CHAPTER 11

Shark Immunoglobulin Light Chains

Michael F. Criscitiello

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SUMMARY

Shark immunoglobulin heavy chains do not always heterodimerize with light chains, but when they do four isotypes are available. In addition to IgLλ and IgLκ, sharks have IgLσ and IgLσ-cart. The IgLλ loci of sharks are always germline-joined, which may shed light on the origins of the V(D)J rearranging.

INTRODUCTION

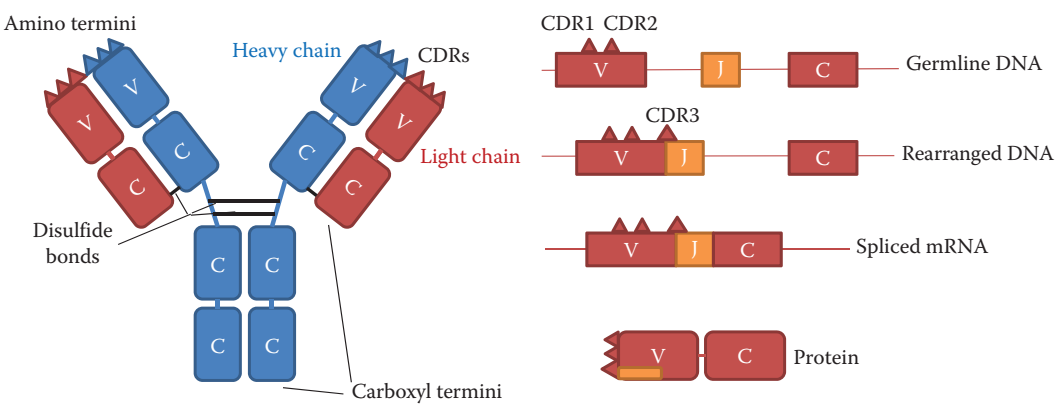
The humoral adaptive immune system of sharks is partly based on the same H_2L_2 heterodimeric antibody structure seen in all jawed vertebrates (reviewed in Flajnik 2002). The constituent vertebrate immunoglobulin (Ig) heavy (H) and light (L) chains provide obligate defense against pathogens and their toxins via neutralization, opsonization, and activation of the classical pathway of the complement cascade. The two IgL are generally identical to one another in any antibody, as are the two IgH, and this antibody is identical to all others made for plasma membrane expression or secretion by a particular B cell at a point in time. However, receptor editing and somatic

hypermutation can genetically alter the IgL (and IgH in the case of hypermutation) during the life of a B cell.

The IgL chain is always comprised of two Ig superfamily domains: one variable (V) and one constant (C) (Figure 11.1). The diverse V domains at the amino terminus of both the IgH and IgL together form the antigen recognition site (paratope) that recognizes the epitope of the antigen. Each V domain has three hypervariable loops called complementarity-determining regions (CDR) connecting the β-strands that form the Ig superfamily domain fold. Both the H and the L chains contribute three CDRs each to each of the two paratopes of the antibody, the region that interacts with the epitope on an antigen. Disulfide bonds link a cysteine in the constant domain of the IgL chain to a cysteine in the first constant domain of the IgH chain. The gross structure of an IgL chain resembles a T-cell receptor (TCR), without the transmembrane or cytoplasmic portions.

In most vertebrates, the genes encoding the V domain of IgL chains (and IgH and TCR chains) are not functional until recombination-activating gene (RAG)-mediated somatic recombination mechanisms act on lymphocyte DNA to assemble the V gene from variable (V) and joining (J) segments (and diversity (D) segments in the case of IgH, TCRβ and TCRδ). Although CDR1 and CDR2 of IgL chains are encoded within the V gene segment, CDR3 is encoded by the juxtaposition and modified ligation of the V and J segments (Figure 11.1). The most common of these modifications at the V-J juncture are N (nontemplate encoded) additions catalyzed by the terminal deoxynucleotidyl transferase (TdT), more common in IgH rearrangements of most species but also contributing to IgL CDR3 diversity. Artemis and the DNA-dependent protein kinase resolve the DNA hairpins at the ends of V(D)J coding segments randomly, which donate P (palindromic) nucleotides. Finally, the exonuclease activity of several DNA repair enzymes removes nucleotides, even while TdT is adding them. Thus, several mechanisms contribute to CDR3 diversity in the IgL protein with immunogenetics at the V-J recombination site.

Although most shark antibodies are of the common H_2L_2 quaternary structure, sharks also use antibodies that contain a pair of H chains without associated L chains, dubbed NAR (Greenberg et al. 1995) for new or nurse shark antigen receptor. IgNAR of cartilaginous fish is not unique in its abandonment of L chains, as mammalian camels, alpacas, and llamas (camelids) have evolved a distinct IgG subclass that functions as IgH dimers (Conrath et al. 2003). There is strong evidence that affinity maturation occurs in the IgNAR isotype (Dooley et al. 2006). Yet other IgH chain isotypes along with their paired IgL somatically hypermutate in sharks as well, albeit possibly



AQ 1 **Figure 11.1** Schematic of IgL chains in a heterodimeric antibody and the genes that encode them. (a) Protein structure: heavy chains are blue and light chains are red. CDR3 is depicted as a larger triangle than CDR1 and CDR2. (b) Corresponding gene structure.

with different mechanisms (more below) (Hinds-Frey et al. 1993). A doubly rearranging form of TCRδ of sharks uses an additional V domain very similar to that of the IgL chain-less IgNAR, called NARTCR (Criscitiello et al. 2006). Nonplacental mammals convergently make TCRs with a similar fundamental structure (Miller 2010), another example of evolution finding similar immune innovations in both cartilaginous fish and more recent vertebrates. More on NAR-TCR can be found in Chapter 12. AQ 2

Mammalian IgL chains were originally identified as Bence-Jones proteins in the serum and urine of lymphoma and myeloma patients (Edelman and Gally 1962). Mammals express two IgL isotypes from distinct genomic loci, λ and κ (Wu and Kabat 1970). Yet four isotypes of IgL have now been recognized in sharks (Criscitiello and Flajnik 2007), this identification being eased by the wealth of molecular sequence data from cartilaginous fish and other vertebrates that became available in the early 2000s.

In this chapter, an older body of formative biochemical work will be reviewed first to set the stage for the molecular cloning to come. Genomic functional work will be discussed that is shedding light on exclusion and hypermutation in sharks, as well as the multiple cluster Ig organization found in bony fish light chain and cartilaginous fish heavy chain genes (reviewed in Dooley and Flajnik 2006; Litman et al. 1999). Finally, discussion will turn to the evolution of IgL chains, building on what we know from these oldest vertebrates that employ them in adaptive immunity. Focus will remain on sharks proper, but knowledge gleaned from other cartilaginous Chondrichthyes will be included when it is likely to have bearing on shark immunobiology (species and references in which they are studied are summarized in Table 11.1).

PIONEERING BIOCHEMICAL STUDIES

Immunological competence had been shown in elasmobranchs as early as 1963 by immunizing lemon sharks *Negaprion brevirostris* with human influenza virus (Sigel and Clem 1963). Anamnesis (as defined by an accelerated antibody response upon secondary immunization) was recognized in horned sharks *Heterodontus francisci* stimulated with hemocyanin (Papermaster et al. 1964), but it was the work of Marchalonis and Edelman that showed in 1965 that IgH/IgL antibody structure existed in sharks (Marchalonis and Edelman 1965). They used urea to reduce immunized smooth dogfish *Musteluscanis* antiserum for resolution on starch gels. Peptide maps indicated that the IgL had different primary structure than the IgH. Continued work in the dogfish model resolved the molecular weight of the IgL to 20 kDa and showed the amino acid composition of IgL associated with the pentameric 17S and monomeric 7S forms of shark IgM to be nearly identical (Marchalonis and Edelman 1966). Similarly, biochemical work on lemon shark sera following bovine serum albumin immunizations showed the established H_2L_2 structure and electrophoretic heterogeneity of the IgL chains (as well as the IgH chains) (Clem et al. 1967). Additionally, various reducing experiments with antibodies of horned shark immunized with *Brucella abortus* showed the necessity of inter-chain disulfide bonds to antibody activity (Frommel et al. 1971). These data suggest that in sharks, like mammals, both chains of Ig are contributing to the diversity of the antigen-binding paratope.

The first amino-terminal protein sequencing of shark IgL was performed by Edman degradation on antibodies of the leopard shark *Triakis semifasciata* (Suran and Papermaster 1967). The sequence of the first five positions of this shark IgL (D/E-I-V-L/V/G-T) suggested Igκ, even though nucleic acid work in this species still has neither confirmed nor refuted the original biochemistry. More extensive amino-terminal IgL sequencing of nurse shark *Ginglymosotma cirratum* identified IgL more related to Igκ than Igλ as well in that species (Sledge et al. 1974), and light chains were later resolved to 23 kDa by reducing polyacrylamide gel electrophoresis (Fuller et al. 1978). Similar to what was seen by Sledge et al. in nurse shark, IgL from the tiger shark *Galeocerdo cuvieri* and Galapagos shark

Table 11.1 **Criscitiello Species Experiment**

			Immunization	Biochemistry	cDNA	Genomic	Expression
Lemon shark	*Negaprion brevirostris*		Sigel (1963)	Clem (1967)			
Smooth Dogfish	*Mustelis canis*		Marchalonis (1965)	Marchalonis (1965), Marchalonis (1966)			
Leopard shark	*Triakis semifasciata*			Suran (1967)			
Horned shark	*Heterodontus francisci*	σ-cart Shamblott (1989), κ l Rast (1994), σ Criscitiello (2007)	Papermaster (1964)	Frommel (1971), Kehoe (1978)	Shamblott (1989), Rast (1994), σ Criscitiello σ Greenberg (1993), Criscitiello (2007)	Shamblott (1989) (σ-cart), Rast (1994)	
Nurse shark	*Ginglymostoma cirratum*	κ, σ-cart, l Greenberg (1993), σ Criscitiello (2007)		Sedge (1974), Fuller (1978)	Greenberg (1993), Criscitiello (2007)	Greenberg (1993), Lee (2000), Fleurant (2004)	Criscitielo (2007)
Tiger shark	*Galeocerdo cuvieri*			Marchalonis (1988), Schluter (1990)			
Galapagos shark	*Carcharhinus galapagensis*			Schluter (1987)			
Sandbar shark	*Carcharhinus plumbeus*	l Schluter (1989), Hohman (1992)		Marchalonis (1988), Schluter (1990)	Schluter (1989) Hohman (1992)	Hohman (1993)	
Little skate	*Raja erinacea*	l Rast (1994)			Rast (1994)	Rast (1994)	
Spotted ratfish	*Hydrolagus colliei*	l Rast (1994)			Rast (1994)	Rast (1994)	
Ratfish	*Callorhinchus Callorhinchus*			De Ioannes (1989)			
Clearnose skate	*Raja eglanteria*						Miracle (2001)
Little skate	*Raja erinacea*	σ-cart Anderson (1995)			Anderson (1995), Criscitiello (2007)	Anderson (1995)	
Spiny dogfish	*Squalus acanthus*	σ Criscitiello (2007)		Smith (2011)	Criscitiello (2007), Smith (2011)	Smith (2011)	Smith (2011)

AQ 3

Carcharhinus galapagensis showed high identity with Igκ of mammals (Marchalonis et al. 1988). This amino-terminal sequencing work was accompanied by isoelectric focusing that showed heterogeneous bands for the sandbar shark *Carcharhinus plumbeus* as well as the other two carcharhine sharks. A second 0-iodosobenzoic acid-liberated peptide was also sequenced from the tiger shark that corroborates the Igκ homology. Binding of rabbit antisera raised to a synthetic human TCRβJ segment cross-reacted to IgL of Galapagos shark, suggesting conservation of the F-G-x-G-T-R-L motif broadly in vertebrate TCR and IgL (Schluter and Marchalonis 1986; Schluter et al. 1987). The amino-terminal sequencing in sandbar and tiger sharks was later complemented by tandem mass spectrometry (Schluter et al. 1990). Amino terminal sequence from the Holocephalian ratfish was analyzed with distant homology noted to κ (De Ioannes and Aguila 1989). Although ratfish are not sharks, the Holocephali they represent is one extant subclass of Chondrichthyes, with the Elasmobranchii being the other (which includes the modern sharks and rays).

MOLECULAR WORK YIELDING cDNA SEQUENCES

Work in the horned shark yielded the first nucleic acid sequence of a shark IgL chain and confirmed the framework and CDR characteristic of vertebrate Ig and TCR proteins (Shamblott and Litman1989), as was suggested by V domain amino acid sequencing of this same isotype (Kehoe et al. 1978). The predicted amino acid sequence showed homology to mammalian Igλ in the more limited database of the day, and the nucleic acid sequence showed over 50% identity with a mammalian TCRβ. Work in the sandbar shark revealed partial cDNAs of an Igλ homologue in that species (Schluter et al. 1989). AQ 4

The next cDNA publication from Marchalonis' sandbar shark team described a complete Igλ sequence (Hohman et al. 1992). This work by Hohman et al. is notable for two reasons, one to be discussed in the next section. It is the first study of shark IgL chains to employ tree-building phylogenetic analysis based on molecular sequence data, using just V sequences to correctly place this sandbar shark IgL with the Igλ of mammals (no small feat, as there were no other poikilothermic or *cold-blooded* sequences in the analysis).

The first IgL cloned from the nurse shark was Igκ (Greenberg et al. 1993), which confirmed both earlier amino-terminal sequencing data of pooled IgL (Sledge et al. 1974) and the emergence of multiple IgL chain isotypes early in vertebrate evolution. This paper by Greenberg et al. also mentions cloning two other IgL isotypes from *Ginglymostoma* and for the first time suggests three or more isotypes in the shark. Evidence for IgL isotypes other than λ and κ had previously only been suggested in the Anuran amphibian *Xenopus* (Hsu et al. 1991).

Rast et al. extended the study of elasmobranch IgL to other cartilaginous fish (Rast et al. 1994). They analyzed the more ancient out-group to the living elasmobranchs, the Holocephali, with the spotted ratfish *Hydrolagus colliei*. They also investigated the other major extant radiation of elasmobranchs besides the sharks (Selachii), the skates and rays (Batoidea), by studying the little skate *Raja erinacea*. An anchored polymerase chain reaction (PCR) strategy on spotted ratfish cDNA resulted in the cloning of Igλ from this ancient group of fishes. Rast et al. used the ratfish Igλ to successfully probe cDNA libraries of the little skate and used a nurse shark κ probe to identify that isotype in the horned shark as well.

GENOMIC ORGANIZATION

The Litman laboratory provided the first evidence that shark IgL genes were organized in a similar multiple cluster organization (Shamblott and Litman 1989) as that seen in the shark IgH (Hinds and Litman 1986) and some bony fish IgL (Bengten et al. 2000; Daggfeldt et al. 1993; AQ 5

Hsu and Criscitiello 2006). They sequenced a cluster containing a single V gene (with intron *split leader* typical of antigen receptors), single J gene, and single C gene occupying a comparatively small 2.7 kb. The conserved heptamer and nonamer recognized by the RAG recombinase were found 3′ of the V gene and were spaced by 12 nucleotides. These motifs 5′ of the J gene were spaced by 23 nucleotides, abiding the rule of 12-bp-spaced recombination signal sequences (RSS) only rearranging with 23-bp-spaced RSS observed in mammals. This orientation (V12/23J) of the RSS is curious, known only at Igκ loci among mammalian antigen receptors. Igλ, all known TCR, and all known Ig V gene segments are flanked by 3′ RSS spaced by 23 nucleotides (Criscitiello and Flajnik 2007). Restriction mapping of genomic clones and genomic Southern blotting with V and C region probes all suggested multiple light chain clusters of very limited complexity (likely 1V-1J-1C). This seminal IgL work did not find evidence for germline-joined V-J genes, a hallmark of many IgH clusters in shark. As discussed in Chapter 10 (Hsu, IgH VDJ), V, multiple D, and J gene segments are often partially or completely germline-joined for many IgH loci in cartilaginous fish (Kokubu et al. 1988).

The description of the complete sandbar shark IgLλ cDNA (Hohman et al. 1992) included a most interesting note added in proof. The addendum shares the sequencing of two sandbar shark IgLλ genomic clones, both of which have the V and J segments fused in the germline. This is the first suggestion of germline-joined V domains encoding genes in IgL chains. Subsequent work by the Marchalonis group confirmed more germline-joined IgLλ in the horned shark (Hohman et al. 1993). In fact, every IgLλ genomic clone or V-J PCR amplicon analyzed in this shark showed fusion. Although IgLκ was originally thought to always be unjoined in cartilaginous fish (Rast et al. 1994), Lee et al. (2000) showed that in the nurse shark, some IgLκ loci are split and some are joined. The joining of V and J segments in the germline is made possible by the expression of RAG in shark germ cells. Genomic Southern blotting of IgLκ in the nurse shark showed the cluster organization now accepted for shark immunoglobulin genes for the first time for this isotype (Greenberg et al. 1993). The RSS orientation of this shark IgLκ is V12/23J, consistent with Igκ in other vertebrates. Genomic V-J PCR suggested that at least one nurse shark IgLκ locus was germline-joined. Later work in *Ginglymostoma* showed that of around sixty IgLκ loci, six are germline-joined but the rest can rearrange (Lee et al. 2000). Phylogenetic dendrograms had been drawn with shark IgL data before (Hohman et al. 1992; Zezza et al. 1992), but Greenberg et al. for the first time showed the distinct patterns C domain data yield compared to that from V domains. Both methods are confounded by different problems: V domains diversify for a broad repertoire (especially problematic if CDR columns of the alignment are included), and C domains tend to cluster as much or more by taxonomic group as by isotype, presumably for heterodimerization of C1 domain peculiarities of the particular taxon. The different physiology of these functional domains of L chains would be expected to exert different evolutionary pressures on the exons encoding them.

Genomic analysis by Southern blotting suggests multiple clusters in *Hydrolagus*. In the same study, PCR and limited sequencing found two V families and no unjoined IgLλ in the spotted ratfish (Rast et al. 1994), consistent with sharks. Rast et al. used the spotted ratfish IgLλ to probe genomic libraries of horned shark and little skate, finding germline-joined IgLλ in both *Heterodontus* and *Raja*.

Genomic library screening and PCR from genomic DNA of the little skate *Raja erinacea* failed to find any of the isotype called Type I (σ-cart, see below) unjoined (Anderson et al. 1995). Some of the exact same sequences (including the CDR3 region) were cloned from cDNA and genomic DNA, proving that these joined loci are at least expressed at the mRNA level. This gives credence to the idea that loci of a particular IgL isotypes may be all joined, all split by intron, or some combination of the two. Additionally, this arrangement may not be consistent among loci of the same isotype when they are examined in different cartilaginous fish species.

CLASSIFICATION

In hindsight, the classification and nomenclature of IgL has been quite confusing across vertebrates, and nowhere more so than in the cartilaginous fish (although bony fish come close). Some comparative studies strove to classify newly discovered IgL as either mammalian IgLλ or IgLκ, some accepted the notion that there could be IgLλ, IgLκ, or other isotypes in these vertebrates, while others designed new independent systems of nomenclature for the isotypes of the particular species or taxonomic group being studied. Recognizing that three cartilaginous fish sequences did not all belong to IgLλ or IgLκ, a system of NS3, NS4, and NS5 was adopted in the nurse shark (Greenberg et al. 1993) simply based on the original monikers of bands in a particularly fruitful PCR experiment (dissertation of Andrew Greenberg, University of Miami 1994). This system was later replaced with type I (NS5), type II (NS3), and type III (NS4) after standardization with other cartilaginous fish (Rast et al. 1994). Several authors recognized that type I were difficult to classify as either IgLκ or IgLλ, whereas type II were more like mammalian IgLλ, and type III were more like IgLκ.

The discovery of a fourth isotype in the nurse shark allowed new connections to be made between isotypes in cartilaginous fish, bony fish, and tetrapods (Criscitiello and Flajnik 2007). A mini-expressed sequence tag (EST) library from spleen and pancreas produced a sequence of an isotype not previously identified in cartilaginous fish, but that shared homology with a teleost isotype called IgL2 and the amphibian isotype IgLσ. The *new* shark sequence was named for the original discovery of its orthologue in frog (Schwager et al. 1991). IgLσ in nurse shark was corroborated by clones from horn shark, dogfish shark, and the little skate (Criscitiello and Flajnik 2007). The cartilaginous fish IgLσ is most similar to the type I/NS5 IgL, yet is distinct. Phylogenetic trees made from V domain, C domains, and RSS orientations suggest that all vertebrate IgL can be placed in one of the four (once more renamed) groups present in sharks: IgLσ, IgLσ-cart (type I/NS5), IgLλ (type II/NS3), and IgLκ (type III/NS4). Because IgLσ, IgLλ, and IgLκ were discovered first and are found in other major vertebrate radiations, these names are used now in shark as well. IgLσ-cart (type I/NS5), however, has now been found outside of the cartilaginous fish in the coelacanth genome (Amemiya et al. 2013).

Recently, studies in the spiny dogfish *Squalus acanthias* demonstrated the existence of these four light chain isotypes in that species as well (Round and Mazmanian 2010). In this molecular and biochemical work on the dogfish immunoglobulins, immunoprecipitations with a monoclonal raised to nurse shark IgLκ preferentially bound to polymeric IgM, whereas monomeric IgM tended to heterodimerize with the other IgL isotypes. Figure 11.2 shows these four isotypes in sharks and their representation in other vertebrate groups.

This system of four ancient clades of IgL present in sharks, three of which are shared with most poikilothermic vertebrates (IgLλ, IgLκ, and IgLσ), two with mammals (IgLλ and IgLκ) and one with birds (IgLλ), has withstood recent discoveries and reevaluations (Das et al. 2008; Edholm et al. 2011) including those taking advantage of recent genomic projects in reptiles and nonplacental mammals (Figure 11.3 adapted from (Round and Mazmanian 2010). However, the next decade will bring high-throughput transcriptomic and genomic analysis within the reach of many more model systems, and this flood of data will determine if other IgL isotypes remain and if nomenclature again may require reanalysis. Table 11.1 organizes the available IgL literature by species.

DIVERSITY GENERATION

The Hsu laboratory took advantage of the relatively few (four) functional IgLλ loci in the nurse shark to rigorously explore IgL somatic hypermutation in this species (Lee et al. 2002). In these V-J fused genes, they found a preponderance of contiguous (sometimes called tandem) mutations

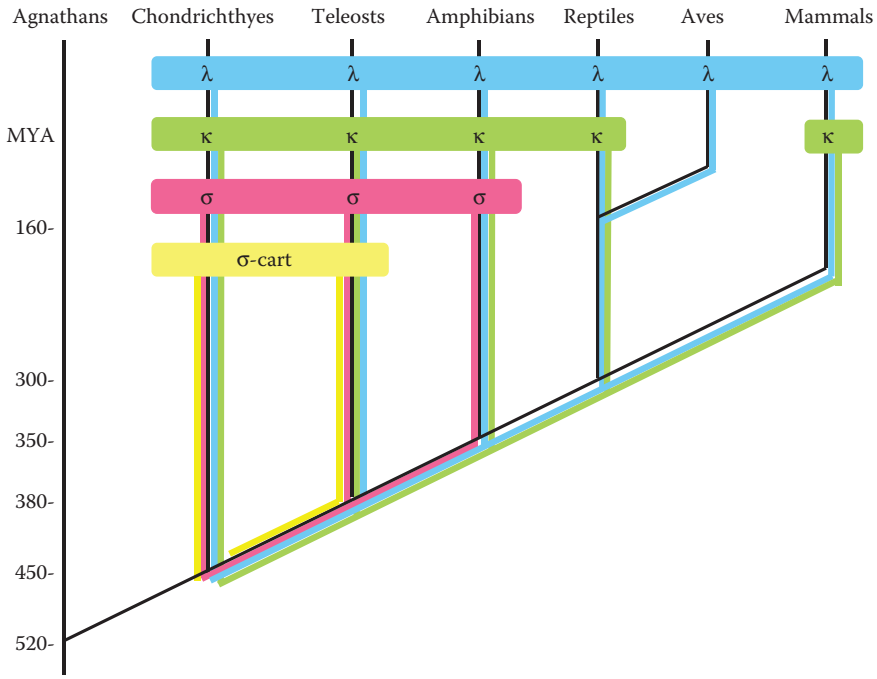


Figure 11.2 Phylogenetic representation of the four IgL isotypes of sharks. These four isotypes have been given other names in other species. These other monikers include for IgLσ-cart (yellow) Type I and NS5 in cartilaginous fish; for IgLσ (magenta) L2 and Type 2 in bony fish; for IgLκ (green) Type III and NS4 in cartilaginous fish, Type 1, Type 3, L1 (L1A and L1B), L3, F and G in bony fish, as well as ρ in amphibians; and for IgLλ (blue) Type II and NS3 in cartilaginous fish, L2 in bony fish, and Type III in amphibians. (Adapted from Criscitiello, M.F., and Flajnik, M.F., *Eur. J. Immunol.*, 37, 2683–2694, 2007.)

occurring in 2–4 bp stretches. The tandem mutations bring to three the count of general mutation patterns that have been seen in cartilaginous fish: point mutations with a GC bias (Du Pasquier et al. 1998), mammalian-like point mutations without the bias (Diaz et al. 1999), and these contiguous or tandem mutations. Lee et al. found no evidence of donor sequences, discounting the possibility of gene conversion.

Junctional diversity was investigated using IgLσ-cart (type 1/NS5) in the nurse shark (Fleurant et al. 2004). The genomic loci encoding this isotype were characterized to enable this comparison of germline sequence with that of somatically joined genes. Fleurant et al. (2004) found only three (two functional) unjoined and one joined loci, a situation this group again exploited to definitively analyze rearrangement at these two functional loci. They found N (nontemplate) extensions at CDR3 to be unusually common and long compared to tetrapod IgL chains; such activity levels are more often associated with IgH chains than with IgL. The authors speculate that in sharks (employing multiple IgH and IgL clusters), simultaneous rearrangement of both IgH and IgL may make the genes encoding both chains accessible to the same processing factors. To date, there is no evidence for a surrogate light chain in sharks, perhaps it is not needed if IgH and IgL are rearranging and being expressed simultaneously. IgLσ in nurse shark shows evidence of N additions and exonuclease activity as well, even in young animals (Criscitiello and Flajnik 2007). TdT is expressed in shark primary lymphoid organs along with RAG and is likely responsible for the N additions (Criscitiello et al. 2010; Rumfelt et al. 2001). Hence, germline-joined genes and 1V-1J-1C clusters do not appear to damn shark IgL genes to limited diversity.

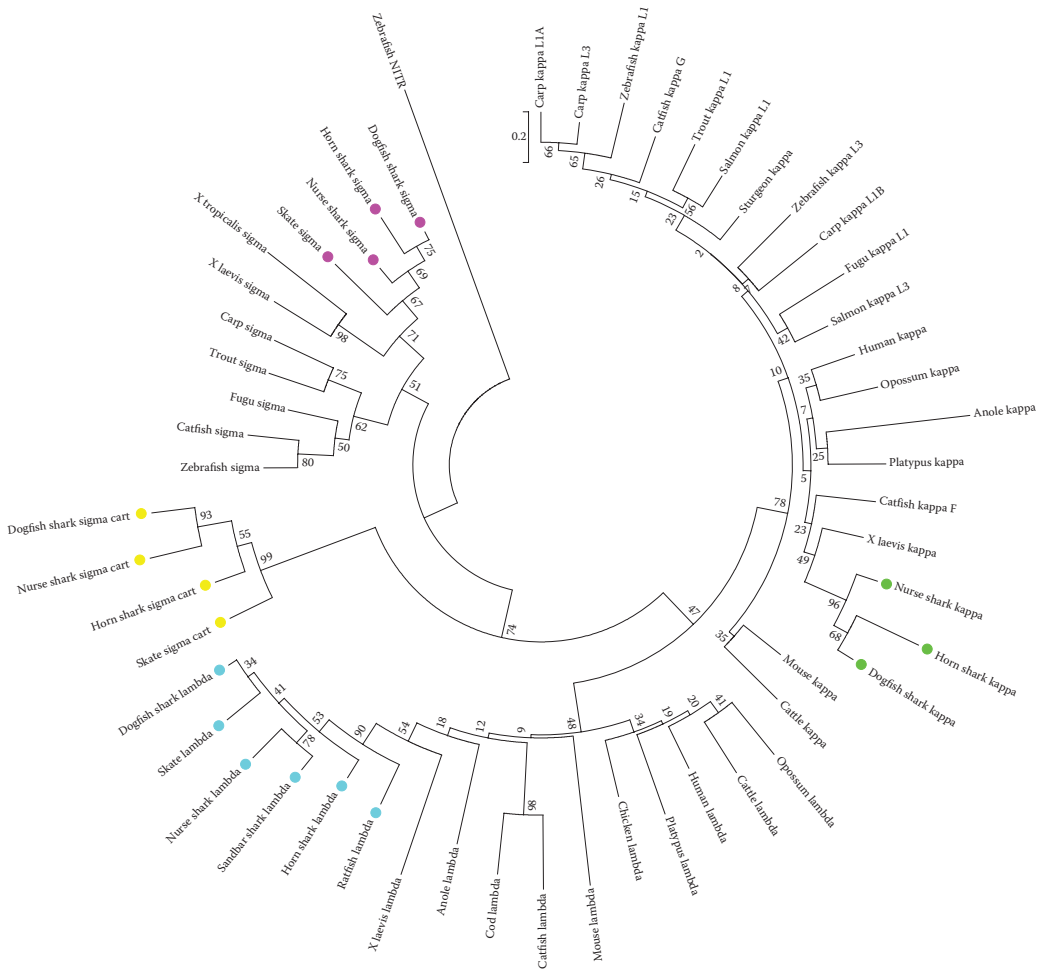


Figure 11.3 Neighbor-joining tree based on IgL variable domain amino acid alignment. Percent bootstrap support after 1000 samplings is shown at each node. A novel immune type receptor (NITR) sequence (GenBank accession: NM001005577) was employed as an out-group. Clusters of sequences in both trees are marked with blocks showing the four ancestral vertebrate isotypes. Spiny dogfish sequences are shaded. Accession numbers of sequences used are as follows: spiny dogfish (*Squalus acanthias*) kappa [JN419107], lambda [JN419108], sigma cart [JN419096], sigma [JN419086]; horn shark (*Heterodontos francisci*) kappa [L25561], lambda [L25560], sigma cart [X15315], sigma [EF114760]; sandbar shark (*Carcharhinus plumbeus*) lambda [M81314]; nurse shark (*Ginglymostoma cirratum*) kappa [GSU15144], lambda (see note), sigma cart [AAV34678.1], sigma [EF114759]; little skate (*Leucoraja erinacea*) lambda [L25566], sigma cart [L25568], sigma [CV222129]; sturgeon (*Acipenser baerii*) kappa [CAB44624]; zebrafish (*Danio rerio*) kappa L1 [AF246185], kappa L3 [AB246193], sigma [AF246183]; spotted ratfish (*Hydrolagus colliei*) lambda [L25549]; pufferfish (*Takifugu rubripes*) kappa L1 [AB126061], sigma [DQ471453]; carp (*Cyprinus carpio*) kappa L1A [AB073328], kappa L1B [AB073332], kappa L3 [AB073335], sigma [AB091113]; salmon (*Salmo salar*) kappa L1 [AF273012], kappa L3 [AF406956]; trout (*Oncorhynchus mykiss*) kappa L1 [X65260], sigma [AAB41310]; cod (*Gadus morhua*) lambda [CAC03754.1]; catfish (*Ictalurus punctatus*) kappa F [U25705], kappa G [L25533], lambda [ACG70845], sigma [CK403931]; African clawed frog (*Xenopus laevis*) kappa [L15570], lambda [L76575], sigma [AAB34863]; western clawed frog (*Xenopus tropicalis*) sigma [from JGI genome project v4.1 scaffold 289]; anole (*Anolis carolinensis*) kappa [ACB45836], lambda [ADB22722]; chicken (*Gallus gallus*) lambda [M24403]; platypus (*Ornithorhynchus anatinus*) kappa [AF116942], lambda [AAO16074]; opossum (*Monodelphis domestica*) kappa [AAF25575], lambda [AAC98667]; cattle (*Bos taurus*) kappa [AA151501], lambda [AAC48564]; mouse (*Mus musculus*) kappa [CAA81787], lambda [AA053422]; human (*Homo sapiens*) kappa [S46371], lambda [AAA59013]. Cartilaginous fish IgLσ sequences are denoted with magenta dots, IgLσ-cart yellow, IgLκ green and IgLλ blue.

EXPRESSION AND FUNCTION

Hohman et al. (1995) found diversity within the noncoding regions of sandbar shark IgLλ genes when many genomic clones were sequenced. Promoter elements in different positions and orientations suggest that there may be differential regulation of antibodies produced by different clusters of the same isotype in this shark. The first application of quantitative real-time PCR to elasmobranchs light chain use was done in the clearnose skate *Raja eglanteria* (Miracle et al. 2001). Developmentally, IgLσ-cart showed moderate expression throughout the first 11 weeks of embryonic life with a peak at week eight, while IgLλ was nearly absent save for a peak at week eight. Differential expression of IgL σ-cart and λ were seen in individual tissues in this skate as well, IgLλ being more abundant in Leydig organ (a B-cell primary lymphoid tissue of some elasmobranchs) whereas IgLσ-cart is higher in the gonad and liver; both isotypes are high in spleen. In all tissues, IgLσ-cart expression dominated IgLλ at the eight-week embryo and hatchling stages, a pattern reversed in the adult skate.

Different mammalian species have different ratios of isotype expression in their mature repertoires. In mice, the average κ:λ ratio is 20:1, in humans it is 2:1, yet in cattle it is 1:20 (Du et al. 2012; Karpati et al. 2011). Not surprisingly, limited analysis of cartilaginous species suggests diversity in relative IgL isotype use as well. IgLκ was found to be dominant in nurse shark (Greenberg et al. 1993) and accounted for 79% of IgL clones in a secondary lymphoid tissue EST library (Criscitiello, M., and Flajnik, MF. unpublished observations). In contrast, IgLσ-cart and IgLλ predominated in cDNA libraries from horn shark (Rast et al. 1994). IgLλ also appears to predominate in the sandbar shark (Hohman et al. 1995).

COMPARATIVE GENOMICS

AQ 6 Although no elasmobranch genomes are available at the time this chapter is being composed, those of the holocephalan elephant shark *Callorhinchus milii* has been reported by Venkatesh et al. (2014). In this more primordial cartilaginous fish lineage, about 20 IgLλ, two IgLκ, and one partial Igσ-cart loci were found in the assembled scaffolds yet no IgLσ. Additionally, although evidence for IgNAR was not found, one unconventional IgM locus was found without canonical residues needed for association with light chains and a duplicated CH2 domain in lieu of CH1. This putative single chain IgM suggests an alternative single-chain antibody alternative to IgNAR in the earliest jawed vertebrates.

EVOLUTION

The genesis of the heterodimeric H_2L_2 antibody system occurred in an ancestor we share with the jawed cartilaginous fishes (Figure 11.2). Both the IgLλ and IgLκ isotypes common in mammals emerged in sharks and their kin, but IgLσ and IgLσ-cart also arose in the cartilaginous fish and have had different evolutionary fates. IgLσ-cart appears to have not been passed to other bony vertebrates, yet IgLσ is found in all non-amniotic jawed vertebrates (cartilaginous fish, bony fish, and amphibians). The orientations of RSS flanking the 3′ of the V gene segment and 5′ of the J segment are consistent in these isotypes across vertebrate groups, as IgLσ, IgLσ-cart, and IgLκ all have V12-23J-spaced RSS, whereas IgLλ is always in the V23-12J orientation (Criscitiello and Flajnik 2007).

The unusual germline joining of shark Ig genes due to gonadal RAG expression may have had several ramifications in IgL evolution. Joined antigen receptor genes occur at least in one other place in vertebrate evolution: the Ig-like supporting V domain of marsupial TCRμ is germline-joined

(Parra et al. 2007), although the monotreme orthologue still rearranges (Vandesompele et al. 2002). Also in the opossum, a IgHV exists (IGHV3.1) with a germline-joined VD (yet not joined D to J) (Livak and Schmittgen 2001). So far, publications with shark IgL have found both joined and unjoined σ-cart and κ, yet only unjoined σ and only joined λ.

Several models for the evolution of vertebrate antigen receptor loci taking shark IgL character states such as RSS orientation and germline-joining into account are presented in Figure 11.4. RAG can act in the germline as a recombinase that will swap the 12- and 23-spaced RSS between two segments (Lewis and Wu 2000; Lewis et al. 1988), a process first recognized in shark Ig (Kokubu et al. 1988). The conservation of RSS orientations not only for IgL but for IgH and TCR as well suggests that this swapping is relatively rare in evolutionary terms, and thus, RSS orientation may be of some value in reconstructing the natural history of antigen receptors. We previously suggested that four possibilities are most parsimonious (Criscitiello and Flajnik 2007). Model *A* suggests the IgLλ isotype evolved from the ancestral common antigen receptor with a V23-12J orientation, with RSS inversion birthing the other IgL chains. This model only invokes one RSS swap and the subsequent fusing of IgLλ and other loci is a shared derived character. Model *B* requires an additional RSS swap but allows the ancestral IgL to be σ/κ, which has been supported in some phylogenies (Criscitiello and Flajnik 2007). A related model that would only require one RSS flip is shown as model *C*. This scheme has the paths of IgH and TCR splitting from the σ/κ-like IgL after the RSS flip along with IgLλ. This model would suggest ancestral IgL function without other chains, perhaps as membrane homodimers with a gross structure similar to TCR. Lastly, perhaps IgLλ has

AQ 7

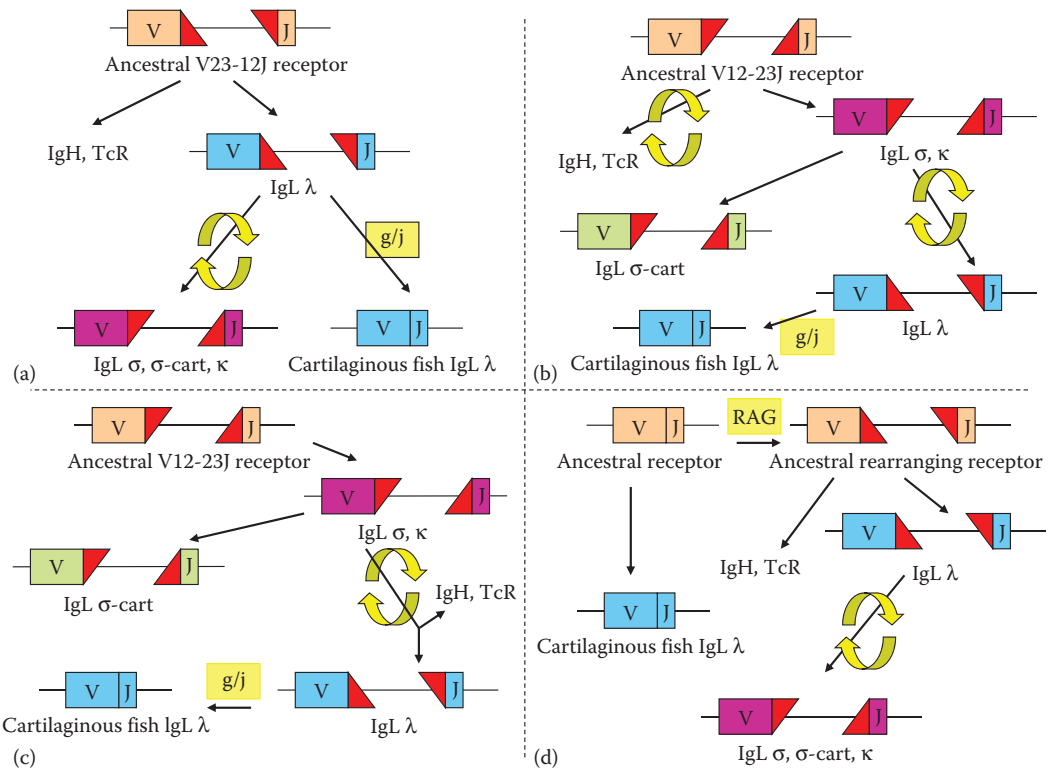


Figure 11.4 Models of shark IgL in the natural history of vertebrate antigen receptors. The two yellow arrows represent RSS swaps (presumable rare and evolutionarily informative). *RAG* symbolizes ancestral RAG transposon insertion event. Yellow *g/j* indicates germline joining of RSS. (Adapted from Criscitiello, M.F., and Flajnik, M.F., *Eur. J. Immunol.*, 37, 2683–2694, 2007.)

not been rejoined in the germline as this was the ancestral state of the antigen receptor. If the RAG insertion event occurred in one site of a multicopy IgLλ locus system, the extant cartilaginous fish IgLλ could be a holdover from the pre-RAG adaptive immune system (model *D*). We look forward to cartilaginous fish genomic projects elucidating the likelihood of these and other models of antigen receptor locus history (Venkatesh et al. 2007).

The maintenance of four distinct isotypes for nearly 500 million years in sharks and apparently at least two isotypes in all vertebrates that do not employ gene conversion (birds) suggests a differential functional basis for their emergence and maintenance. Ample evidence is lacking for distinct physiology between mammalian IgLλ and IgLκ (Stanfield et al. 2006; Sverremark et al. 2000), though it exists (Hershberg and Shlomchik 2006). We have suggested that the distinct CDR length patterns between different IgL isotypes may be a clue (Criscitiello and Flajnik 2007). IgLσ and IgLσ-cart have shorter CDR1 and longer CDR2, whereas IgLκ and IgLλ have the opposite (longer CDR1 and shorter CDR2). These different IgL isotypes may have paired differentially with different VH domains, accommodating different paratopes that are not sterically hindered by the very different CDR loops of the IgL. Long CDR2 such as that found in IgLσ would be expected to pair with IgHV with a short CDR3 (Padlan 1994). Alternatively, different IgL isotypes may have evolved for different IgH isotype heterodimerization. IgLσ in the frog *Xenopus laevis* was found to associate with only two of the three IgH (IgM and IgX, but not IgY) (Hsu et al. 1991). Additionally, work in the cartilaginous skate showed a large disparity in relative IgLσ-cart and IgLλ expression from intestine (Miracle et al. 2001). These two hypotheses are not mutually exclusive, different IgL isotypes could have evolved for both binding paratope structure in the V domain and distinct IgH isotype IgHC1 domain pairing with the IgL C domain.

Much work is left to do in shark IgL biology. It is anticipated that the new accessibility to genomics will direct novel hypothesis testing back in the animal, where basic science should be able to reveal fundamental properties of the antibodies of sharks and all vertebrates.

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