jawed vertebrates. The divergence time of animals shown in Mya (million years ago) is based on Blair and Hedges.[12] Abbreviations: BCR, B cell receptor; MHC; major histocompatibility complex; RAG, recombination-activating gene; TCR, T cell receptor; VLR, variable lymphocyte receptor.

Jawless vertebrates represented by hagfish and lamprey are equipped with rearranging antigen receptors that are clonally expressed on lymphocyte-like cells.[13,14] However, their receptors, known as variable lymphocyte receptors (VLRs), generate diversity through somatic recombination of leucine-rich repeat (LRR) modules, and are hence structurally unrelated to TCRs or B cell receptors (BCRs).[15–19] In invertebrate chordates, such as urochordates (represented by sea squirts *Ciona intestinalis*) and cephalochordates (represented by amphioxus *Branchiostoma floridae*), draft genome sequence analysis has provided no evidence for the presence of the AIS.[9,20] Thus, accumulated evidence indicates that T cells as defined by the expression of TCRs are unique to jawed vertebrates and that authentic T cell immunity arose in a common ancestor of jawed vertebrates.

Less well understood is how T cell immunity, and more generally the AIS, emerged in evolution. In terms of molecular components, the cartilaginous fish have fully developed AISs essentially identical to those of mammals; they have not only TCRs of $\alpha/\beta$ and $\gamma/\delta$ types and BCRs,[2,21,22] but also MHC class I and class II molecules,[23–26] recombination-activating gene (RAG) recombinases,[27] and the components of the classical pathway of complement activation.[28] By sharp contrast, jawless vertebrates have none of these components, giving the impression that the TCR/BCR/MHC-based AIS emerged abruptly in a jawed vertebrate lineage.[3,29,30]

One event widely believed to have contributed to the emergence of the jawed vertebrate-type AIS is the acquisition of RAG recombinases that cut double-stranded DNA at the recombination signal sequence (RSS) and mediate V(D)J recombination in TCR/BCR loci.[31,32] Not only does the site-specific recombination process mediated by RAG share mechanistic similarities with the integration and excision process of transposable elements,[33] but also, RAG proteins can transpose an RSS-containing cleavage product to an unrelated target DNA *in vitro*.[34,35] Furthermore, the DNA-binding region of RAG1 shows sequence similarity to that of a *Transib* superfamily of DNA transposons.[36] Collectively, these observations have provided strong evidence that RAGs originated from transposons. The horizontal transfer of RAG transposons may have taken place multiple times or only once during deuterostome evolution.[8] However, the insertion of RAG transposons in an appropriate context ("appropriate" in the sense that the insertion disrupted an ancestral antigen receptor gene and eventually conferred upon it the ability to rearrange) seems to have taken place only in a common ancestor of jawed vertebrates. Exploitation of RAG transposons as V(D)J recombinases was most likely accidental, thus explaining why combinatorial antigen receptors such as TCRs and BCRs emerged abruptly in jawed vertebrates.

Another event assumed to have played a pivotal role in the emergence of the jawed vertebrate-type AIS is two rounds of whole-genome duplication (2R-WGD) that occurred early in vertebrate evolution.[3,37] The importance of

this event in the evolution of T cell immunity was initially suggested by the observation that many of the genes encoded in the MHC, including those involved in antigen presentation, arose as a result of large-scale chromosomal duplication that presumably took place as part of WGD.[38,39] With the accumulation of genomic data from key vertebrate and invertebrate species, it is becoming increasingly clear that WGD was an important event that enabled the ancestor of jawed vertebrates to evolve highly sophisticated AISs.[7] Here I review the role of WGD in the emergence of the AIS, with particular emphasis on the evolution of T cell immunity. I then review the latest advances in our understanding of the immune system of jawless vertebrates. Surprisingly, the overall design of the agnathan AIS is similar to that of the gnathostome AIS, despite the fact that jawed and jawless vertebrates use completely different antigen receptors.

## II. WGD: From a Hypothesis to the Fact

Exactly 40 years ago, Susumu Ohno proposed that the vertebrate genome underwent one or two rounds of WGD at the stage of fish or amphibians through a tetraploidization process.[40] This proposal was based mainly on the comparison of DNA content and karyotypes in various organisms, and the observation that tetraploid species occur naturally in fish and amphibians. Ohno argued that WGDs, which duplicate all genes in the genome simultaneously, were more effective than cumulative tandem duplications in bringing about major evolutionary changes because they would free an entire set of genes from purifying selection and allow it to coevolve, thus providing a unique opportunity to form novel genetic networks required for biologic innovations.

Although his proposal was quite influential from its inception, it was viewed with skepticism until the mid-1990s because of the paucity of experimental evidence. However, with the progress of genome projects, observations supporting Ohno's hypothesis, which became known as the 2R (two-round) hypothesis after some refinement,[41] accumulated exponentially. The major supporting evidence is twofold.[42–45] First, a gene, which occurs only in a single copy in invertebrate chordates such as urochordates and cephalochordates, often has multiple, typically up to four, copies (paralogous copies or paralogs) in jawed vertebrates, indicating that there were waves of gene duplication during the transition from invertebrates to jawed vertebrates. Second, such paralogs, often called ohnologs in honor of Ohno,[46] are not distributed randomly in the vertebrate genome, but tend to occur in clusters (called paralogons) on multiple, and typically four, separate chromosomes.[47] For example, the human genome contains four *HOX* clusters[48] (Fig. 2). Here, not only is the *HOX* gene cluster quadruplicated on four separate chromosomes, but also, many of

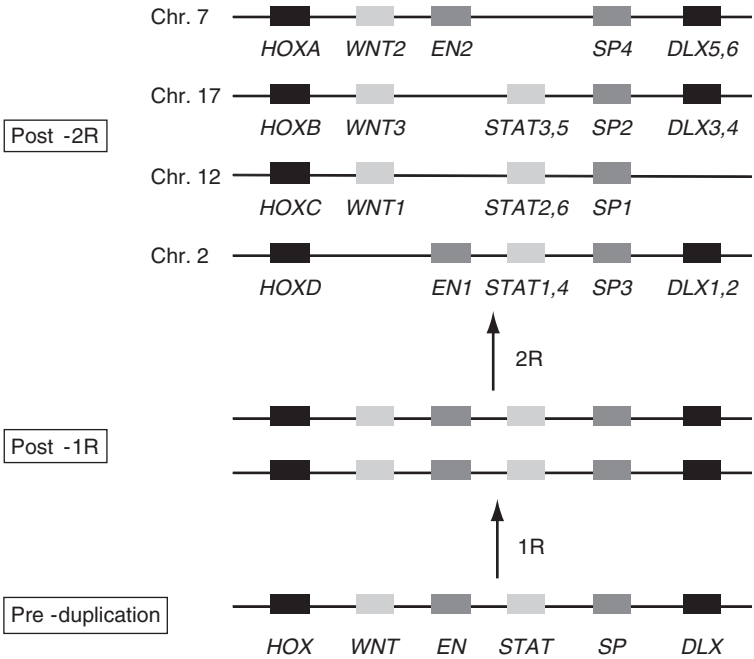


FIG. 2. Origin of the *HOX* paralogy group. Genes are arranged arbitrarily to emphasize corresponding paralogs. The upper panel shows four sets of paralogons constituting the human *HOX* paralogy group. Invertebrate chordates such as amphioxus have only a single *HOX* gene cluster.[49] "1R" and "2R" indicate the first and second rounds of WGD, respectively. Abbreviations: Chr, chromosome; DLX, distal-less homeobox; EN, engrailed homeobox: HOX, homeobox; SP, specificity protein transcription factors; STAT, signal transducer and activator of transcription; WNT, wingless-type MMTV integration site family member.

the genes adjacent to the *HOX* gene cluster are quadruplicated, triplicated, or duplicated on the same four sets of chromosomes, indicating that this unique arrangement of paralogs, known as genome paralogy, arose not as a result of individual gene duplications, but as a consequence of two rounds of large-scale block duplication.

A close inspection of the vertebrate genome indicates that genome paralogy is by no means an exceptional observation. Dehal and Boore[50] systematically identified ohnologs by comparing human and sea squirt genomes and examined their locations in the human genome; their analysis showed that ~25% of the human genome is covered by four sets of paralogons, indicating that genome paralogy is an essential feature of human genome architecture. More recently, comparison of the human and amphioxus genomes revealed widespread occurrence of quadruple conserved synteny, where four sets of human

paralogons corresponded to one set of linked genes in amphioxus.[51] These observations provided incontrovertible evidence for the 2R hypothesis. It is now widely accepted that 2R-WGD took place in the vertebrate lineage after its separation from invertebrate chordates, but before the radiation of jawed vertebrates[41,45,52,53] (Fig. 1). Apart from the 2R-WGD discussed earlier, an ancestor of the majority of ray-finned fish experienced a lineage-specific WGD $\sim$320 million years ago.[11] This duplication is often called 3R (the third round of WGD).

## III. Roles of Ohnologs in Adaptive Immunity

The function of the jawed vertebrate-type AIS depends on the participation of a large number of genes. Klein and Nikolaidis[4] have classified the genes deployed by the AIS into three categories. The first category includes genes that evolved long before the emergence of the AIS. Because these genes evolved for other biologic systems and were subsequently recruited to the AIS, they usually have functions not restricted to adaptive immune responses. For example, the proteasome, a proteolytic enzyme complex,[54] evolved as protein degradation machinery essential for cell survival and was later recruited as a supplier of peptides to MHC class I molecules.[55] Thus, most of the proteasome subunits are well conserved throughout eukaryotes, and their functions are not specialized for the AIS.[54]

The second category includes paralogs that emerged by duplication from preexisting genes and acquired functions involved in or specialized for adaptive immune responses. Many of these genes appear to be ohnologs generated by 2R-WGD.[56] For example, jawed vertebrates have a specialized type of proteasomes, called immunoproteasomes, that facilitates the production of peptides that serve as MHC class I ligands.[57,58] Instead of β1, β2, and β5 subunits found in regular proteasomes, immunoproteasomes contain three interferon (IFN)-γ-inducible subunits called β1i, β2i, and β5i.[55] These subunits alter the cleavage specificities of the proteasome so that peptides suitable for binding to MHC class I molecules are produced more efficiently. The genes coding for β1i, β2i, and β5i are related to those coding for β1, β2, and β5 subunits, respectively, and the former set of genes are ohnologs that arose by WGD from the latter set of evolutionarily more ancient genes with housekeeping functions.[55]

The third category includes a relatively small number of genes, such as those coding for MHC class I and class II molecules, TCRs, and BCRs, with functions dedicated to immune responses. These genes appear to have emerged by mechanisms other than simple duplication of preexisting genes; in the case of MHC class I and class II molecules, peptide-binding domains of

unknown origin appear to have been grafted to the immunoglobulin (Ig)-like constant domains.[59,60] In the case of antigen receptors, an invasion by RAG transposons was instrumental in their emergence.[31]

Here, representative examples of ohnologs are discussed to highlight the importance of WGD in the emergence of the jawed vertebrate-type AIS.

## A. Molecules of the MHC System

The MHC system is a cornerstone of T cell immunity because conventional α/β TCRs recognize antigen only in the form of peptides bound to MHC class I or class II molecules. Accumulated evidence indicates that many molecules involved in antigen presentation by class I and class II molecules are encoded by ohnologs[7,61] (Table I). Peptides presented by class I molecules are produced by proteasomes and transported to the endoplasmic reticulum by transporters associated with antigen processing (TAP), where they bind to nascent class I molecules with the help of tapasin.[62] Immunoproteasome subunits, β1i, β2i, and β5i, are encoded by ohnologs as discussed earlier, and so are the TAP and tapasin molecules.[7] Recently, a novel form of proteasomes, designated thymoproteasomes, has been identified in mice[63] and man.[64] Thymoproteasomes, expressed specifically in cortical thymic epithelial cells, are involved in positive selection of $CD8^+$ T cells.[65] β5t, a β-type subunit unique to thymoproteasomes, is also encoded by an ohnolog (Table I).

Peptides presented by MHC class II molecules are produced by endosomal/lysosomal proteases. Important among such proteases are cathepsins[66,67]; accumulated evidence indicates that cathepsins S, D, and L play particularly important roles in antigen presentation by class II molecules and that cathepsin L is involved in thymic selection of $CD4^+$ T cells and degradation of invariant chains.[68] As described below, the majority of cathepsin isoforms are encoded by ohnologs mapping to paralogons (Table I). Other examples of ohnologs directly related to the function of MHC molecules are *RXRB* (retinoid X receptor β) and *RFX5* (regulatory factor X, 5) genes, which regulate the expression of class I and class II molecules, respectively.[69,70]

The MHC is a prototypical region exhibiting genome paralogy.[61,71] Initially, the MHC paralogy group was defined as a set of paralogons located on human chromosomes 1, 6, 9, and 19.[37,39] Recent evidence indicates that the MHC paralogy group and the neurotrophin paralogy group[72] are partially overlapping and that they descended from a neighboring region on the same ancestral chromosome[41,73] (Fig. 3). It is remarkable that almost all of the ohnologs discussed earlier are located in the paralogons of the MHC/neurotrophin paralogy group.[7] This suggests that a preduplicated region that existed in the genome of our invertebrate chordate ancestor contained precursors of many genes coding for the components of the MHC system.[71,75]

TABLE I
REPRESENTATIVE HUMAN OHNOLOGS INVOLVED IN ANTIGEN PRESENTATION

| Gene family | Genes | Location[a] | Gene products | Function | Other closely related ohnologs | Location[a] |
|---|---|---|---|---|---|---|
| Ohnologs involved in class I antigen presentation | | | | | | |
| 20S proteasome | *PSMB8* | 6p21.3 (MHC) | β5i | Component of immunoproteasomes: production of MHC class I-binding peptides | *PSMB5* | 14q11.2 |
| β-subunits | *PSMB9* | 6p21.3 (MHC) | β1i | Component of immunoproteasomes: production of MHC class I-binding peptides | *PSMB6* | 17p13 |
| | *PSMB10* | 16q22.1 | β2i | Component of immunoproteasomes: production of MHC class I-binding peptides | *PSMB7* | 9q34.11–q34.12 |
| | *PSMB11* | 14q11.2 | β5t | Component of thymoproteasomes: positive selection of $CD8^+$ T cells | | |
| TAP | *TAP1* | 6p21.3 (MHC) | TAP1 | TAP1/TAP2 heterodimer transports peptides into the endoplasmic reticulum | *ABCB9 (TAPL)* | 12q24 |
| | *TAP2* | 6p21.3 (MHC) | TAP2 | | | |
| Tapasin | *TAPBP* | 6p21.3 (MHC) | Tapasin | Promotes association of TAP and MHC class I molecules | *TAPBPL* | 12p13.3 |
| Retinoid X receptor | *RXRB* | 6p21.3 (MHC) | RXRβ | Binds to the MHC class I promoter and regulates class I expression | *RXRA*<br>*RXRG* | 9q34.3<br>1q22–q23 |

Ohnologs involved in class II antigen presentation

| | | | | | | |
|---|---|---|---|---|---|---|
| Cathepsins | *CTSL1* | 9q21–q22 | Cathepsin L1 | $CD4^+$ T cell and NKT cell development | *CTSH* | 15q24–q25 |
| | *CTSL2* | 9q22.2 | Cathepsin L2 | $CD4^+$ T cell and NKT cell development | *CTSK* | 1q21 |
| | *CTSS* | 1q21 | Cathepsin S | Removal of invariant chains in B cells and dendritic cells | *CTSG* | 14q11.2 |
| | *CTSD* | 11p15.5 | Cathepsin D | Production of MHC class II-binding peptides | *CTSC* | 11q14.1–q14.3 |
| | | | | | *CTSF* | 11q13.1 |
| | | | | | *CTSW* | 11q13.1 |
| Regulatory factor X | *RFX5* | 1q21 | RFX5 | A component of RFX involved in MHC class II expression | *RFX1* | 19p13.1 |
| | | | | | *RFX2* | 19p13.3–p13.2 |
| | | | | | *RFX3* | 9p24.2 |
| | | | | | *RFX4* | 12q24 |

[a]Chromosomal localization of human genes is based on the OMIM database (http://www.ncbi.nlm.nih.gov/omim) or Entrez gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

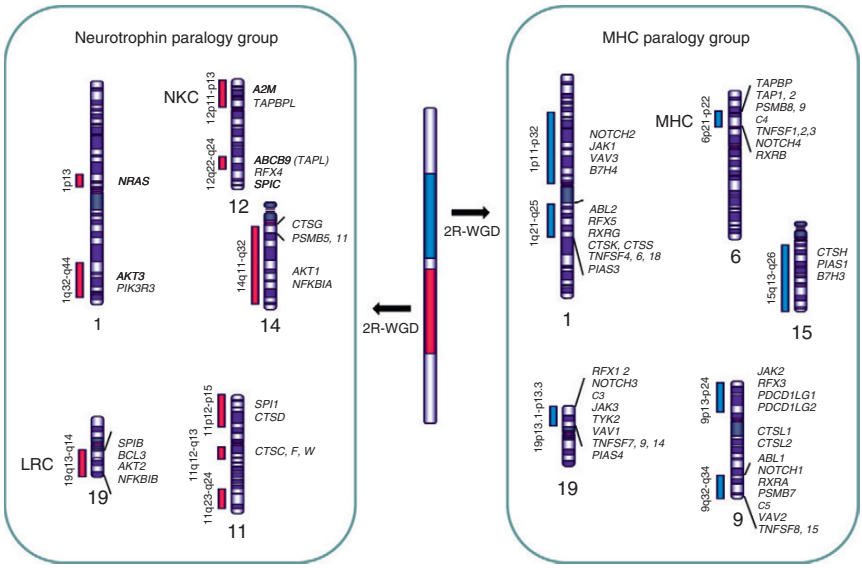


FIG. 3. The MHC/neurotrophin paralogy group in the human genome. The MHC paralogy group (right) is made up of four sets of paralogons located on human chromosomes 1, 6, 9, and 19. A number of smaller-sized MHC paralogons, which presumably originated from fragmentation and subsequent translocation of the major paralogons, have been identified. Among them, the paralogon located on 15q13-q26 appears to have been broken off from the paralogon on chromosome 6. Four sets of major neurotrophin paralogons (left) are located on chromosomes 1, 11, 12/14, and 19. The MHC- and neurotrophin-paralogy groups are partially overlapping and are thought to have descended from neighboring regions on a single ancestral chromosome. Hence, some paralogs are distributed across the two paralogy groups. In mammals, only two proteasome β-type subunit genes *PSMB8* and *PSMB9*, which encode β5i and β1i, respectively, are located in the MHC (Table II). However, β2i is also encoded in the MHC in the bony fish.[74] Abbreviations: A2M, α2-macroglobulin; ABL, Abelson murine leukemia viral oncogene homolog; AKT, V-AKT murine thymoma viral oncogene homolog; B7, B7 family; CTSC, CTSD, CTSF, CTSG, CTSH, CTSK, CTSL, CTSL2, CTSS, and CTSW, cathepsins C, D, F, G, H, K, L, L2, S, and W; JAK, Janus kinase; LRC, leukocyte receptor complex; MHC, major histocompatibility complex; NFKBIA, nuclear factor κ-B inhibitor; NKC, natural killer complex; NRAS, neuroblastoma RAS viral oncogene homolog; PD, programmed cell death 1 ligand; PIAS, protein inhibitors of activated STAT; PIK3R, phosphatidylinositol 3-kinase, regulatory subunit; PSMB, proteasome subunits, β-type; RFX, regulatory factor X; RXRA, RXRB, and RXRG, retinoid X receptors α, β, and γ; SPIC, SPIC transcription factor; SPI1, spleen focus forming virus proviral integration oncogene; TAP, transporter associated with antigen processing; TAPBP, TAP-binding protein (tapasin); TAPBPL, TAP-binding protein-like; ABCB9 (TAPL), transporter associated with antigen processing-like; TNFSF, tumor necrosis factor ligand superfamily; and 2R-WGD, two rounds of whole-genome duplication. This figure was modified from Flajnik and Kasahara.[7]

## B. Signaling Molecules

Okada and Asai[76] systematically performed phylogenetic analysis of signaling molecules and came to the conclusion that 2R-WGD played a major role in the generation of over 100 ohnologs involved in lymphocyte signaling.

An example of ohnologs in this category is a family of genes coding for transcription factors known as signal transducers and activators of transcription (STAT) that mediate signal transduction in response to various cytokines[77] (Table II). STAT4 and STAT6 mediate transcriptional activation of target genes in response to IL-12 and IL-4, respectively. STAT4 deficiency causes a defect in T helper 1 (Th1) cell development,[78,79] whereas STAT6 deficiency impairs the development of T helper 2 (Th2) cells and IL4-dependent Ig class switching.[80,81] When cytokines are bound to cytokine receptors, Janus kinases (JAKs) are activated, and the activated JAKs phosphorylate STAT proteins, which then move to the nucleus and activate transcription of cytokine-responsive genes.[82] Four known members of the *JAK* family, *JAK1*, *JAK2*, *JAK3*, and *TYK2*, are ohnologs mapping to the MHC paralogy group (Table II, Fig. 3). The JAK/STAT pathway is negatively regulated by protein inhibitors of activated STAT (PIAS)[83] and suppressors of cytokine signaling (SOCS).[84] Four known members of the *PIAS* family are ohnologs, with three of them encoded in the MHC/neurotrophin paralogy group. It has also been suggested that four of the *SOCS* family members, *SOCS1*, *SOCS2*, *SOCS3*, and *CIS*, diverged by 2R-WGD.[85] These observations indicate that WGD was highly effective in creating a network of interacting molecules constituting the JAK/STAT pathway.

Other notable ohnologs involved in signal transduction include VAV family proteins[86] (Table II, Fig. 3), Abl tyrosine kinases[87] (Table II, Fig. 3) and TEC family kinases.[88]

## C. Cytokines and Cytokine Receptors

Cytokine and cytokine receptor families are also known to have increased their family members by WGD (Table III). The best known example is a tumor necrosis factor (TNF) superfamily of cytokines with crucial roles in both adaptive and innate immunity. Most members of the TNF ligand superfamily, including CD40 ligand and 4-1BBL that function as co-stimulators for T cells,[89,90] are encoded by ohnologs mapping to the MHC paralogy group[61,91] (Fig. 3), indicating that 2R-WGD played a critical role in the diversification of TNF ligand genes.[92] It has been suggested that WGD was also involved in the diversification of TNF receptor superfamily genes.[93]

Most chemokine receptors and chemokines are encoded in the *HOX* paralogons.[94] Not only do chemokines recruit leukocytes including T and B cells to sites of infection, but they also regulate physiological migration of lymphocytes to and within various lymphoid tissues.[95] Detailed analysis of

TABLE II
REPRESENTATIVE HUMAN OHNOLOGS INVOLVED IN SIGNAL TRANSDUCTION

| Gene family | Genes | Location[a] | Gene products | Function |
|---|---|---|---|---|
| Ohnologs encoded by the *HOX* paralogy group | | | | |
| STAT transcription factors | *STAT1* | 2q32.2 | STAT1 | Th1 cell development, cytokine signaling (IFN-α/β, IFN-γ) |
| | *STAT2* | 12q13.2 | STAT2 | Cytokine signaling (IFN-α/β) |
| | *STAT3* | 17q21.31 | STAT3 | Cell growth, suppression and induction of apoptosis, cytokine signaling (IL-6, IL-10) |
| | *STAT4* | 2q32.2-q32.3 | STAT4 | Th1 cell development, cytokine signaling (IL-12) |
| | *STAT5A* | 17q11.2 | STAT5A | Cytokine signaling (IL-2, prolactin) |
| | *STAT5B* | 17q11.2 | STAT5B | Cytokine signaling (IL-2, IL-15, growth hormone) |
| | *STAT6* | 12q13 | STAT6 | Th2 cell development, cytokine signaling (IL-4, IL-13) |
| Ohnologs encoded by the MHC/neurotrophin paralogy group | | | | |
| Janus kinases | *JAK1* | 1p31.3 | JAK1 | Response to IFNs, γc-dependent cytokines and gp130-dependent cytokines, involved in lymphopoiesis |
| | *JAK2* | 9p24 | JAK2 | Response to erythropoietin, thrombopoietin, IL-3, GM-CSF and IFNγ |
| | *JAK3* | 19p13.1 | JAK3 | γc-dependent lymphoid development |
| | *TYK2* | 19p13.2 | TYK2 | Required for IL-12-induced T cell function |
| PIAS (Protein inhibitor of activated STAT) | *PIAS1* | 15q22 | PIAS1 | Inhibitor of activated STAT1 |
| | *PIAS2*[b] | 18q21.1 | PIAS2 | Inhibitor of activated STAT2 |
| | *PIAS3* | 1q21 | PIAS3 | Inhibitor of activated STAT3 |
| | *PIAS4* | 19p13.3 | PIAS4 | Inhibitor of activated STAT4 |
| VAV guanine nucleotide exchange factor | *VAV1* | 19p13.3-p13.2 | VAV1 | T cell signaling |
| | *VAV2* | 9q34.1 | VAV2 | BCR-induced proliferation, T cell-dependent antibody response |
| | *VAV3* | 1p13.3 | VAV3 | B cell signaling |

(*Continues*)

TABLE II (*Continued*)

| Gene family | Genes | Location[a] | Gene products | Function |
|---|---|---|---|---|
| Abl (Abelson tyrosine kinases) | *ABL1* | 9q34.1 | ABL1 (ABL) | T cell signaling and T cell development |
| | *ABL2* | 1q24-q25 | ABL2 (ARG) | T cell signaling and T cell development |

[a]Chromosomal localization of human genes is based on the OMIM database (http://www.ncbi.nlm.nih.gov/omim) or Entrez gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)

[b]*PIAS2* seems to have been translocated secondarily; it is encoded outside the MHC/neurotrophin paralogy group.

chemokine and chemokine receptor genes indicated that they increased their copy number not only by tandem duplication but also by cluster duplication mediated by 2R-WGD.[96]

It has been shown recently that the transforming growth factor-β pathway also increased their complexity through 2R-WGD.[97]

## D. Transcription Factors Involved in Lymphocyte Development

Lymphocyte development requires the participation of a number of transcription factors.[98] Many transcription factors critically involved in T cell development are encoded by ohnologs. For example, GATA3 is a transcription factor indispensable for Th2 cell development.[99] Phylogenetic analysis indicates that three *GATA* genes, *GATA1*, *GATA2*, and *GATA3*, map to *GATA* paralogons and diverged by 2R-WGD from a common ancestral *GATA1/2/3* gene.[100] Similarly, detailed analysis of the Ikaros-related family of zinc finger transcription factors showed that four of its members, *Ikaros*, *Aiolos*, *Helios*, and *Eos*, most likely diverged by 2R-WGD.[101–103] Mice lacking *Ikaros* ohnolog display multiple defects in T cell development.[104] Helios is also essential for T cell differentiation and homeostasis.[105]

Transcription factors of the IFN regulatory factor (IRF) family play essential roles in Th1-type immune responses, IFN-induced antiviral and antibacterial responses, and the development of natural killer (NK) cells.[106] Recent work has shown that they also increased their members by 2R-WGD.[107]

## E. Co-stimulatory Molecules

Effective activation of naïve T cells requires a second signal known as the co-stimulatory signal. The best characterized co-stimulators for T cells are the B7 family of molecules expressed on antigen presenting cells.[108] Many

TABLE III
THE HUMAN TNF AND THE CHEMOKINE/CHEMOKINE RECEPTOR FAMILY

| Gene family | Ohnologs located in the paralogons[a] | Location[b] | Gene products | Function | Other ohnologs | Location[b] |
|---|---|---|---|---|---|---|
| TNF superfamily ligands | *LTA (TNFSF1)* | 6p21.3 (MHC) | LT α | Lymphoid organ, γ/δ T and NKT cell development | *TNFSF5* | Xq26 |
| | *TNF (TNFSF2)* | 6p21.3 (MHC) | TNF | Lymphoid organ development | *TNFSF10* | 3q26 |
| | *LTB (TNFSF3)* | 6p21.3 (MHC) | LT β | Lymphoid organ, γ/δ T and NKT cell development | *TNFSF11* | 13q14 |
| | *TNFSF4* | 1q25 | OX40-L | Control of T cell function, and activation of B cells | *TNFSF12* | 17p13 |
| | *TNFSF6* | 1q23 | Fas-L | Apoptosis of Fas-expressing cells | *TNFSF13* | 17p13.1 |
| | *TNFSF7* | 19p13 | CD27-L, CD70 | Control of T cell function | *TNFSF13B* | 13q32–q34 |
| | *TNFSF8* | 9q33 | CD30-L | Th1 response, B cell proliferation | *EDA* | Xq12–q13.1 |
| | *TNFSF9* | 19p13.3 | 4-1BB-L | Control of T cell function | | |
| | *TNFSF14* | 19p13.3 | LIGHT | Control of T cell function | | |
| | *TNFSF15* | 9q33 | TL1A | Control of T cell function | | |
| | *TNFSF18* | 1q23 | GITRL | Modulation of T cell survival | | |
| CC chemokines | *CCL1*, *2*, *3*, *4*, *5*, *7*, *8*, *11*, *13*, *14*, *15*,*16*, *18*, *23* | 17q11-q12 | CCL1, 2, 3, 4, 5, 7, 8, 11, 13, 14, 15, 16, 18, 23 | Recruitment of leukocytes to sites of infection. Regulation of the traffic of leukocytes including T cells, B cells and dendritic cells. Development of nonlymphoid organs | *CCL19*, *21*, *27* | 9p13 |

| | | | | | |
|---|---|---|---|---|---|
| | *CCL20* | 2q33-q37 | CCL20 | *CCL17*, *22* | 16p13 |
| | | | | *CCL24*, *26* | 7q11.23 |
| | | | | *CCL25* | 19p13.2 |
| | | | | *CCL28* | 5p12 |
| CC chemokine receptors | *CCR1*, *2*, *3*, *5*, *8*, *9* | 3p21-p22 | CCR1, 2, 3, 5, 8, 9 | *CCR6* | 6q27 |
| | *CCRL2* | 3p21-p22 | CCRL2 | | |
| | *CCR4* | 3p24 | CCR4 | | |
| | *CCR7*, *10* | 17q12-q21.2 | CCR7, 10 | | |
| CXC chemokine receptors | *CXCR1*, *CXCR2* | 2q35 | IL8RA, IL8RB | *CXCR3* | Xq13 |
| | *CXCR4* | 2q21 | CXCR4 | *CXCR5* | 11q23.3 |
| | *CXCR6* | 3p21 | CXCR6 | | |
| | *CXCR7* | 2q37.3 | CXCR7 | | |
| Other chemokine receptors | *CX3CR1*, *XCR1* | 3p21.3-p21.1 | CX3CR1, XCR1 | | |

[a]For the TNF superfamily, copies mapping to the classical MHC paralogy group (chromosomes 1, 6, 9, and 19) are listed. For the chemokine/chemokine receptor family, copies mapping to the *HOX* paralogy group (chromosomes 2, 3, and 17) are listed. Some of the members listed here most likely arose by tandem duplication after WGD, and hence are not ohnologs in a strict sense.

[b]Chromosomal localization of human genes is based on the OMIM database (http://www.ncbi.nlm.nih.gov/omim) or Entrez gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

members of the B7 family, including B7-1, B7-2, ICOS, B7-H1, and B7-DC, are encoded by paralogous regions located on human chromosomes 1, 3, 11, 21, and 19, and are thought to have diverged by 2R-WGD.[109,110]

### F. Complement

Genes coding for complement components C3, C4, and C5 are classic examples of ohnologs and are encoded in the MHC paralogons.[37] C4 and C5 are the components of the classical pathway of complement activation initiated by binding of antigen–antibody complexes to the C1 molecules. These components are present only in jawed vertebrates,[111] and are thought to have diverged from a C3-like precursor protein.[28,112]

## IV. Controversies Surrounding the Timing of WGD

The genomes of invertebrate chordates show no evidence of WGD,[49,51,113,114] whereas those of jawed vertebrates exhibit clear evidence of 2R-WGD.[50,51] Thus, it is widely accepted that 2R-WGD took place in the vertebrate lineage after its divergence from invertebrate chordates. However, the exact timing of WGD in relation to the emergence of jawless vertebrates is still controversial. Three major possibilities have been suggested concerning the timing of WGD (Fig. 4): (i) the first round of WGD in a common ancestor of jawed and jawless vertebrates, and the second round in a common ancestor of jawed vertebrates (scenario A), (ii) both rounds before the separation of jawed and jawless vertebrates (scenario B); and (iii) both rounds in the jawed vertebrate lineage after its separation from the jawless vertebrate lineage (scenario C).

Initial analysis of 33 gene families by Escriva *et al.*[115] supported scenario A. This scenario is consistent with the observation that lampreys have single-chained hemoglobins whereas jawed vertebrate hemoglobins are composed of α and β chains.[116] It is also consistent with recent analysis of lamprey blood coagulation factors, using a draft genome assembly, which showed a simpler clotting system in jawless vertebrates.[117] Subsequent analysis of 358 gene families using sea lamprey expressed sequence tag sequences also favored scenario A, but it also raised the possibility that one or both rounds of WGD occurred nearly coincident with the lamprey lineage divergence.[51] More recently, Kuraku *et al.*[118] suggested, on the basis of the analysis of 55 gene families, that scenario B was most likely. However, this scenario appears inconsistent with the observation that lamprey genes can be assigned to one of the four human ohnologs in only 58% of gene families.[51] The absence of lamprey genes orthologous to one of the four human ohnologs may be

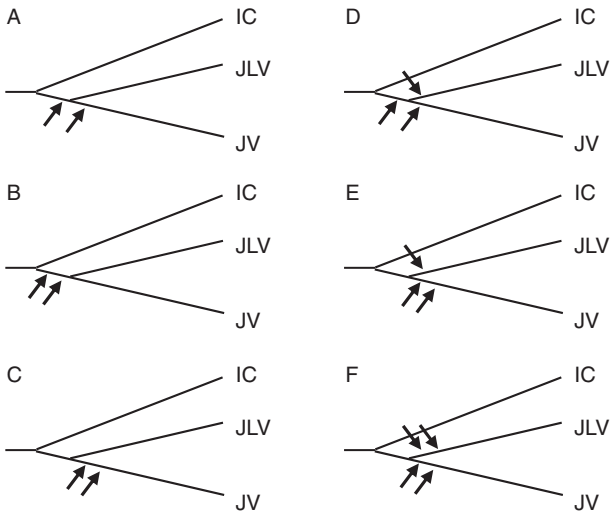


FIG. 4. Timing of WGDs in vertebrate evolution. Arrows indicate WGDs. Scenarios D–F assume independent WGD in a jawless vertebrate lineage. If scenario B is the case, jawed and jawless vertebrates should share corresponding ohnologs. In all other cases, they are in principle expected not to share strictly orthologous ohnologs. Two extant members of jawless vertebrates, hagfish and lamprey, are thought to have diverged 470–390 million years ago and are highly divergent from each other.[10] For simplicity, they are shown as a single group, but it is possible that their genome complexity is not the same as a result of independent lineage-specific WGDs. The third round of WGD, known to have occurred in some bony fish,[11] is not shown. Abbreviations: IC, invertebrate chordates; JLV, jawless vertebrates; and JV, jawed vertebrates.

accounted for by assuming extensive gene loss in the lamprey lineage; however, the presence of lamprey genes not orthologous to any of the four human ohnologs is difficult to explain.

Scenarios other than those discussed earlier are also possible (Fig. 4). For example, shortly after the separation of jawed and jawless vertebrate lineages, WGD may have taken place in both lineages independently, thus contributing to reduced gene orthology between the two lineages of vertebrates (scenarios D–F). Analysis of some gene families is consistent with these scenarios.[119,120] An ultimate resolution of the controversies surrounding the timing of WGD must await comprehensive analysis of the lamprey draft genome sequence.

So far as the genes involved in T cell and B cell immunity are concerned, there is ample evidence that jawless vertebrates lack many important ohnologs. For example, hagfish do not have authentic *GATA3* gene; their *GATA3*-like gene is equidistant from *GATA2* and *GATA3* and qualifies as a preduplicated form of *GATA2* and *GATA3*.[121] Likewise, hagfish do not have authentic *BTK* (gene coding for Bruton's tyrosine kinase), a member of the TEC family of

tyrosine kinases required for B cell maturation; their *BTK*-like gene is equidistant from *BTK* and *BMX*.[121] Similarly, two *Ikaros*-like transcription factor genes of lampreys, *IKFL1* and *IKFL2*, are almost equidistant from *Ikaros*, *Helios*, *Eos*, and *Aiolos* of jawed vertebrates and not related to any specific members.[103] Also, lamprey *SPI*, a member of the Ets family of transcription factors, is not orthologous to any of the gnathostome genes *SPI1* (PU.1), *SPIB*, or *SPIC*,[122,123] which are encoded by the neurotrophin paralogy group (Fig. 3). These observations are more consistent with scenario A and some of the scenarios that assume independent WGD in the jawless vertebrate lineage.

It should be emphasized that the importance of 2R-WGD remains unchanged regardless of which scenario turns out to be the case, because it is clear that many ohnologs with essential roles in T cell and B cell immunity owe their existence to 2R-WGD. If scenario B is the case, 2R-WGD provided a basis required for the emergence of the jawed vertebrate-type AIS. On the other hand, if scenario A is the case as has been generally favored,[7] the second round of WGD likely played an important role as a trigger to the emergence of the jawed vertebrate-type AIS, along with the acquisition of RAG transposons. All the other scenarios are compatible with the idea that the second round of WGD served as a trigger to the emergence of the jawed vertebrate-type AIS.

## V. The AIS of Jawless Vertebrates

Studies conducted in the 1960s and 1970s showed that lampreys were capable of producing specific agglutinins against particulate antigens and rejecting skin allografts with immunological memory,[124–130] suggesting that they are equipped with the AIS. In the following decades, extensive efforts were made to identify TCR, BCR, and MHC molecules in jawless vertebrates, but without any success.[121,131,132] This apparent paradox was resolved by the recent discovery that jawless vertebrates are equipped with a unique form of adaptive immunity that does not rely on TCR, BCR, or MHC molecules.[15,16]

### A. Rearranging Antigen Receptors of Jawless Vertebrates

VLRs are antigen receptors of jawless vertebrates expressed clonally on lymphocyte-like cells; they generate diversity comparable to that of antigen receptors of jawed vertebrates by somatically rearranging LRR modules.[15,16] The germ line *VLR* gene has an incomplete structure incapable of encoding functional proteins (Fig. 5). In its vicinity are a large number of LLR-encoding modules with highly diverse sequences. During the development of lymphocyte-like cells, these modules are sequentially incorporated into the *VLR* gene

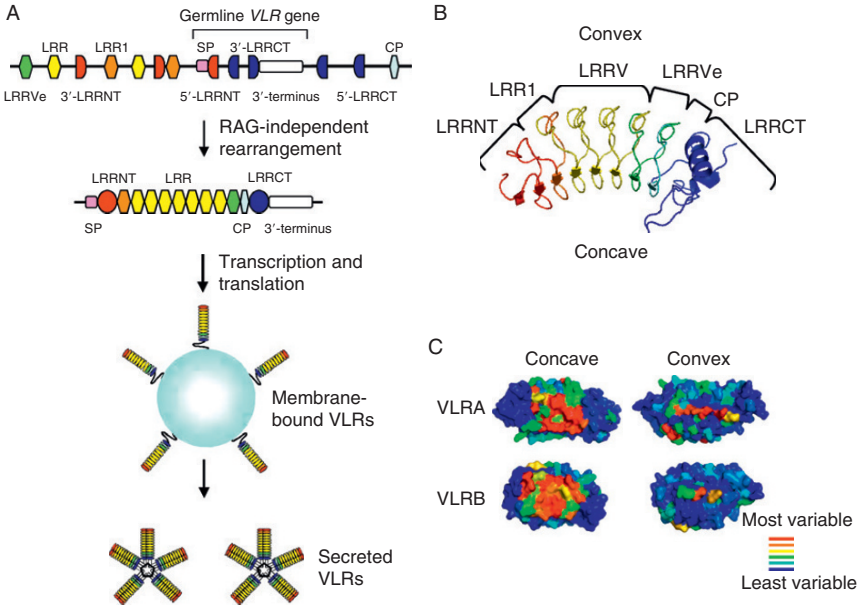


FIG. 5. Organization of the *VLR* gene and the structure of VLR proteins. (A) The germ line *VLR* gene has a structure incapable of encoding proteins. Modules coding for N-terminal caps (LRRNT), leucine-rich repeats (LRR), connecting peptides (CP) and C-terminal caps (LRRCT) occur in multiple copies adjacent to the germ line *VLR* gene. During the development of lymphoid cells, these modules are incorporated into the *VLR* gene. The rearranged *VLR* gene encodes a membrane-bound protein. The product of the *VLRB*, but not *VLRA*, gene is secreted and functions as antibodies. Whether membrane-bound VLRs occur as a monomer or multimer is not known. LRR1 and LRRVe denote LRR modules located at the N- and C-termini, respectively. The organization of the *VLR* locus shows considerable variation depending on loci and species. This figure, modified from Flajnik and Kasahara,[7] is intended to show salient features of *VLR* genes and does not accurately reproduce the organization of a specific *VLR* locus. (B) Crystal structure of hagfish VLRB molecules (PDB ID: 2o6S). The figure was generated using the PyMOL graphics tool (http://pymol.sourceforge.net/). (C) Sequence variability of hagfish VLR proteins. Variability is indicated by the color gradation from red to blue, where the most variable and the least variable patches are indicated in red and blue, respectively. Concave view (left); convex view (right). This figure was reproduced from Kim *et al.*[133]

by a process called "copy choice,"[134,135] presumably assisted by cytidine deaminases of the AID-APOBEC family.[136] The rearranged, mature *VLR* gene encodes a glycosylphosphatidylinositol-anchored polypeptide composed of an N-terminal cap (LRRNT), multiple LRR modules, a connecting peptide (CP), a C-terminal cap (LRRCT), an invariant threonine/proline-rich stalk, and a C-terminal hydrophobic tail. Because the sequence of individual LRR modules is highly diverse, and the number of LRR modules incorporated into a

rearranged gene shows considerable variation, a single *VLR* gene can generate combinatorial diversity comparable to that of BCR[137] (Fig. 5A). The crystal structures of hagfish VLR monomers indicate that they adopt a horseshoe-like solenoid structure characteristic of LRR family proteins (Fig. 5B), where seven-amino-acid LXXLXLX repeats of LRR modules form parallel β-strands in the concave surface (where L and X stands for leucine and any amino acid).[133] The majority of variable residues in VLR are located on the concave surface, suggesting that this surface is involved in antigen binding[133] (Fig. 5C). This suggestion was recently confirmed by *in vitro* mutagenesis experiments[138] and the crystal structure analysis of VLR–antigen complexes.[139,140]

## B. Independent Evolution of Antigen Receptors in Jawed and Jawless Vertebrates

TCR/BCR and VLR are similar in that they both rely on combinatorial joining of gene segments to generate diversity. However, these receptors are evolutionarily unrelated and their structures are completely different. An analogous situation is found in NK cell receptors of mammals.[7] In primates, NK receptors interacting with classical MHC class I molecules are members of the killer cell Ig-like receptor (KIR) family. By contrast, the corresponding receptors in rodents are C-type lectin-like molecules known as Ly49.[141,142] Thus, primates and rodents use totally unrelated molecules as NK receptors. Available evidence indicates that a common ancestor of primates and rodents possessed precursor genes for both types of NK receptors and that the primate and rodent lineages adopted distinct gene families as their NK receptors.[143] The occurrence of two radically different antigen receptors in vertebrates appears to be accounted for in a similar manner.[7]

## C. Two Types of Lymphoid Cells in Lamprey

Soon after the discovery of *VLR* in the sea lamprey,[13] it was shown that hagfish have two *VLR* genes.[14] These genes, named *VLRA* and *VLRB*, map to the same chromosome, but are distant from each other.[144] This suggested that they function as separate recombination units, with potentially distinct roles in host defense.

Subsequently, lampreys were shown to have two *VLR* genes orthologous to hagfish *VLRA* and *VLRB*.[136] Both genes are expressed exclusively on lymphocyte-like cells and seem to exhibit allelic exclusion.[145] In response to stimulation with antigen, $VLRB^+$ cells undergo clonal expansion and begin to secrete VLRs in a manner analogous to the secretion of Igs by B cells.[13,146] The secreted VLRB molecules, referred to as "VLRB antibodies," occur as pentamers or tetramers of dimers and have 8–10 antigen binding sites, thus resembling IgM in subunit organization[138] (Fig. 5A). Like IgM, VLRB binds antigens

carrying repetitive epitopes with high avidity and specificity, and displays strong agglutinating activities,[146] accounting for the earlier observations that immunized lampreys produce specific agglutinins.[124–130] Thus, VLRB$^+$ cells resemble B cells in that they both expand clonally and secrete antibodies in response to antigen challenge (Fig. 6).

Specific agglutinins are exclusively or almost exclusively derived from the *VLRB* gene.[146] This suggested a role other than the production of antibodies for lamprey VLRA$^+$ cells. Remarkably, recent evidence indicates that VLRA$^+$ cells resemble T cells[145]; VLRA$^+$ cells not only undergo blastoid transformation in response to a T cell mitogen, but they also express such genes as *IL17*,

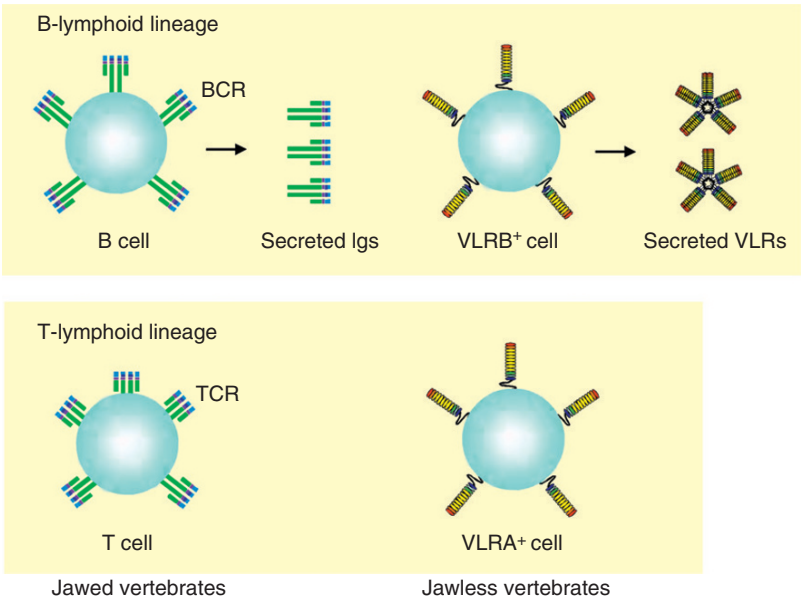


FIG. 6. Two lymphoid lineages in jawed and jawless vertebrates. VLR$^+$ cells of jawless vertebrates resemble lymphocytes of jawed vertebrates in that they clonally express specific antigen receptors and proliferate in response to antigen challenge. In both jawed and jawless vertebrates, two major populations of lymphoid cells have been identified. Like B cells, VLRB$^+$ cells secrete the antigen receptors as antibodies. By contrast, like T cells, VLRA$^+$ cells do not secrete the receptors. The gene expression profiles of VLRA$^+$ and VLRB$^+$ cells are remarkably similar to those of T cells and B cells, respectively.[145] Hence, it is likely that the two lineages of lymphoid cells emerged before the divergence of jawed and jawless vertebrates. After the divergence, jawed and jawless vertebrates appear to have adopted distinct molecules as their antigen receptors. If scenario A or D turns out to be the case (Fig. 4), it is possible that the first round of WGD was involved in the divergence of T-lymphoid and B-lymphoid lineages.

*GATA2/3*, and *NOTCH* whose jawed vertebrate counterparts are expressed in T cells and involved in their development and differentiation. Thus, lampreys have two major types of lymphocyte-like cells, with $VLRA^+$ and $VLRB^+$ cells likely involved in cellular and humoral arms of adaptive immunity, respectively[18,19,145] (Fig. 6).

More recently, a third VLR molecule, designated VLRC, was identified in the lamprey.[147] VLRC is expressed on a population of lymphocyte-like cells distinct from $VLRA^+$ or $VLRB^+$ cells. Because VLRC is more closely related in sequence to VLRA than to VLRB and is apparently not secreted, it was suggested that $VLRC^+$ cells might resemble T cells rather than B cells.[147] Interestingly, all classes of jawed vertebrates have two major lineages of T cells: αβ and γδ T cells.[148] It remains to be examined whether $VLRA^+$ and $VLRC^+$ cells are functionally specialized in a manner analogous to α/β and γ/δ T cells.

## D. Convergent Evolution or Common Ancestry?

In evolutionary biology, convergent evolution is defined as the process whereby distantly related organisms independently evolve similar traits to adapt to similar necessities. VLRs and TCRs/BCRs both serve as antigen receptors, but are evolutionarily unrelated. Thus, the use of distinct receptors in jawed and jawless vertebrates can be regarded as a prime example of convergent evolution.[13] However, the overall design of the AIS in jawed and jawless vertebrates seems too similar to be accounted for solely by convergent evolution. Particularly striking is the observation that both jawed and jawless vertebrates have two major populations of lymphoid cells presumed to have similar specialized immune functions[145] (Fig. 6). To account for this, it seems more reasonable to assume that $VLRA^+$ cells and T cells evolved from a common ancestor and that, likewise, $VLRB^+$ cells and B cells shared common ancestry; most likely, a common ancestor of all vertebrates was equipped with two lineages of lymphoid cells.[7] Recent evidence indicates that, contrary to a commonly held belief, T cells and B cells do not share an immediate common ancestor, but differentiate from myeloid-T and myeloid-B progenitors, respectively.[149,150] If T cells and B cells are distantly related as suggested by these studies, it is not surprising if the two lineages of lymphoid cells diverged at an earlier stage in evolution than previously thought.[151]

In summary, authentic T cells and B cells, as defined by surface expression of TCRs and BCRs, are unique to jawed vertebrates (Fig. 1). However, jawless vertebrates have at least two populations of lymphoid cells that likely share common ancestry with T cells and B cells of jawed vertebrates.

## VI. Concluding Remarks

The idea that the vertebrate genome underwent 2R-WGD close to the origin of vertebrates stirred hot debate for more than a decade.[41] This debate was finally settled in favor of the 2R hypothesis by systematic synteny comparison of human and amphioxus genomes.[51] As discussed earlier, many molecules that play essential roles in T cell and B cell immunity are encoded by ohnologs. Thus, the emergence of the AIS centered on T cells and B cells was critically dependent on WGD. A major unresolved issue at the moment is whether jawless vertebrates experienced 2R-WGD or not.[118] Despite this uncertainty, it is clear that jawless vertebrates lack many ohnologs indispensable for the function of the jawed vertebrate-type AIS.

It has been debated whether the AIS of jawed vertebrates emerged abruptly or gradually.[4,152] Evidence emerging from the studies of jawless vertebrates indicates that the two lineages of lymphoid cells that became T cells and B cells were already present in a common ancestor of all vertebrates.[7] Furthermore, the function of the gnathostome AIS is dependent on the participation of many genes that clearly evolved prior to the emergence of the AIS. In this sense, the AIS of jawed vertebrates emerged gradually, taking advantage of the resources already available in a common vertebrate ancestor.[4] On the other hand, it is also true that the two sudden accidents, the acquisition of RAG transposons[153] and the birth of a large number of ohnologs triggered by genome doubling,[30,37] transformed the nature of the immune system fundamentally, leading to the emergence of the jawed vertebrate-type AIS. In this regard, a novel form of immunity did emerge abruptly in a jawed vertebrate ancestor. Therefore, the evolutionary processes leading to the emergence of the jawed vertebrate-type AIS were gradual in some aspects, but abrupt in other aspects. This appears to be a more balanced view.

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