CHAPTER 12

Shark T-Cell Receptors

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CONTENTS

SUMMARY

Sharks possess the four canonical T-cell receptor (TCR) chains known from other vertebrates: α, β, γ, and δ. The loci encoding these chains employ recombination-activating gene (RAG)-mediated somatic cell V(D)J rearrangement mechanisms for diverse repertoires. Sharks have some additional immunogenetic TCR capacity, including the doubly rearranging NAR-TCR δ, somatic hypermutation, and trans-rearrangements that use immunoglobulin (Ig) variable segments.

T-CELL RECEPTORS

The cellular arm of the adaptive immune system of sharks depends upon lymphocytes derived from the thymus with hallmark T-cell receptors (TCRs) of the immunoglobulin superfamily. Lymphocytes of the only older vertebrates, the jawless lampreys and hagfish, use a very different

receptor system based on leucine-rich repeat domain molecules more akin to toll-like receptors than immunoglobulins (reviewed in Boehm et al. 2012). Thus, together with antibodies and the major histocompatibility complex (MHC) appearing in cartilaginous fish and all more recent vertebrates, but not in the jawless fishes, the existence of TCRs in sharks suggests that the adaptive immune system evolved in cartilaginous fish with the same fundamental major components that exist in warm-blooded vertebrates such as humans (Flajnik and Rumfelt 2000).

Canonical TCRs are heterodimers of proteins each consisting of a variable (V) and constant (C) region. Each of the two protein chains of the TCR contains an amino-terminal immunoglobulin superfamily V domain and an immunoglobulin superfamily C domain (Figure 12.1). TCRs are type I transmembrane glycoproteins, with extracellular V and C domains and a short cytoplasmic tail. Unlike the immunoglobulin that is secreted as antibody and serves as the B-cell receptor, TCRs are always membrane bound. It is these V domains of both protein chains that interact with ligand. Similar to the B-cell receptor, the short cytoplasmic tails of TCR chains are incapable of transducing a signal, so TCR heterodimers cluster with a complex of accessory molecules called CD3 that recruit cytoplasmic kinases to signal receptor ligation. There are usually only two TCR chain heterodimerization partnerships, αβ and γδ, and individual T cells bear receptors of one or the other form. The ligand of the αβ TCR is peptide antigen in the context of MHC. The γδ TCR binds a more diverse set of poorly characterized ligands and is not restricted to classical antigen presentation by MHC.

A great deal is known about the physiology of mammalian T cells bearing the αβ receptor. These subfunctionalize into $CD8^+$ cytotoxic T cells, $CD4^+$ regulatory T cells, and $CD4^+$ helper (T_H) cells. These T_H can be further classified as inflammatory T_H1 that activate macrophages, T_H2 that stimulate B cells to produce antibodies, and T_H17 that recruit neutrophils to sites of infection (to name but a few). Much less is known about γδ T cells. They develop from a distinct fate decision in the thymus and sometimes recognize antigen directly, in a manner more akin to antibody than the αβ TCR (Allison et al. 2001). Use of the comparative method to query the function of these cells in sharks may provide a clearer picture of the fundamental role of these cells in broader vertebrate immunity.

As we shall see, TCR genes of cartilaginous fish are organized much like TCR genes of other vertebrates. This is not the case with the Ig heavy and light chain gene loci that encode shark

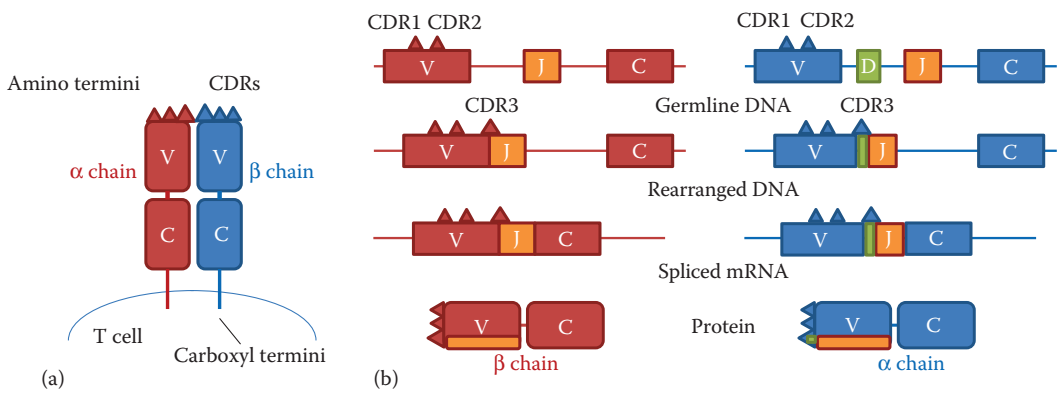

Figure 12.1 Schematic of protein chains in a heterodimeric TCR and the genes that encode them. (a) Domain structure of TCR heterodimer: rounded rectangles represent immunoglobulin superfamily domains and triangles represent complementary determining regions (CDR) that are most variable and articulate most with MHC/peptide antigen. (b) Simplified schematic of genetic elements that combine to form mature TCR gene and cartoons showing what those elements encode in the translated proteins. TCRδ rearrangement is similar to that of TCRβ (including the use of D segment), and TCRγ is similar to that of TCRα.

antibodies. The Ig gene multiple cluster organization (Hinds and Litman 1986) and frequent germline joining (Kokubu et al. 1988) are deviations from the vertebrate norm. Much of what is known of shark TCR is consistent with their form and function in *higher* vertebrates, but shark TCR has provided some surprising deviations whose functional consequences for their immune system are just starting to be explored.

PIONEERING FUNCTIONAL STUDIES

Early work studying adaptive immunity in sharks yielded neither robust responses nor conclusive results. The first work by Robert Good's group found evidence for memory to allografts in Chondrichthyes (Perey et al. 1968) but a study in the horned shark *Heterodontus francisci* by Bill Hildemann's team determined that the response to skin allografts was chronic (Borysenko and Hildemann 1970; Hildemann 1970). It has since been suggested that maintaining the animals at 22°C may have marred these results described in the nurse shark *Ginglymostoma cirratum*. Although the adherent, cold-loving effectors were likely macrophages (Pettey and McKinney 1983), they may have been regulated by T cells (although MHC restriction was lacking) (Haynes and McKinney 1991). Now that we are better prepared with immunogenetic data and experimental tools, T-cell function in shark can and should be reexamined.

MOLECULAR WORK YIELDING CANONICAL TCR cDNA SEQUENCES

A series of publications by Gary Litman's group in the 1990s elucidated the expressed TCR of cartilaginous fishes. Then, a student in the lab, Jonathan Rast, devised short, minimally degenerate PCR primers that exploited the conserved WYRQ and YYCA motifs of the framework 2 and framework 3 regions of immunoglobulin light chains and TCR. This approach yielded an amplicon from horned shark genomic DNA that was used as a probe to screen a spleen cDNA library, resulting in the discovery of mature TCRβ homologue transcripts (Rast and Litman 1994). Four complete and one partial V sequences showed diversity in this domain and the C region sequence clearly showed highest homology to tetrapod TCRβ versus other antigen receptor chains.

A second, more extensive cDNA study of horned shark TCRβ characterized 55 clones from the spleen (Hawke et al. 1996). This work, first-authored by Noel Hawke (yes, brother of actor Ethan), found seven diverse V families, a consensus D sequence, and 18 J sequences used in the TCRβ repertoire of this species. The dataset showed V family multiplicity that was more suggestive of a translocon arrangement of TCR loci for the horned shark, in contrast to the multicluster organization described for shark antibody genes. Diverse length was found in the CDR3 regions formed by the juxtaposition of V, D, and J segments, indicative of both complex shark TCR loci and complex resolution of the coding joints in elasmobranch RAG-mediated recombination (specifically, the existence of nontemplate additions by terminal deoxynucleotidyl transferase (TdT) and removal of bases by exonuclease activity).

This same PCR approach was successful in identifying chicken TCRγ, *Xenopus* TCRα, pufferfish TCRα, and horned shark TCRδ from genomic and cDNA libraries (Rast et al. 1995). At the time, the authors were conservative in assigning the horned shark sequences to α or δ and left open the possibility that the two loci had yet to diverge to form separate chains in the cartilaginous fish. Time would tell, however, that their suggestion that these sequences encoded TCRδ was indeed correct.

Rast's last publication while in Litman's group definitively showed that cartilaginous fish share all four TCR chains found in other vertebrates. In the clearnose skate *Raja eglanteria*, he refined

his minimally degenerate framework 2 and 3 PCR primers to clone α, β, γ, and δ from this batoid elasmobranch (Rast et al. 1997) in which lymphoid tissue architecture and development had already been defined by Carl Luer's group (Luer et al. 1995). The sequences described in this work placed α, β, γ, and δ TCR at the dawn of jawed vertebrate adaptive immunity, suggesting chains of similar structure to their mammalian counterparts and similar repertoire diversity. This manuscript described complete cDNAs for each of the four chains as well as representative V and J gene segment and junctional diversity.

Thirteen years passed after Rast's *Immunity* paper before the characterization of TCR α, β, γ, and δ was completed in a shark. All four chains were identified in the nurse shark (by four different approaches) detailing hark sequences and repertoire diversity that are fundamentally similar to that found in the skate, and with a couple of additional surprises (Criscitiello et al. 2010) that are described below.

GENOMIC ORGANIZATION

In Rast and Litman's first horned shark TCR study, genomic Southern blots with V and C probes showed much concordance in hybridizing bands, though not absolutely (Rast and Litman 1994). These data were seen as consistent with the existence of multiple V-C loci in the shark genome, an organization akin to that found for the immunoglobulin genes. Screening of a horned shark genomic phage library with these V and C probes identified nearly 300 hybridizing clones. Twelve unique V-containing clones were restriction mapped, eight contained two Vs, and eleven hybridized to a J probe (Rast and Litman 1994). Twelve C clones were similarly mapped and ten found to be unique, five bearing J sequences. This same publication described genomic V genes lacking the intron-split leader peptide typical of vertebrate antigen receptor genes but found in chicken TCRα (Gobel et al. 1994).

Deeper cDNA analysis of horned shark TCRβ made the multicluster organization less obvious. Multiple, diverse V families and many J sequences with relative uniformity in C sequences suggested the combinatorial mechanisms of the translocon genomic organization (Hawke et al. 1996). This led to the conclusion that TCR genes of sharks may be more similar between sharks and men than immunoglobulin genes. This latter idea was corroborated by work in the skate and nurse shark, where genomic Southern blotting with constant region probes yielded at most two bands and often one for any TCR chain, again suggesting translocon organization (Criscitiello et al. 2010; Rast et al. 1997).

The translocon genomic organization was definitively confirmed for the sandbar shark *Carcharhinus plumbeus* TCRγ locus by the group of Jack Marchalonis (Chen et al. 2009). The 32 kb locus containing five Vs, three Js, and a C gene is about one-fifth the size of the human locus, which contains more Vs, Js, and nonfunctional V pseudogenes. The locus was assembled from a combination of PCR products and chromosomal walking fragments. The careful assembly of this TCRγ locus in the sandbar shark set the stage for a major immunogenetic discovery that would depend upon a well-defined germline genomic sequence.

GENERATION OF DIVERSITY

The trailblazing horned shark TCR work identified canonical recombination signal sequences (RSS) flanking germline genomic elements used in the construction of mature variable domain encoding exons (Rast and Litman 1994). V segments had 23-bp-spaced RSS 3′ of their coding sequences and J genes had 12-bp-spaced RSS 5′ of their coding sequence. It is predicated that

D elements flanked by 5′ 12-bp-spaced and 3′ 23-bp-spaced RSS could be contributing to the horned shark TCRβ diversity at CDR3. AQ 1

The clonal selection theory requires a single antigen receptor specificity for a given lymphocyte, though we know this is not a steadfast rule (Brady et al. 2010). Much work has been performed by Ellen Hsu's group elucidating isotypic and allelic exclusion at B-cell receptor loci in shark (Malecek et al. 2005, 2008; Zhu et al. 2011), yet comparatively little is understood of these mechanisms at the shark TCR loci. At the antibody genes of shark (as at those genes of chicken, rabbit, and other vertebrates), there does not appear to be the need for the rigid, sequential, stepwise process for maintenance of isotypic and allelic exclusion at the cellular level yet a diverse repertoire in the organism (Hsu 2009).

The group of Jack Marchalonis identified both recombination-activating genes RAG1 and RAG2 in the sandbar shark. Interestingly, in shark as in humans, the RAG lack introns and are closely linked (Bernstein et al. 1994, 1996; Schluter and Marchalonis 2003). This is consistent with their origin in the vertebrate adaptive immune system via horizontal transfer as the transposase system of a prokaryotic transposon, with RSS evolving from the terminal repeats of the ancestral transposon.

Nontemplate nucleotides are found at the V(D)J junctures of rearranged B- and T-cell receptor CDR3 regions. TdT, the X family polymerase responsible for these additions, has been identified in both shark and skate (Bartl et al. 2003). In elasmobranchs as in bony vertebrates, the predicted structure of TdT suggests both a lack of substrate nucleotide specificity and template independence. Phylogenetically, the TdT of cartilaginous fish appears to be more akin to ancient polymerases than to polymerases lambda and beta.

The outcome of RAG-mediated V(D)J recombination with CDR3-extending TdT action is a diverse shark repertoire for all four TCR chains (Criscitiello et al. 2010). The range of CDR3 lengths are larger in the limited shark sampling than those seen for mouse and man, suggesting large diversity in this most important loop for antigen recognition. Significant diversity is also added to the TCR paratope at CDR1 and CDR2 by diverse V genes, and these V genes display trans-species evolutionary maintenance (Criscitiello et al. 2010) as has been noted for MHC alleles (Klein 1987).

Our understanding of shark TCR repertoire development, diversity, and dynamics is still very much in its infancy, but two distinct themes are beginning to emerge. As detailed thus far in this chapter, the first is that sharks are capable of making αβ and γδ TCR similar to *higher* vertebrates with just as much diversity and employing similar immunogenetic mechanisms of rearrangement. The second theme, to be described in the next three sections, is that shark TCR loci perform some extraordinary feats very much at odds with mouse-centric immune dogma.

NAR TCR AQ 2

Nurse shark TCR characterization work in the lab of Martin Flajnik revealed a subset of unheralded, longer TCR δ cDNA sequences by 5′ RACE (rapid amplification of cDNA ends) PCR (Criscitiello et al. 2006). These sequences encoded two V domains and a TCRδ C domain, which at the time was the first occurrence of a lymphocyte antigen receptor chain containing more than one V domain. Further inquiry determined that about 20% of nurse shark TCRδ rearrangements held to this longer, two-V form. Some TCRδV gene families were always found to have a canonical leader peptide and existed in mature cDNAs with only one V and TCRδ C, whereas other TCRδV gene families were exclusively found as the membrane proximal V domain in the larger TCRδ products, supporting an additional membrane distal V domain. The additional membrane distal V domain encoded 5′ of the TCRδV was always closely related to the V domains of IgNAR, an

immunoglobulin heavy chain peculiar to the cartilaginous fish that does not heterodimerize with light chain (Greenberg et al. 1995). Thus, the special TCRδ chains employing the additional V domain were given the moniker of NARTCR. This was satisfying as the hypothesized NARTCR δ is expected to heterodimerize with a TCRγ chain that lacks the additional domain, and therefore, both the IgNAR V domain and the NARTCR δ V domains would be expected to lack a pairing partner.

Both V domains in the NARTCR δ chain are the products of RAG-mediated V(D)J recombination. Genomic DNA sequencing by long-range PCR identified several blocks of NARTCRV-NARTCRD-NARTCRJ-TCRδV separated by short introns of ~300 bp (Criscitiello et al. 2006). Importantly, there is no split leader peptide encoded between the NARTCRJ and supporting TCRδV, yet standard GT/AG intron splicing does splice the J of one V domain to the V segment encoding the other at the RNA level. Mature NARTCR would always be constructed from the NARTCR V-D-J and supporting TCRδV all from one of these genomic blocks, whereas the J used in the supporting TCR V domain could be from any of the ~30 that also are used in rearrangements to canonical (non-NARTCR supporting) TCRδV. Adding further diversity, the CDR3 regions of both the NARCRV and the supporting TCRV were diversified with palindromic and N nucleotide additions. Thus, a model emerged where V(D)J rearrangement of the membrane and C domain proximal domain encoding V is instructive for the generation of a canonical (one V) TCRδ or a NARTCRδ (with two V). If this initial rearrangement selects a canonical TCRδV (with a leader peptide 5′ of it) a canonical TCRδ will result. If the initial rearrangement selects a supporting TCRδV (with a NARTCRV-D-J block 5′ of it) that block will subsequently rearrange (or has concurrently rearranged) and will result in the NARTCRδ.

Data from the elephant shark *Callorhinchus milli* genome project suggests that NARTCR has similar diversity and genomic organization in that more ancient Holocephalian fish (Venkatesh et al. 2007), which suggests that NARTCR may be widespread in the cartilaginous fish. Curiously, TCR with two V domains is now known not to be restricted to cartilaginous fish. Nonplacental mammals have a fifth TCR chain called TCRμ that appears to have converged on a similar, two V domain structure as shark NARTCRδ (reviewed in Miller 2010). Rob Miller's group has elucidated the evolution of this fifth TCR chain in early mammals that is most closely related to TCRδ and uses V domains more akin to those of the IgH locus. In marsupials, the supporting V is germline-joined yet in the monotreme platypus it diversifies as in shark NARTCR (Parra et al. 2007, 2012).

TRANS-REARRANGEMENTS

AQ 3 The Flajnik group's characterization of nurse shark TCR also unearthed a second unusual subset of TCRδ chain rearrangements, these using Ig heavy chain V segments rearranged with D, J and spliced to C of TCR δ (Criscitiello et al. 2010). The first of these cDNA's was isolated by Rebecca Lohr in an IgWV library screen (IgW is the shark orthologue of IgD) (Ohta and Flajnik 2006), when she found TCRδ with a clone of IgWV-TCRδD-J-C from neonatal shark. These trans-rearrangements between immunoglobulin and TCR antigen receptor locus elements employ only a few V genes from IgM and IgW, never the IgNAR that shun Ig light chains. Since that publication, we have characterized many such chimeric rearrangements from thymus, spleen, and spiral valve of sharks of adult sharks. The CDR3 encoding regions of these sequences are almost always in frame, at a much higher frequency than if they were not being selected for functional protein products on the surface of T cells. Quantitative real-time PCR suggests that these chimeric Ig-TCR mRNAs are being expressed with a tissue distribution consistent with T-cell expression (most signal in thymus) and at a level less than but on the same order of magnitude as the canonical TCRδ rearrangements using TCRδV.

At the same time the trans-rearrangements were discovered in shark, the Miller group at the University of New Mexico described the amphibian *Xenopus* TCRα/δ locus, finding that it contains IgH V genes that are used in TCRδ rearrangements (Parra et al. 2010). Subsequent studies have unearthed seemingly related combinations of IgHV and the TCRδ loci in birds (Parra and Miller 2012; Parra et al. 2012), platypus (Parra et al. 2012), and coelacanth (Amemiya et al. 2013). Although more shark genomic antigen receptor locus characterization is required to understand if the Ig/TCR trans-rearrangements in shark are orthologous or convergent with the IgHV use in the TCRδ loci of Sarcopterygii (lobe-finned fish and tetrapods), it is intriguing to think that vertebrates old and (relatively) new employ antibody V gene segments in their TCRδ repertoires.

SOMATIC HYPERMUTATION

Shark B cells also use the activation-induced cytidine deaminase (AID) (Conticello et al. 2005) in what Tonegawa called the *fourth somatic diversifier* after gene segment combinatorial diversity, imprecise coding ends, and nontemplate additions: somatic hypermutation (SHM) (Tonegawa 1983). In addition to SHM in all jawed vertebrate immunoglobulins, AID now has been implicated in the processes of heavy chain class switch recombination in sharks (Zhu et al. 2012) as well as tetrapods, and immunoglobulin gene conversion in birds and mammals as well (Barreto and Magor 2011).

The notion of SHM at TCR loci was recently given credence by work in the sandbar shark driven by Sam Schluter. Sequencing of the entire TCRγ translocon in *Carcharhinus plumbeus* allowed definitive determination that SHM is occurring at that locus (Chen et al. 2009). The shark TCRγ SHM occurs in two distinct patterns: point mutations and tandem mutations characteristic of SHM in cartilaginous fish (Anderson et al. 1995; Lee et al. 2002; Zhu and Hsu 2010), possibly suggesting two different mechanisms (Chen et al. 2012). The sandbar shark analysis found targeted nucleotide motifs of AID activity at the TCRγ locus and evidence for SHM being used for repertoire diversification rather than affinity maturation. Our study of TCR expression in the nurse shark showed some evidence for SHM in that species, in both the γ and α chains (Criscitiello et al. 2010), although it was not recognized as such before Schluter's seminal discovery. This preliminary nurse shark mutation data mandated a more rigorous analysis of the TCRα expression in our nurse shark model.

Additional cloning and sequencing of TCR from multiple sharks found evidence for somatic hypermutation not only in the TCRα sequences isolated from peripheral lymphoid tissues such as spleen and spiral valve, but in the thymus as well. In the absence of a fully assembled TCRα locus, somatic hypermutation was confirmed by mutated sequences with the same CDR3 rearrangement. Interestingly for the nurse shark TCRα set, more mutations are found in the framework regions compared to the CDR regions, yet more tandem mutations are found in the CDRs. There are more nonsynonymous mutations that change the amino acid coded for by a codon than synonymous ones, and more of these nonsynonymous mutations in the CDR compared to the framework regions. We found no transition, transversion, or particular nucleotide bias in the mutations and nearly no evidence of mutation at TCRβ, TCRδ nor in the constant region exons of these clones.

We were curious if this SHM was due to AID activity in the shark thymus and found evidence for just that via quantitative PCR and *in situ* hybridization (nurse shark AID sequence kind gift of Ellen Hsu). Intriguingly, Niels Jerne suggested over forty years ago that the thymus was a *mutant-breeding organ* in which mutation was used to modify overly self-reactive cells to generate both self-tolerance and diversity in repertoire specificity (Jerne 1971). Much work remains to

determine definitively whether TCR SHM is used for repertoire diversification, rescue for passage of thymic selection, affinity maturation in the periphery, or something entirely novel altogether.

THYMUS AND T-CELL DEVELOPMENT

In the clearnose skate, real-time PCR showed expression of all four TCR chains in the thymus of eight-week-old embryos, then an apparent shut down of that thymic expression in after hatching (Miracle et al. 2001). Northern blotting of adult nurse shark with TCR C region probes gave a more expected robust signal (Criscitiello et al. 2010). *In situ* hybridization experiments in nurse shark thymus generally showed great conservation of thymic architecture and gene expression between shark and mammal. RAG1 and TdT were strongest in the subcapsular region and expressed throughout the cortex, indicating active V(D)J recombination and junctional diversification there. MHC class I and II were generally expressed in the medulla yet more sparsely in the cortex. TCRα and β were brightest in the central cortex but weaker in the subcapsular region and medulla, γ and δ were also in the cortex but brighter in the medulla, with delta having the greatest medullary expression of the four. There is evidence for greater γ and δ expression relative to α and β in shark thymus versus that of mammals (Criscitiello et al. 2010).

PERIPHERAL EXPRESSION AND FUNCTION

In the clearnose skate, both northern blotting and real-time PCR were used to track antigen receptor, TdT, and RAG expression in different tissues and developmental stages (Miracle et al. 2001). At eight weeks of development, all four TCR genes appear to be expressed, beta perhaps a week earlier and delta in a bell curve of expression over several weeks. This expression is exclusively in the thymus in the eight-week-old embryo but showed diverse, chain-specific expression
AQ 4 profiles in the spleen, intestine, and liver of hatchling skates. In addition to skate thymus spleen adult TCR was expressed heavily in the rectal gland, intestine, and liver.

Adult nurse shark relative TCR expression in peripheral tissues by northern blotting was generally consistent amongst the four chains: spleen > spiral valve > gill > peripheral blood leukocytes > pancreas > liver. Epigonal organ, ovary, and brain were negative (Criscitiello et al. 2010).

No data are available describing actual function of these receptors in shark cell systems or mam-
AQ 5 malian transfections. It is of note that a glutamine (position 136 in human) in the TCRβ-connecting peptide, thought to be crucial for signaling efficiency, is present in shark, skate, and mammal but generally not bony fish, amphibians, reptiles, and birds (Backstrom et al. 1996).

COMPARATIVE GENOMICS

The recent publication of the elephant shark *Callorhinchus milii* genome gives much insight into the evolution of TCR loci and T-cell biology in the holocephalans and broader cartilaginous fish as well (Venkatesh et al. 2014). Major findings of the elephant shark genome include the close linkage of Ig and TCR genes, NARTCR yet no IgW or IgNAR, and no canonical CD4 and evidence for a primitive, very limited helper T-cell capacity. These results are consistent with the trans-rearrangements seen in nurse shark being enabled by the close proximity of immunoglobulin V genes and TCR D segments. Additionally, they suggest that the NAR V domain was originally a TCR component that was secondarily co-opted for B-cell receptor use in evolution. However, the distinction between T- and B-cell receptor immunogenetics appears to have been weaker 450 million years ago and in extant cartilaginous fish.

EVOLUTION

When all four TCR chains were found in the skate without obvious multicluster or germline-joined genes, the authors suggested the possibility of a longer or more stable evolutionary history of TCR genes compared to those of Ig (Rast et al. 1997). I like this notion, as vertebrates have evolved myriad Ig isotypes (I think it is likely that at least IgM, IgD, IgT, IgA, IgY, IgG, and IgE are distinct classes) and divergent organs for B-cell development (Leydig organ, epigonal, bursa, bone marrow, fetal liver, ileal Peyers patch, anterior kidney, and appendix are all primary B-cell tissues in some species), yet it appears most have held fast to α, β, γ, and δ TCR developing in the thymus (save some recent notable developments). That should be one take-home message for the evolution of vertebrate TCR, and it is anchored by shark data as oldest group with TCR. However, sharks have also introduced a second theme in TCR evolution that TCR may be able to employ some antigen receptor immunogenetic tricks thought only to be associated with B cells. The doubly rearranging NARTCR with its presumably partnerless V domain paratope, SHM at TCR loci, and the use of IgH V in TCRδ are all exciting findings that will help us reevaluate the physiological boundaries of TCR and T-cell physiology in mammals (Figure 12.2). AQ 6

AID is a member of the APOBEC family of nucleic acid mutators, some of which have been found to diversify the variable lymphocyte receptor (VLR) system in the more ancient vertebrate lineages of lamprey and hagfish (Guo et al. 2009). There is an emerging connection between the use of AID as a diversifying agent for adaptive immunity now in shark B- and T-cell repertoires with APOBEC family members being used to diversify the older VLR system in lamprey and hagfish (Rogozin et al. 2007). More functional work in shark may shed light on the relative importance of RAG versus AID-mediated diversification mechanisms for the genesis of the gnathastome adaptive immune system.

We have every reason to believe that the V genes of αβ T cells have evolved with MHC for 450 million years (Yin et al. 2012), as sharks have polygenic, polymorphic MHC (Kasahara et al. 1992). Shark MHC/TCR physiology may help answer the chicken-egg conundrum of how the system began (Kurosawa and Hashimoto 1997). Although a smoking gun, non-rearranging receptor gene that was clearly the recipient of a RAG transposon invasion one half billion years ago has yet to be found (Rast and Litman 1998), studies of TCR in sharks and other lower chordates will continue to elucidate the natural history of man's immune system and expose new potential paths of clinical intervention for its future.

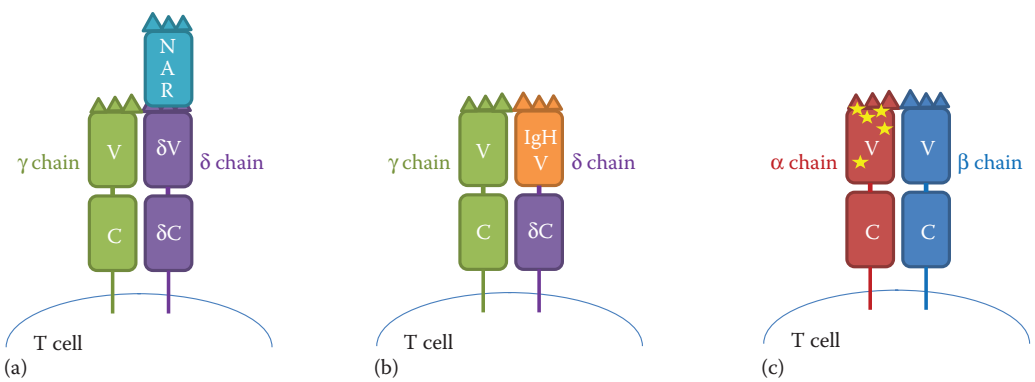

Figure 12.2 Three immunogenetic tricks available to shark T-cell receptors that depart from the mouse/human norm. (a) NARTCR extends the TCRδ chain with a second somatically rearranged V domain supported by special TCRδV domains. (b) Trans-rearrangements allow TCRδ to draw from Ig heavy chain V segments to create V domains on T cells that are largely the product of antibody genes (the D and J are contributed by δ gene segments). Mounting evidence suggests that many vertebrate groups have a similar capability. (c) Somatic hypermutation, known as a B-cell phenomenon, acts upon the light chains of in sharks TCR, α and γ.

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REFERENCES

Allison, T.J., Winter, C.C., Fournie, J.J., Bonneville, M., and Garboczi, D.N. Structure of a human gammadelta T-cell antigen receptor. *Nature* 2001; 411:820–824.

Amemiya, C.T., Alfoldi, J., Lee, A.P., Fan, S.H., Philippe, H., MacCallum, I., Braasch, I. et al. The African coelacanth genome provides insights into tetrapod evolution. *Nature* 2013; 496:311–316.

Anderson, M.K., Shamblott, M.J., Litman, R.T., and Litman, G.W. Generation of immunoglobulin light chain gene diversity in Raja erinacea is not associated with somatic rearrangement, an exception to a central paradigm of B cell immunity. *J. Exp. Med.* 1995; 182:109–119.

Backstrom, B.T., Milia, E., Peter, A., Jaureguiberry, B., Baldari, C.T., and Palmer, E. A motif within the T cell receptor alpha chain constant region connecting peptide domain controls antigen responsiveness. *Immunity* 1996; 5:437–447.

Barreto, V.M., and Magor, B.G. Activation-induced cytidine deaminase structure and functions: A species comparative view. *Dev. Comp. Immunol.* 2011; 35:991–1007.

Bartl, S., Miracle, A.L., Rumfelt, L.L., Kepler, T.B., Mochon, E., Litman, G.W., and Flajnik, M.F. Terminal deoxynucleotidyl transferases from elasmobranchs reveal structural conservation within vertebrates. *Immunogenetics* 2003; 55:594–604.

Bernstein, R.M., Schluter, S.F., Bernstein, H., and Marchalonis, J.J. Primordial emergence of the recombination activating gene 1 (RAG1): Sequence of the complete shark gene indicates homology to microbial integrases. *Proc. Natl. Acad. Sci. U.S.A.* 1996; 93:9454–9459.

Bernstein, R.M., Schluter, S.F., Lake, D.F., and Marchalonis, J.J. Evolutionary conservation and molecular cloning of the recombinase activating gene 1. *Biochem. Biophys. Res. Commun.* 1994; 205:687–692.

Boehm, T., McCurley, N., Sutoh, Y., Schorpp, M., Kasahara, M., and Cooper, M.D. VLR-based adaptive immunity. *Annu. Rev. Immunol.* 2012; 30:203–220.

Borysenko, M., and Hildemann, W.H. Reactions to skin allografts in the horn shark, Heterodontis francisci. *Transplantation* 1970; 10:545–551.

Brady, B.L., Steinel, N.C., and Bassing, C.H. Antigen receptor allelic exclusion: An update and reappraisal. *J. Immunol.* 2010; 185:3801–3808.

Chen, H., Bernstein, H., Ranganathan, P., and Schluter, S.F. Somatic hypermutation of TCR gamma V genes in the sandbar shark. *Dev. Comp. Immunol.* 2012; 37:176–183.

Chen, H., Kshirsagar, S., Jensen, I., Lau, K., Covarrubias, R., Schluter, S.F., and Marchalonis, J.J. Characterization of arrangement and expression of the T cell receptor gamma locus in the sandbar shark. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:8591–8596.

Conticello, S.G., Thomas, C.J., Petersen-Mahrt, S.K., and Neuberger, M.S. Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Mol. Biol. Evol.* 2005; 22:367–377.

Criscitiello, M.F., Ohta, Y., Saltis, M., McKinney, E.C., and Flajnik, M.F. Evolutionarily conserved TCR binding sites, identification of T cells in primary lymphoid tissues, and surprising trans-rearrangements in nurse shark. *J. Immunol.* 2010; 184:6950–6960.

Criscitiello, M.F., Saltis, M., and Flajnik, M.F. An evolutionarily mobile antigen receptor variable region gene: Doubly rearranging NAR-TcR genes in sharks. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:5036–5041.

Flajnik, M.F., and Rumfelt, L.L. The immune system of cartilaginous fish. *Curr. Top. Microbiol. Immunol.* 2000; 248:249–270.

Gobel, T.W., Chen, C.L., Lahti, J., Kubota, T., Kuo, C.L., Aebersold, R., Hood, L., and Cooper, M.D. Identification of T-cell receptor alpha-chain genes in the chicken. *Proc. Natl. Acad. Sci. U.S.A.* 1994; 91:1094–1098.

Greenberg, A.S., Avila, D., Hughes, M., Hughes, A., McKinney, E.C., and Flajnik, M.F. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. *Nature* 1995; 374:168–173.

Guo, P., Hirano, M., Herrin, B.R., Li, J., Yu, C., Sadlonova, A., and Cooper, M.D. Dual nature of the adaptive immune system in lampreys. *Nature* 2009; 459:796–801.

Hawke, N.A., Rast, J.P., and Litman, G.W. Extensive diversity of transcribed TCR-beta in phylogenetically primitive vertebrate. *J. Immunol.* 1996; 156:2458–2464.

Haynes, L., and McKinney, E.C. Shark spontaneous cytotoxicity: Characterization of the regulatory cell. *Dev. Comp. Immunol.* 1991; 15:123–134.

Hildemann, W.H. Transplantation immunity in fishes: Agnatha, Chondrichthyes and Osteichthyes. *Transplant. Proc.* 1970; 2:253–259.

Hinds, K.R., and Litman, G.W. Major reorganization of immunoglobulin VH segmental elements during vertebrate evolution. *Nature* 1986; 320:546–549.

Hsu, E. V(D)J Recombination: Of Mice and Sharks. V(D)J recombination. 2009; 650:166–179. AQ 8

Jerne, N.K. The somatic generation of immune recognition. *Eur. J. Immunol.* 1971; 1:1–9.

Kasahara, M., Vazquez, M., Sato, K., McKinney, E.C., and Flajnik, M.F. Evolution of the major histocompatibility complex: Isolation of class II A cDNA clones from the cartilaginous fish. *Proc. Natl. Acad. Sci. U.S.A.* 1992; 89:6688–6692.

Klein, J. Origin of major histocompatibility complex polymorphism: The trans-species hypothesis. *Hum. Immunol.* 1987; 19:155–162.

Kokubu, F., Litman, R., Shamblott, M.J., Hinds, K., and Litman, G.W. Diverse organization of immunoglobulin VH gene loci in a primitive vertebrate. *EMBO J.* 1988; 7:3413–3422.

Kurosawa, Y., and Hashimoto, K. How did the primordial T cell receptor and MHC molecules function initially? *Immunol. Cell Biol.* 1997; 75:193–196.

Lee, S.S., Tranchina, D., Ohta, Y., Flajnik, M.F., and Hsu, E. Hypermutation in shark immunoglobulin light chain genes results in contiguous substitutions. *Immunity* 2002; 16:571–582.

Luer, C., Walsh, C.J., Bodine, A.B., Wyffels, J.T., and Scott, T.R. The Elasmobranch Thymus: Anatomical, histological, and preleminary functional characterization. *J. Exp. Zool.* 1995; 273:342–354.

Malecek, K., Brandman, J., Brodsky, J.E., Ohta, Y., Flajnik, M.F., and Hsu, E. Somatic hypermutation and junctional diversification at Ig heavy chain loci in the nurse shark. *J. Immunol.* 2005; 175:8105–8115.

Malecek, K., Lee, V., Feng, W., Huang, J.L., Flajnik, M.F., Ohta, Y., and Hsu, E. Immunoglobulin heavy chain exclusion in the shark. *PLoS Biol.* 2008; 6:e157.

Miller, R.D. Those other mammals: The immunoglobulins and T cell receptors of marsupials and monotremes. *Semin. Immunol.* 2010; 22:3–9.

Miracle, A.L., Anderson, M.K., Litman, R.T., Walsh, C.J., Luer, C.A., Rothenberg, E.V., and Litman, G.W. Complex expression patterns of lymphocyte-specific genes during the development of cartilaginous fish implicate unique lymphoid tissues in generating an immune repertoire. *Int. Immunol.* 2001; 13:567–580.

Ohta, Y., and Flajnik, M. IgD, like IgM, is a primordial immunoglobulin class perpetuated in most jawed vertebrates. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:10723–10728.

Parra, Z.E., Baker, M.L., Schwarz, R.S., Deakin, J.E., Lindblad-Toh, K., and Miller, R.D. A unique T cell receptor discovered in marsupials. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:9776–9781.

Parra, Z.E., Lillie, M., and Miller, R.D. A model for the evolution of the Mammalian T-cell receptor alpha/delta and mu Loci based on evidence from the Duckbill Platypus. *Mol. Biol. Evol.* 2012; 29:3205–3214.

Parra, Z.E., and Miller, R.D. Comparative analysis of the chicken TCR alpha/delta locus. *Immunogenetics* 2012; 64:641–645.

Parra, Z.E., Mitchell, K., Dalloul, R.A., and Miller, R.D. A second TCRdelta locus in Galliformes uses antibody-like V domains: Insight into the evolution of TCRdelta and TCRmu genes in tetrapods. *J. Immunol.* 2012; 188:3912–3919.

Parra, Z.E., Ohta, Y., Criscitiello, M.F., Flajnik, M.F., and Miller, R.D. The dynamic TCRdelta: TCRdelta chains in the amphibian Xenopus tropicalis utilize antibody-like V genes. *Eur. J. Immunol.* 2010. AQ 9

Perey, D.Y., Finstad, J., Pollara, B., and Good, R.A. Evolution of the immune response. VI. First and second set skin homograft rejections in primitive fishes. *Lab. Invest.* 1968; 19:591–597.

Pettey, C.L., and McKinney, E.C. Temperature and cellular regulation of spontaneous cytotoxicity in the shark. *Eur. J. Immunol.* 1983; 13:133–138.

Rast, J.P., Anderson, M.K., Strong, S.J., Luer, C., Litman, R.T., and Litman, G.W. Alpha, beta, gamma, and delta T cell antigen receptor genes arose early in vertebrate phylogeny. *Immunity* 1997; 6:1–11.

Rast, J.P., Haire, R.N., Litman, R.T., Pross, S., and Litman, G.W. Identification and characterization of T-cell antigen receptor-related genes in phylogenetically diverse vertebrate species. *Immunogenetics* 1995; 42:204–212.

Rast, J.P., and Litman, G.W. T-cell receptor gene homologs are present in the most primitive jawed vertebrates. *Proc. Natl. Acad. Sci. U.S.A.* 1994; 91:9248–9252.

Rast, J.P., and Litman, G.W. Towards understanding the evolutionary origins and early diversification of rearranging antigen receptors. *Immunol. Rev.* 1998; 166:79–86.

Rogozin, I.B., Iyer, L.M., Liang, L., Glazko, G.V., Liston, V.G., Pavlov, Y.I., Aravind, L., and Pancer, Z. Evolution and diversification of lamprey antigen receptors: Evidence for involvement of an AID-APOBEC family cytosine deaminase. *Nat. Immunol.* 2007; 8:647–656.

Schluter, S.F., and Marchalonis, J.J. Cloning of shark RAG2 and characterization of the RAG1/RAG2 gene locus. *FASEB J.* 2003; 17:470–472.

Tonegawa, S. Somatic generation of antibody diversity. *Nature* 1983; 302:575–581.

Venkatesh, B., Kirkness, E.F., Loh, Y.H., Halpern, A.L., Lee, A.P., Johnson, J., Dandona, N. et al. Survey sequencing and comparative analysis of the elephant shark (Callorhinchus milii) genome. *PLoS Biol.* 2007; 5:e101.

Venkatesh, B., Lee, A.P., Ravi, V., Maurya, A.K., Lian, M.M., Swann, J.B., Ohta, Y. et al. Elephant shark genome provides unique insights into gnathostome evolution. *Nature* 2014; 505:174–179.

Yin, L., Scott-Browne, J., Kappler, J.W., Gapin, L., and Marrack, P. T cells and their eons-old obsession with MHC. *Immunol. Rev.* 2012; 250:49–60.

Zhu, C., Feng, W., Weedon, J., Hua, P., Stefanov, D., Ohta, Y., Flajnik, M.F., and Hsu, E. The multiple shark Ig H chain genes rearrange and hypermutate autonomously. *J. Immunol.* 2011; 187:2492–2501.

Zhu, C., and Hsu, E. Error-prone DNA repair activity during somatic hypermutation in shark B lymphocytes. *J. Immunol.* 2010; 185:5336–5347.

AQ 10 Zhu, C., Lee, V., Finn, A., Senger, K., Zarrin, A.A., Du Pasquier, L., and Hsu, E. Origin of immunoglobulin isotype switching. *Curr. Biol.* 2012.

Author Query Sheet

Chapter No: 12

Query No	Queries	Response
AQ 1	The terms '12-base-spaced and 23-base-spaced RSS' have been changed to '12-bp-spaced and 23-bp-spaced RSS' Please check and confirm.	
AQ 2	Please suggest whether this section heading "NAR TCR" can be changed to 'NARTCR'	
AQ 3	Please check the clarity of the following sentence "The Flajnik group's.... with D, J and spliced to C of TCR δ (Criscitiello et al. 2010)"	
AQ 4	The usage of the following phrase "to skate thymus spleen adult" is not clear. Please check.	
AQ 5	Please check whether the edit done in the following sentence "It is of note that a ... reptiles and birds" is correct.	
AQ 6	Please check whether the inserted citation for Figure 12.2 is correct.	
AQ 7	Please suggest whether the following phrase 'support from the National Science Foundation (IOS1257829)' can be changed to 'the National Science Foundation (IOS1257829) for their support.'	
AQ 8	Please provide journal title for reference Hsu (2009).	
AQ 9	Please provide volume number and page range for reference Parra et al. (2010).	
AQ 10	Please provide volume number and page range for reference Zhu et al. (2012).	