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What the shark immune system can and cannot provide for the expanding design landscape of immunotherapy

Michael F Criscitiello
Texas A&M University, College of Veterinary Medicine and Biomedical Sciences, Texas A&M Health Science Center, Comparative Immunogenetics Laboratory, Department of Veterinary Pathobiology, College Station, TX, USA

Introduction: Sharks have successfully lived in marine ecosystems, often atop food chains as apex predators, for nearly one and a half billion years. Throughout this period they have benefitted from an immune system with the same fundamental components found in terrestrial vertebrates like man. Additionally, sharks have some rather extraordinary immune mechanisms which mammals lack.

Areas covered: In this review the author briefly orients the reader to sharks, their adaptive immunity, and their important phylogenetic position in comparative immunology. The author also differentiates some of the myths from facts concerning these animals, their cartilage, and cancer. From thereon, the author explores some of the more remarkable capabilities and products of shark lymphocytes. Sharks have an isotype of light chain-less antibodies that are useful tools in molecular biology and are moving towards translational use in the clinic. These special antibodies are just one of the several tricks of shark lymphocyte antigen receptor systems.

Expert opinion: While shark cartilage has not helped oncology patients, shark immunoglobulins and T cell receptors do offer exciting novel possibilities for immunotherapeutics. Much of the clinical immunology developmental pipeline has turned from traditional vaccines to passively delivered monoclonal antibody-based drugs for targeted depletion, activation, blocking and immunomodulation. The immunogenetic tools of shark lymphocytes, battle-tested since the dawn of our adaptive immune system, are well poised to expand the design landscape for the next generation of immunotherapy products.

Keywords: antibody, antigen receptor, immunology, shark, T cell receptor

1. Introduction: sharks and the evolution of immunity

1.1 Sharks

Nearly 450 species of shark have radiated to most marine and a few of the freshwater environments of our planet. They range from wee (the 7-inch dwarf lanternshark *Etmopterus perryi*) to mammoth (the 39-foot whale shark *Rhincodon typus*, the largest fish). They have keen olfaction and electroreception. They use diverse reproductive strategies that include oviparity, polyandry, viviparity and parthenogenesis [1]. Sharks have many sets of replaceable teeth. Sharks are carnivores that collectively have evolved a variety of feeding strategies: filter-feeding, cooperative pack hunting, ambush sucking, cookie-cutting, ram feeding and protrusible jaw catapulsion. And yes, a very few species of shark have been involved in a small number of unprovoked, fatal attacks upon humans. These rare events created the popular

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Article highlights.

- Sharks get cancer and shark cartilage is no cancer panacea.
- Cartilaginous fish (such as sharks) are the oldest group of animals with the basic components of the vertebrate adaptive immune system.
- Sharks employ a single-chain antibody isotype (two heavy chains, no light chain) that have much utility.
- There are additional immunogenetic tricks available to shark lymphocytes (single-variable domain T cell receptor (TCR) binding, Ig/TCR trans-rearrangements and somatic hypermutation at TCR loci), which may be translationally useful.

This box summarizes key points contained in the article.

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misconception of sharks as indiscriminate eating machines. Although far from certain, it is possible that the evolution of their infamous *Jaws* may have been important in the genesis of the adaptive immune system upon which most of our global public health efforts are based.

1.2 Adaptive immunity

The adaptive immune system is distinguished from the innate system it was built upon by its fine molecular specificity for antigen and memory for that specific antigen long after an initial immune response. Lymphocytes are the cells that mediate adaptive immunity: B cells making antibodies for humoral immunity and T cells effecting cell-mediated immunity. The B and T lymphocytes develop in the primary lymphoid tissues, the thymus for T cells and (in humans) the bone marrow for B cells. There they stochastically diversify the genes that encode the antigen receptors on the cell surface. The antigen receptors on any individual lymphocyte all have the same antigen-binding structure based on the unique somatic cell gene rearrangements that occurred at the antigen receptor loci (**Figure 1**). Vast repertoires of lymphocytes with distinct antigenic specificities circulate in the organism. A mature lymphocyte activated in the secondary lymphoid tissues (e.g., lymph node, spleen, Peyer's patch) can then be clonally expanded, mobilizing an army of cells specific for a particular antigen. The mitotic lymphocyte expansion will retain the rare antigenic specificity encoded by the antigen receptor genes of the maternal activated cell. These cells will produce specific antibody, kill virally infected cells, direct other immune cells to act and remove neoplastic cells all based on the specificity of their antigen receptor. Some of the expanded lymphocyte clones differentiate into long-lived memory cells that can greatly augment the quality and quantity of response to challenges by the same antigen in years or decades to come.

The hallmark characteristics of adaptive immunity (specificity and memory) reside in the antigen receptors: the B cell receptor (surface antibody or immunoglobulin [Ig]) and the T cell receptor (TCR). The specificity in the lymphocyte antigen receptors has been utilized to preemptively engineer an animal's

immune repertoire through immunization. Successful immunization with attenuated virus, killed pathogen or irradiated cancer cell (or parts thereof) increases the frequency of antigen specific lymphocytes by several orders of magnitude. Therefore, if the same antigen is seen again in the context of the actual pathogen or cancer, the kinetics, quality and amplitude of the response is much better than it would be on first encounter with the antigen. Ideally this memory effect from immunization will result in a secondary response upon challenge that will provide both asymptomatic clearance and a dead end for an infectious pathogen with no further spread in the population.

1.3 The evolution of 'our' immune system

All life has innate immune mechanisms, but adaptive immunity has for long been thought to be an innovation of vertebrate animals (**Figure 2**) [2]. Antigen challenge and allogeneic graft experiments in lamprey [3], shark [4-6] and bony fish [7] suggested that these species were capable of making antigen-specific memory responses long before any molecular or genetic mechanisms were elucidated. A wealth of data from several model species suggests that many if not most of the fundamental components of the adaptive immune system found in mouse and man had evolved in our most recent common ancestor with sharks [8]. As the shark adaptive immune system is germane and central to the discussions of drug development that follows, our current knowledge of this system will be briefly reviewed here. Much of this has been learned from the study of shark immunology, but also from the study of other cartilaginous fish such as skates and chimeras. Thus, I will review these data too where appropriate.

Those early graft and challenge studies gave way to biochemical and molecular work to determine many genes and proteins responsible for immune responses in these fish. Primate and rodent immunologists would recognize many of the immune mechanisms of sharks. Innate immunity is fully competent and includes lysozyme [9], acute phase proteins [10], a complement system with tripartite activation pathways [11-15], and nitric oxide production in leukocytes stimulated with pathogen associated molecular patterns [16]. Importantly, the adaptive immune system is just as well developed with multiple Ig heavy chain isotypes (including IgM and IgD) [17,18], antibody multimerization by the joining (J) chain [19], four isotypes of Ig light chains (including λ and κ) [20-22], B cell differentiation to Blimp-1$^+$ plasma cells [23], well-characterized affinity maturation [24], Ig-mediated opsonization and cytotoxicity [25], four TCR chains (α, β, γ and δ) [26,27], antigen receptor signal transduction via accessory molecule immunoreceptor tyrosine based activation motifs [28], polygenic and polymorphic major histocompatibility complex (MHC) Class I [29] and Class II [30], β_2-microglobulin genomically linked to the MHC [31], MHC-linked immunoproteasome and peptide transport components [32], MHC Class II antigen processing employing the invariant chain (Ii) [33] and phagolysosomal acid [34], the B7 family of lymphocyte costimulatory molecules [35], well-defined secondary lymphoid tissue architecture [36] and
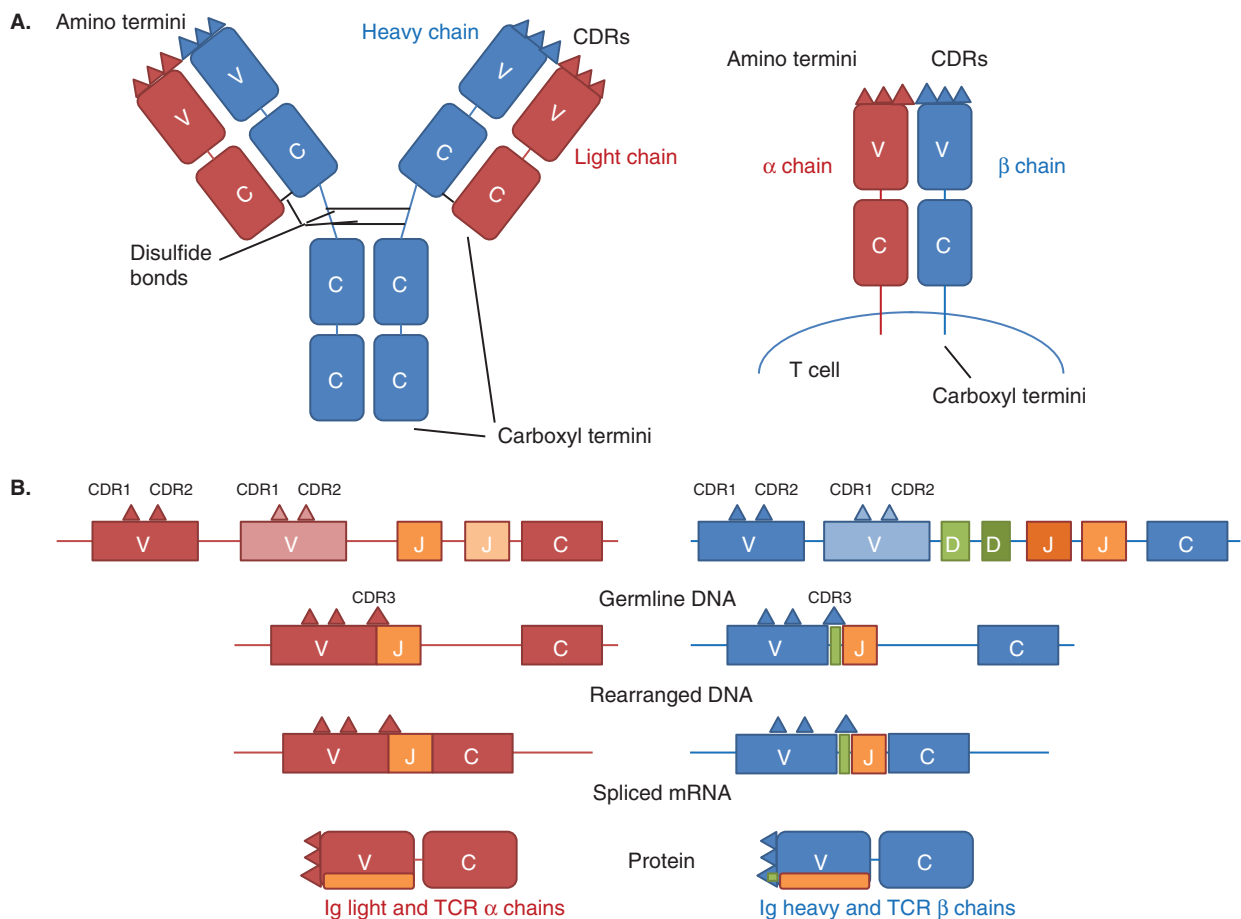
Figure 1. Schematic of antigen receptor quaternary structure and the genes that encode it. A. Protein structure, immunoglobulin (antibody) heavy chains and TCR β chains are in blue, and immunoglobulin light chains and TCR α chains are in red. CDR3 is depicted as a larger triangle than CDR1 and CDR2. **B.** Corresponding gene structure. At both the protein and genetic levels, TCR δ and γ are similar to TCR β and α, respectively. Importantly, there are many more V(D)J segments than shown at the antigen receptor loci, all with slight sequence differences that encode structurally different antigen receptor variable domains if used in somatic gene rearrangement.

CDR: Complementarity determining region; TCR: T cell receptor.

rigorously confirmed memory responses [37]. Hence, cartilaginous fish have a very similar fundamental immunological toolbox to the one that humans employ.

Much has been written about how and why the complex, Ig/TCR/MHC-based adaptive immune system appears evolutionarily in the earliest jawed vertebrates [38]. The emergence of adaptive immunity has been likened to the cosmological 'Big Bang' [39] noting that the combinatorial genetics are mediated by recombination activating genes (RAG) that were horizontally acquired from a prokaryotic transposon [40]. Other theories invoke the life histories of early gnathostomes shifting from 'r selection' (small body size, high fecundity, early maturity, fast generation time, wide offspring dispersal) towards 'K selection' (large body size, long life expectancy, fewer offspring with more parental investment) for favoring expensive defense mechanisms with mnesic properties [41].

Some have wondered whether the evolution of opposable jaw articulations in vertebrates that allowed biting (rather than slurping and sucking) may have made adaptive immunity worthwhile [42]. Ingesting foodstuff with broken shards of invertebrate exoskeletons must have been a challenge for the gut of these organisms. Innate and adaptive immune systems have coevolved with mutualistic flora as well as pathogenic microbes [43], and containing gut flora to the lumen would likely have been much more challenging for a biting carnivore compared with an extracting parasite. A second line of evidence also supports a jaw hypothesis. Many primary and secondary lymphoid tissues are developmentally or anatomically associated with the gastrointestinal tract [44]. B cells develop in the Leydig organ of many cartilaginous fishes' esophagus [45], the thymus develops partially from branchial gill arches in the pharynx and ruminant Peyer's patches, rabbit appendix and bird bursa all diversify
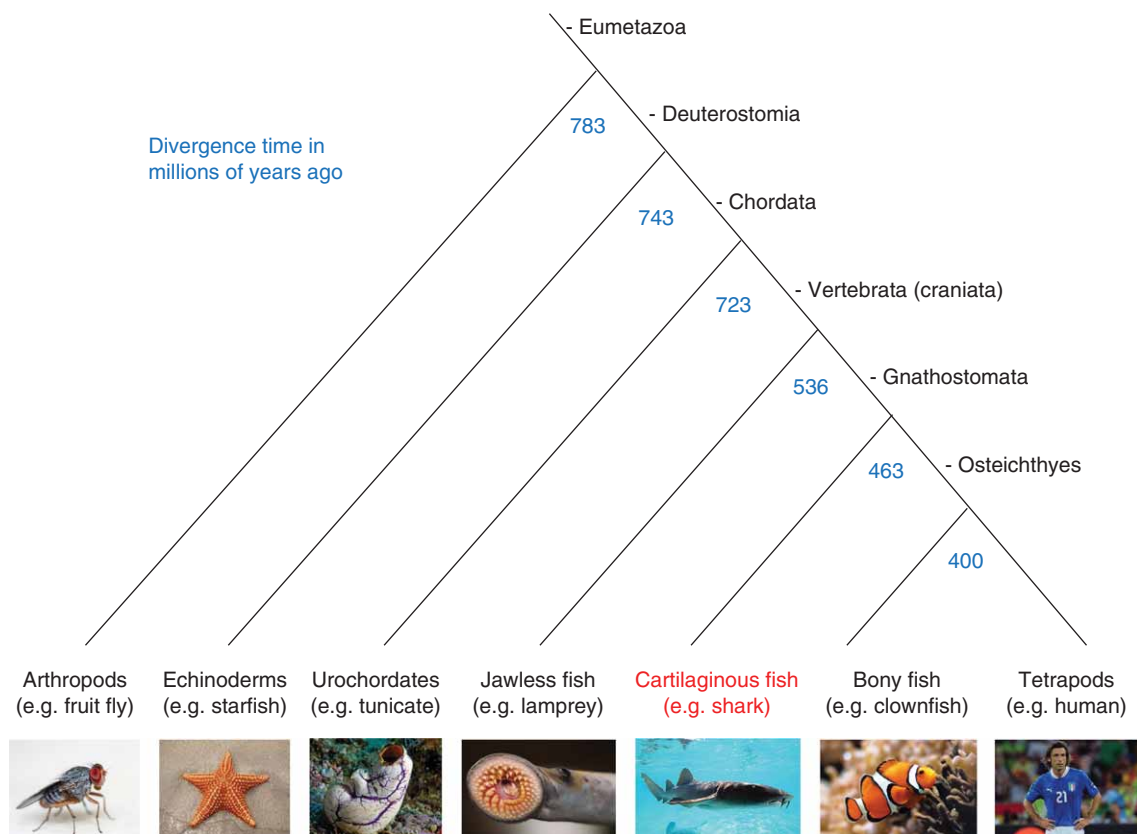
Figure 2. Sharks and their place in vertebrate phylogeny. Phylogeny showing the relationship and approximate divergence times of various animal clades with a tetrapod such as man. Divergence times calculated with TimeTree [143].

B cell repertoires [46]. The association between jaws and adaptive immunity has even been explored in a natural loss of function experiment: gut-associated immune tissues have been found to be lacking in seahorses that (belong to a group of bony fish that) have secondarily abandoned biting [47].

But the jaw hypothesis was dealt a blow by the elucidation that those *jawless* vertebrates (the lamprey and hagfish) have lymphocytes using a totally different somatically diversifying antigen receptor system called variable lymphocyte receptors (VLR) [48]. These leucine-rich repeat VLRs are diversified by members of the same APOBEC family of nucleic acid mutators to which activation-induced cytidine deaminase (AID) belongs [49]. AID is responsible for immunoglobulin chain class switch recombination, somatic hypermutation (SHM) and gene conversion. 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 APO-BEC family members being used to diversify the older VLR system in lamprey and hagfish [50].

So now it appears that two very different forms of adaptive immunity evolved in vertebrates: a VLR-based system in the jawless agnathans and an immunoglobulin superfamily-based system in the gnathostomes (**Figure 3**).

As we continue to explore the defense mechanisms of other species, it is possible that we may find that specificity and memory in immune mechanisms have evolved many times with different components [51], all of which will provide fodder for biotechnological innovations in targeted therapeutics.

2. What shark immunity cannot offer us: simple cancer cures

2.1 Cartilage and cancer

The vertebrate endoskeleton is composed of connective tissue cartilage that is usually replaced during development by ossified bone. In extant chondrichthyes, ossification does not appear to take place and the skeleton remains cartilaginous through adulthood. The ancestral state of the jawed-vertebrate endoskeleton may have included the capacity for bone, and Chondrichthyes such as sharks secondarily evolved to lose it. The bizarre 'spine-brush complex' dorsal appendage of the primitive shark *Stethacanthus* appears to have had endochondral bone [52] and others have described osseous tissue in living sharks [53]. Dermal bone is clearly present back in the jawless fishes. It has been suggested that an abandonment of ossification may have enhanced spring capacity in the axial skeleton,
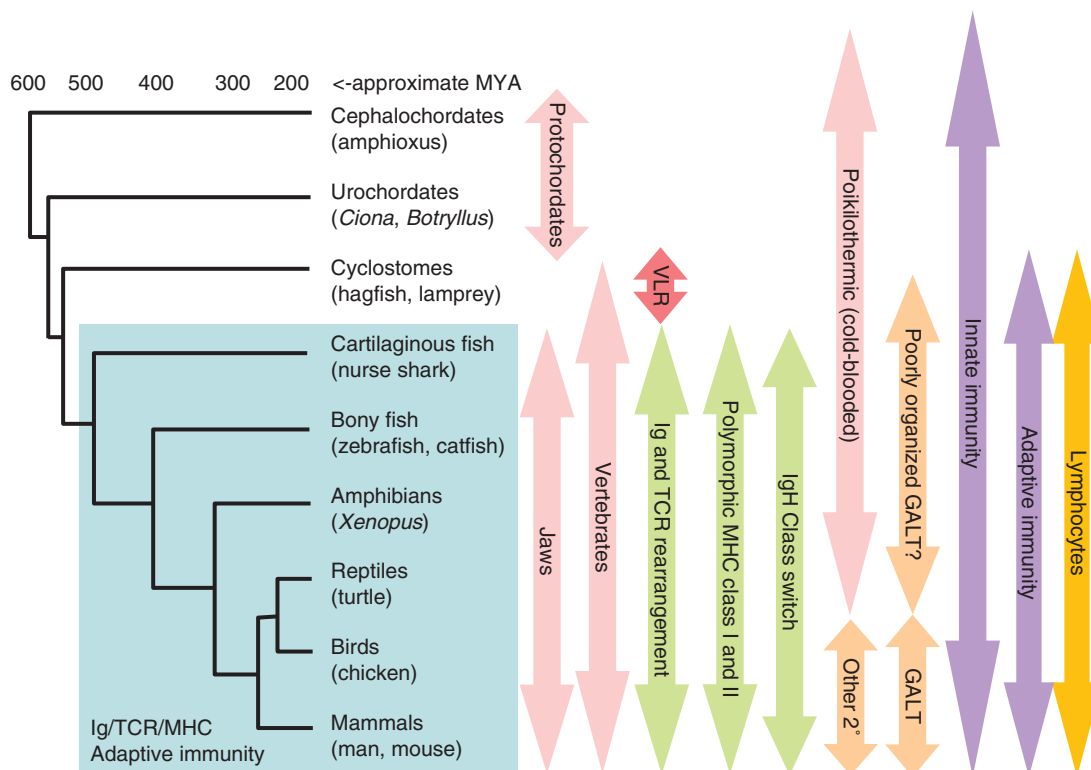
Figure 3. The evolution of adaptive immunity. Comparative immune mechanisms show that sharks represent the oldest animals sharing the Ig/T cell receptor/major histocompatibility complex-based adaptive immune system with mammals (green arrows and blue box). All life has innate immunity and all vertebrates have lymphocyte-mediated (orange) adaptive immunity as well (purple), but the Agnatha or cyclostomes use a very different antigen receptor system (red). Some general characteristics of these groups are also shown (pink). So far well-defined secondary (or peripheral) lymphoid tissues in addition to the white pulp of spleen have only been identified in birds and mammals (light orange).
GALT: Gut associated lymphoid tissue; MYA: Millions of years ago.

contributing to sharks distinctive swimming form compared with bony fish [54]. However, recent genomic data have suggested that the capacity to make a fully ossified endoskeleton was enabled by a duplication of the *Sparc* gene during gnathastome whole genome duplication allowing for a tandem duplication in osteichthyes to give rise to the *SCPP* gene family that have a crucial role in the formation of bone [55].

Angiogenesis is new blood vessel development from existing vasculature. It was recognized by Judah Folkman to be necessary for any substantial tumor growth [56], sparking a drive for antiangiogenesis factors not only for oncology but also inflammatory and metabolic diseases where new vascularization is implicated in pathology (e.g., arthritis, psoriasis, diabetic retinopathy) [57]. Cartilage is an avascular tissue and chondrosarcomas are among the least vascularized of solid tumors [58]. Cartilage of rabbit [59] and calf [60] was shown to inhibit tumor angiogenesis. These three observations (sharks have cartilaginous skeletons, cartilage is poorly vascularized and angiogenesis is key in tumor growth) conspired to prime fraught oncology patients for shameful exploitation by pseudoscience and the supplement industry with the addition of just one myth.

2.2 Sharks and cancer

Recognizing that large sharks are a ready source of cartilage, Robert Langer repeated his calf cartilage experiment [60] using cartilage from basking shark (*Cetorhinus maximus*, a species that can exceed 40 feet) [61]. The authors found similar antiangiogenic properties in the shark cartilage as in the mammalian analog, in preparations that were cruder. They went on to suggest that with the size and hypothesized cartilage content of the basking shark, 'sharks may contain as much as 100,000 times more angiogenesis inhibitory activity on a per animal basis' compared with calves.

Toxicological studies by Carl Luer in the nurse shark (*Ginglymostoma cirratum*) showed the species to be resistant to high levels of carcinogenic aflatoxin, not showing visible tumors 50 days after exposure (perhaps not that long for tumor growth in a 'cold-blooded' poikilotherm) [62]. Subsequent biochemistry implicated lower levels of shark (and clearnose skate *Raja eglanteria*) liver monoxygenase activity in this fungal aflatoxin resistance phenomenon [63,64].

These independent lines of research on tumor angiogenesis, cartilage and elasmobranch oncotoxicology propelled William

Lane to publish *Sharks Don't Get Cancer: How Shark Cartilage Could Save Your Life* in 1992 (wherein it is admitted that sharks do get cancer) [65]. The book was a bestseller, spurred a television segment on the program *60 minutes* documenting improbable cancer remissions in a nonrandomized trial in Mexico that have yet to be published, made Lane Labs (manufacturer of BeneFin) much profit and was followed by a second book in 1996 (*Sharks Still Don't Get Cancer* [66]). Shark cartilage has been marketed as a cancer cure ever since, despite a warning from the FDA in 1997. In 2004, a US District Court in New Jersey issued an injunction against Lane Labs to refund purchasers of illegally marketed, unapproved drugs and in 2000 the Federal Trade Commission fined Lane as well.

Importantly, sharks do get cancer. Elasmobranchs get neoplasms both malignant and benign, 42 of which were categorized from 21 species [67]. Systemic surveys are needed to determine whether the rates are higher, lower or the same as those of other vertebrate groups, and studies of cytotoxic T cell and natural killer cells of shark will define just what immune surveillance these animals have against neoplasms.

2.3 Shark cartilage and mammalian cancer

There is reason to study shark cartilage. One of the more promising early studies showed two cartilage components from hammerhead shark, sphyrnastatin 1 and 2, to be antineoplastic and able to extend the life of leukemic mice [68]. An often-quoted study from the Massachusetts Institute of Technology showed basking shark cartilage inhibited tumor angiogenesis and did so better than other sources [61]. Doses of Japanese shark cartilage inhibited angiogenesis on tumor and embryonic tissues in a linear relationship [69]. Shark cartilage has been shown to have antiproliferative effects on human umbilical vein endothelial cells, with the active components residing in a fraction of < 10 kDa [70].

Other studies *in vivo* also showed promise. A commercially available preparation from blue shark (*Prionace glauca*) cartilage dubbed U-995 displayed potent antiangiogenic activity in the chick chorioallantoic membrane (CAM) assay. Oral administration (up to 200 μg 4× a day) did not affect the growth of sarcoma in mice, although intraperitoneal delivery did in a dose-dependent manner [71]. Intraperitoneal delivery of U-995 also showed a marked reduction of lung metastases of B16F10 melanoma when given at 200 μg per mouse, and metastases were not seen at 1 mg orally. This raises questions of how the active constituent is not denatured or hydrolyzed in the stomach. The liquid shark cartilage product AE-941 (Neovastat) has been shown to inhibit neovascularization in the CAM model as well. It has prevented skin irritation in humans, showing promise as a possible treatment for psoriasis [72] AE-941 is a standardized, water-soluble extract that represents < 5% of crude cartilage and was set to be tested in Phase III trials in Europe and North America for metastatic nonsmall cell lung [73]. Orally administered shark cartilage has been shown to inhibit fibroblast growth factor-induced angiogenesis in a rabbit cornea model [74].

Two persistent problems raised by critics of oral shark cartilage are the lack of satisfactory patient outcomes and data that correlates bioavailability with pharmacological effects with oral administration [75]. However, on this latter point there is evidence of some gastrointestinal adsorption of intact proteins into the blood [76], especially smaller ones. Studies in mice have demonstrated that low molecular weight (14 – 15 kDa) proteins from shark cartilage can augment delayed-type hypersensitivity responses against sheep erythrocytes, inhibit angiogenesis and increase tumor-infiltrating T cells when injected intraperitoneally [77,78]. And a water-soluble shark cartilage fraction was found to stimulate B cells and macrophages in BALB/c mice, yet had no significant effect on T cells. The bioactive molecules in the aqueous solution were found to be thermally stable proteoglycans with mass > 100 kDa [79]. Commercially available Sharkilage and MIA Shark Powder were tested for antitumor and antimetastatic effects in mice. After oral doses of 5 – 100 mg daily for 25 days after implantation of a SCCVII primary carcinoma, no decrease in primary tumor growth or inhibition of lung metastases was found [80].

Phase I and II human clinical trials found no beneficial effects of oral shark cartilage in patient quality of life [81]. Sixty adults with previously treated cancers (16 breast, 16 colorectal, 14 lung, 8 prostate, 3 non-Hodgkin's lymphoma, 1 brain, 2 with unknown primary) were given 1 g/kg daily spread over three doses. In total, 13 patients were lost or refused further treatment, 5 experienced toxicity or intolerance, 22 had progressive disease at 6 and 5 at 12 weeks, and there were neither complete remissions nor partial responses. Overall there was a 16.7 rate of stable disease, similar to supportive care alone [82].

Even more disappointing, a two-arm, randomized, placebo-controlled, double-blind clinical trial by the Mayo Clinic of 83 patients with incurable colorectal or breast carcinoma found no difference in overall survival between patients receiving standard care with Benefin shark cartilage or standard care and a placebo [82]. These better-controlled clinical studies have led to the conclusion that 'no convincing data exists for advocating shark cartilage in cancer' [83]. More disappointing still, chemoradiotherapy with or without AE-941 was administered to 379 patients with stage III nonsmall cell lung cancer. This randomized Phase III trial found no differences in time to progression, tumor response rates or survival [84]. These clinical studies have led most to conclude that shark cartilage is not just unproven as a cancer remedy, it is actually well disproven [85].

The negative data for oral shark cartilage in human oncology trials should not deter future investigations to determine the mechanisms and possible translational potential of promising *in vitro* data. Commercial shark cartilage extracts induce inflammatory T_H1 cytokines (TNF-α, IL-1β, IFN-γ, but not IL-4) and chemokines (IL-8) from human peripheral blood leukocytes [86]. Shark cartilage induced more TNF-α than bovine cartilage, collagen or chondroitin sulfate. This activity was diminished with addition of protease. It has been suggested that the type II collagen α1 protein may be the

bioactive component in commercial shark cartilage for TNF-α production [87]. If so, the authors suggest that the slaughterhouse may be a more sustainable cartilage source.

Interesting cell growth control data have also come from shark research not involving cartilage. Cytokine-like factors from cultured bonnethead (*Sphyrna tiburo*) epigonal (primary B lymphoid tissues) cells inhibit the growth of many human cell lines [88]. Epigonal conditioned media causes apoptosis in the Jurkat T cell leukemia line via the mitochondrial caspase-mediated pathway [89].

With the benefit of hindsight, the emergence of shark cartilage can be seen as a series of good scientific studies being unfortunately misconstrued, occasionally in a profane business model that has exploited the most desperate patients while devastating marine species that, in part due to an inaccurate media representation, are just as easily abused.

3. What shark immunity can offer us: useful antigen receptor genetics and structure

Although their cartilage is not a panacea for cancer, shark lymphocyte antigen receptors are capable of some special abilities. I now turn to these immunoglobulins and TCRs and the genes that encode them, aiming to provide an understanding of the ways their immunogenetics are distinct and can be harnessed to serve our laboratory, diagnostic and clinical needs.

3.1 Locus organization

The Ig heavy chain and each Ig light chain isotype are each encoded by one locus (one IgH, one IgLλ, one IgLκ) in mammalian genomes. The many V(D)J gene segments in these loci provide much potential combinatorial diversity for the antibody repertoire. Cartilaginous fish, however, use a different approach to achieve a diverse repertoire. Instead of one very complex IgH locus and one for each IgL isotype, they employ many less complex loci for each [90]. Sharks have tens to hundreds of these loci generally with one V, a few D (for IgH) and one J [91]. Sequence differences in these elements between loci and junctional diversification by nontemplate (N) and palindromic (P) nucleotide additions create a repertoire as diverse as that of other vertebrates [92-94]. Having many IgH and IgL loci spread throughout the genome may have afforded shark antigen receptor immunogenetics some of the plasticity described below. All evidence to date suggests that shark TCR loci are traditional translocon [26,55], rather than the multicluster Ig gene organization.

More bizarre still, some of these IgH and IgL loci have rearranged in the shark germline. Thus, there are many un-rearranged loci available for the RAG to act upon for V(D)J rearrangement, but sharks have some that the shark is born with ready to express a prescribed variable domain with germline encoded third complementarity determining region (CDR) [95]. Studies need to determine whether these germline-encoded rearrangements recognize antigens of particularly common pathogens of elasmobranchs. One such

germline-joined IgM gene is preferentially used by young nurse sharks [96], and it is known that RAG expression in the gonads of shark is responsible for these fused loci [97].

Remarkably, the many shark IgH loci still exclude (akin to allelic and isotypic exclusion in mammals) to maintain clonal antigen receptor expression on each B lymphocyte [98]. Bony fish appear transitional with IgH loci largely following the single translocon model of higher vertebrates but IgL existing in the multicluster organization of sharks [99]. Shark AID can isotype switch a mature variable gene exon encoding rearrangement between at least some IgM (μ) and IgW (ω) loci [100] and even in between two distinct IgW loci [101]. The task of heavy chain class switch to distinct mucosal-functioning isotypes is managed at different steps of V(D)J recombination in different bony fish [102], and AID-mediated IgH switch at a single locus appears not to have evolved until tetrapods [103].

Now that we have covered some unique aspects of shark immunoglobulin gene organization and expression, we have the fundamentals in place to appreciate four special cases of shark lymphocyte antigen receptor biology.

3.2 Heavy chain-only antibodies: IgNAR

Sharks have distinct functional IgH isotypes, and even distinct functional C region encoding loci for individual isotypes [101]. In addition to IgM and IgW (orthologous to mammalian IgD [104]), sharks employ an additional isotype called IgNAR. Interestingly, this isotype is a homodimer of IgH chains that does not use IgL chains. The variable domains used by IgNAR share as much homology with TCR as Ig [105], which has caused questions as to its origin [106] (and spurred the moniker new antigen receptor (NAR), before it was confirmed as a B cell receptor). Originally discovered in nurse shark, IgNAR has been studied in divergent species such as the catshark (*Scyliorhinus canicula*), suggesting that IgNAR evolved in an elasmobranch ancestor at least 200 millions of years ago [107]. IgNAR can exist as a monomer but has been found to multimerize in the spiny dogfish [108]. We know a great deal about how the IgNAR paratope binds antigen epitope, and an induced fit model has been demonstrated with surface plasmon resonance [109] and in the solved structure of IgNAR binding lysozyme [110].

Both the simpler immunogenetics of a single-variable domain and the relative stability of the heavy chain homodimer (compared with a homodimer of IgH/IgL heterodimers) have fueled great interest in the adaptation of IgNAR antibodies to biotechnology and immunotherapy. The IgNAR variable domains are also unusual in that hypervariable region 2 (HV2) can form a belt around the side of the domain for a structure akin to C1-type immunoglobulin family domains [24]. However, the HV2 of IgNAR can have selected hypermutations [111] and this loop can be important for antigen binding [112]. Phage display technologies are easily adapted to analyze IgNAR antibodies from immunized sharks [113] and the immunization protocols necessary for a robust, affinity matured humoral IgNAR response have been described [114]. These technologies have been used to create

recombinant shark antibodies to many relevant pathogens such as Ebola [115]. The pharmaceutical industry has taken full advantage of the unique binding structure and simpler single-chain genetics of shark IgNAR for the development of targeting reagents [116].

It is interesting that heavy chain-only antibodies have evolved (at least) twice in vertebrate evolution. Although elasmobranchs use the dedicated IgNAR isotype at committed loci for IgL-less antibodies, camelids employ an IgG variant that has structural modifications allowing the eschewing of the light chain [117]. I doubt whether cartilaginous fish and camelids are the only two vertebrate groups to have evolved this innovation, but currently these are the only two known examples of this structural convergence upon single-variable domain Ig paratopes, employing different genetics in each case [118].

3.3 Single-variable binding, doubly-rearranging NAR-TCR

Surprisingly, a domain sharing high identity to IgNAR was found at the amino terminus of TCRδ [119]. This diversified NAR-TCR variable domain is expressed in addition to the canonical TCRδV domain. The NAR-TCR δ chain is unusual among lymphocyte antigen receptor chains in containing two variable domains, and both of these variable domains are the product of V(D)J genetic rearrangement. The fundamental immunogenetics of the NAR-TCR receptor chain are understood, and the doubly-rearranging receptor is older than IgNAR – conserved in both the older holocephalian and the more modern elasmobranch chondrichthyes [120]. Dedicated supporting TCRδV domains buttress each NAR-TCRV domain in a 1-1 relationship and are linked in the TCRδ locus of sharks. It is predicted that NAR-TCR (like the B cell IgNAR) affords γδ T cells with single-variable domain antigen recognition. Neither the predicted structure of NAR-TCRV nor the solved structure of IgNAR V contains hydrophobic faces to pack against another domain. Thus, NAR-TCR V is not predicted to associate with an additional V domain on the TCRγ chain of shark, and no biochemical or genetic evidence for one has been found. So both B and T cells of cartilaginous fish have the ability to make diverse repertoires of antigen receptors that recognize antigen with a projecting, free variable domain, as opposed to the more planar paratope of traditional heterodimeric receptors. These binding structures are very different from what is naturally available to the lymphocyte repertoires of mouse and man.

Just as shark IgNAR has structural analogs in camelids, TCRs similar to shark NAR-TCR have been found in more ancient clades of mammals. Both marsupials and monotremes (e.g., platypus) make a fifth TCR called TCRμ (in addition to TCR α, β, γ and δ) [121]. Although the enabling genetics are different in these lower mammals than sharks, TCRμ is a TCR chain with two consecutive membrane-distal variable domains [122]. Thus, the tertiary structure of mammalian TCRμ is essentially similar to that of shark NAR-TCRδ.

3.4 Ig-TCR trans-rearrangements

Lymphocytes of the adaptive immune system are defined by their antigen receptor: T cells have the TCR and B cells the surface Ig. Yet sharks express transcripts from V(D)J rearrangements in which components of B and TCRs have combined. The V gene segments of Ig μ and ω loci can rearrange to D and J segments from the TCRδ locus and the trans-rearrangement is expressed with the TCRδC exons [26]. Much is known of the individual IgH loci in the nurse shark [123]. These trans-rearrangements are expressed without frameshift mutations in the thymus, spleen and spiral valve (shark intestine) at quantitative levels and functional frequencies too high for them not to be physiologically selected, and are detected by anti-IgHV antibodies on the surface of thymocytes (unpublished data). Although the Ig-TCR trans-rearrangements are expressed at lower levels than canonical IgV-D/J/C or TCRV-D/J/C transcripts, they are on the same order of magnitude and are thought to contribute additional binding specificities to the γδ T cell repertoire.

Chimeric Ig-TCR rearrangements are not restricted to sharks. The TCRδ locus of the African clawed frog *Xenopus* contains IgHV gene segments that are used in IgV-TCRδD/J/C transcripts [124]. Similar genomic organizations have also been described in lower mammals [125] and galliforme birds [126]. Hence, there is significant antibody/TCR structural plasticity in TCRδ chains of vertebrates and this appears to be an ancestral character state of the jawed vertebrate immune system.

3.5 TCR somatic hypermutation

Much is known of the mechanics and outcomes of cartilaginous fish Ig SHM. Although AID is suspected of mediating this process [127] autonomously at different Ig loci [128], studies of SHM at IgL loci showed a propensity for contiguous mutations [129], and there is evidence for the contributions of the nontemplate polymerase η [130].

SHM is considered a B cell phenomenon. Yet careful study of the sandbar shark (*Carcharhinus plumbeus*) TCRγ locus and expressed transcripts allowed the recognition of somatic hypermutation in this TCR gene [131]. The mutations at sandbar shark TCRγ target the classical DGYW/WRCH AID targeting motif [132]. My laboratory has subsequently found mutation at the TCRα locus as well, and this is occurring in the primary T lymphoid tissue (the thymus) before antigen exposure (data not shown). Thus, many questions are raised about the role of SHM in shaping the primary αβ TCR repertoire, its role in diversification and/or the passing of positive and negative selection. Importantly, this demonstrates the capability of both classes (αβ and γδ) of T cells to employ SHM for molding their TCR repertoires.

Much remains to be learned of shark thymic development and selection, for example, the autoimmune regulator gene *AIRE* is known to function at least down to bony fish but we do not yet know whether it upregulates the expression of self-tissue antigens in the shark thymus for central

tolerance [133]. The recent genome of the elephant shark (*Callorhinchus milli*) showed that the CD4 coreceptor is absent in that cartilaginous fish and many of the transcription factors and cytokines characteristic of mammalian helper T cell subsets are absent (save the T_H1 lineage) [55].

Very little is known of TCR SHM beyond sharks, but there is evidence that the dromedary camel TCRγ locus can undergo SHM [134].

4. Sharks and the future of immunotherapeutics

The specificity and memory of adaptive immunity have been recruited through immunization to provide arguably our greatest tool against infectious pathogens for global public health. That same specificity has also been successfully harnessed in a passive approach through monocolonal antibody therapy to target specific molecules key in the pathology of a wide swath of other conditions. For example, belimumab (Benlysta) targets B cell activating factor to treat systemic lupus erythromatosis, rituximab (Rituxan) targets CD20 in non-Hodgkin's lymphoma and infliximab (Remicade) targets TNF-α for several inflammatory autoimmune disorders, including Crohn's disease, ankylosing spondylitis, rheumatoid arthritis and ulcerative colitis.

Antibodies make such useful therapeutics because they can be generated to bind almost anything. But of course there are target antigen conformations that are more impervious to the creation of an effective blocking or neutralizing antibody. That has contributed to the appeal of the single-variable domain binding of shark IgNAR for the development of therapeutic binders that recognize epitope recesses that may not be accessible to the flatter paratope of IgH+IgL antibodies. For example, shark IgNAR have been made to cholera toxin [135] and the precore antigen of hepatitis B virus [136]. IgNAR to other targets are being actively developed by both academic and industrial laboratories worldwide.

What has not been as widely pursued are the B cell immunogenetics that shark TCR use. A newer arena for immunotherapy is engineered TCR therapeutics [137]. Now sharks allow the TCR diversification toolbox to be expanded to include a single binding variable domain (NAR-TCR), use of IgH V gene segments (Ig/TCR chimeric trans-rearrangements) and further diversification and possibly affinity maturation via somatic hypermutation (that shark TCR 'light chains' γ and α employ) (**Figure** 4). The immunogenetics of sharks have successfully broken many rules: i) that Ig must use light chains; ii) that antigen receptor chains have only one V domain; iii) that TCR bind heterodimerically; iv) that B and TCRs do not mix; and v) that SHM is just for Ig and not to be used by TCR. These significantly expand the arsenal of immunogenetic tricks available to the antibody/TCR engineer in finding a binder to a difficult structural moiety and expands the functions that can be exacted once targeted (e.g., opsonization, neutralization, T cell killing or regulation). Partial or full humanization of antibodies is available on multiple platforms to avoid xenotypic reactions [138] and this will follow for TCR. More genomic and transcriptomic tools for cartilaginous species are becoming available, including a nurse shark bacterial artificial chromosome library [139], elephant shark genome [55] and developing resources in skate, dogfish shark, catshark and bamboo shark. These resources will accelerate the use of shark antigen receptor loci genetics in therapeutic development.

I have highlighted several examples where the immunogenetic oddities of shark have been discovered in other vertebrate groups as well. These examples demonstrate that the utility of single-variable antibody and TCR paratopes, Ig/TCR chimeric molecules and somatic hypermutation of TCR genes is not limited to elasmobranch immunity. Whenever an unheralded antigen receptor structure or mechanism is discovered, there is new potential for a binder of a difficult epitope to be generated. The discovery of an exceptionally long, knob-and-stalk IgH CDR3 structure in cattle antibodies is one such recent example [140], and the therapeutic binding capabilities of the aforementioned VLR of lamprey and hagfish are being actively pursued [141,142]. Sharks are just one of many sources of immunotherapeutic tools and inspiration that we must fully utilize. The comparative method not only teaches us what is fundamentally conserved in an evolutionary system but also shows the many diversifications that have been found to be successful.

5. Conclusion

Shark antigen receptors offer much that is not found in traditional mammalian models (**Figure** 4). The stability, tissue penetrance, B cell source, relatively simple single binding domain and small size of shark IgNAR antibodies have led to its translational development faster than the TCRs. IgNAR have even now been humanized onto an IgLκ scaffold [112], suggesting that immunogenicity issues can be overcome with immunotherapeutics from very diverse taxa. Much work remains to be done, however, to see these technologies adapted fully to translation in the clinic.

6. Expert opinion

Shark cartilage continues to create income for the supplement industry despite robust evidence of its ineffectiveness against cancer in humans. Meanwhile, shark single-variable antibodies are being developed for many antigens of oncologic, inflammatory and biodefense importance. Novel shark TCR immunogenetics are the next to be exploited in the development of immunotherapeutics. Basic knowledge of shark immunobiology is developing fast, and dovetailing this with structural biology and biotechnology development is necessary to target the candidate clinical antigens that have structure and binding requirements that will be most addressable by the shark immunogenetic tools. I do not think it is fantasy that genes conferring domains and diversification mechanisms from other species will
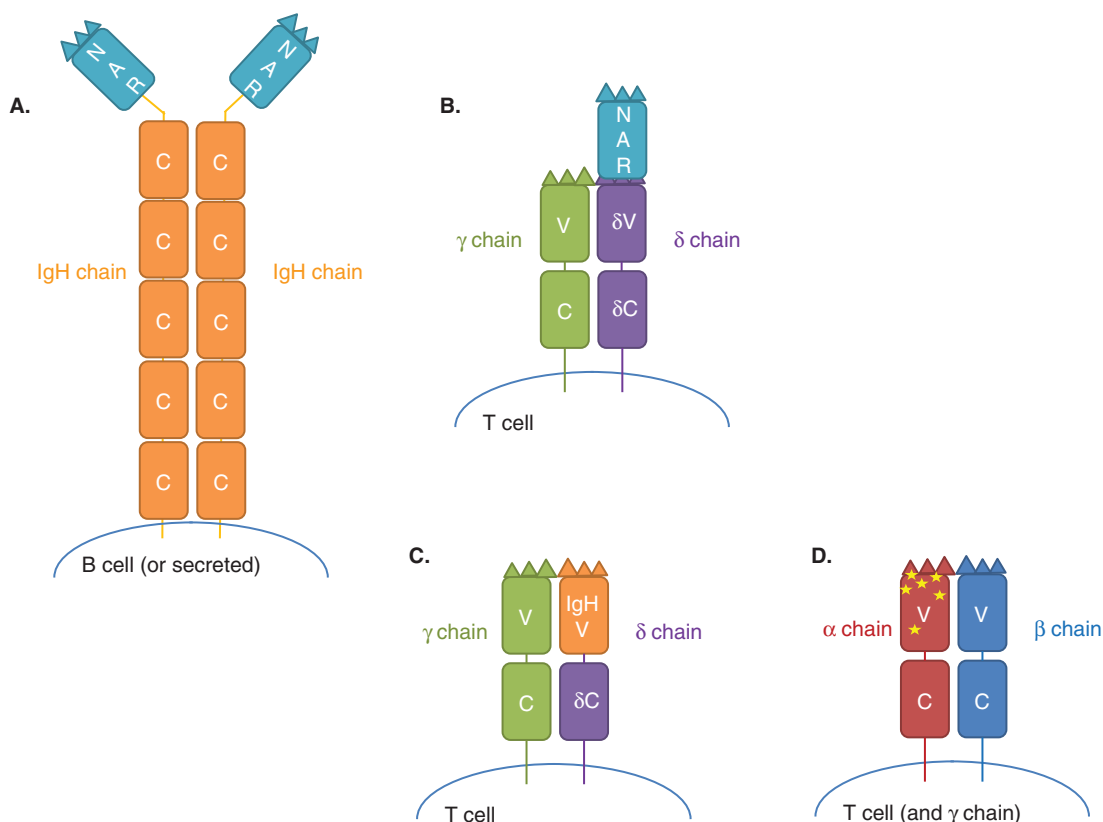
Figure 4. Immunogenetic tricks of shark lymphocyte antigen receptors that are or can be exploited for immunotherapeutics.
A. IgNAR binds antigen with a single-variable domain. **B.** NAR-TCR is a doubly-rearranging TCRδ chain that is expected to bind antigen as a single TCR V domain. **C.** Trans-rearrangements between Ig and TCR loci create chimeric TCRs employing (predominantly, save the D and J contributions) IgHV domains. **D.** Somatic hypermutation at TCR loci further diversifies TCR repertoires and may allow T cell affinity maturation. All of these could be exploited in humoral or membrane-bound molecules as positive or negative effectors.
MHC: Major histocompatibility complex; TCR: T cell receptor.

be engineered into the antigen receptor loci of an individual's lymphocytes *ex vivo* to contain autoimmunity or combat a neoplasm. In the shorter term, the dogma-defying shark TCR technologies have the most potential to avail paratopes that, when paired with the proper scaffold antibody or receptor, will create truly vertical advancements in clinical translation.

The immunology of cartilaginous fish (while fascinating) is certainly not unique in offering tools for the antigen receptor engineer. I predict that many more surprising and useful mechanisms and utensils will be discovered as the antigen receptor loci and immunogenetics of more species are explored. As in the example of the guy looking for his keys under the streetlamp, I suspect the rich discoveries in shark, camelids and (what are incorrectly called) nonplacental mammals are in part due to rigorous research in these species by open-minded comparative immunologists. There will be additional good things to come as more nonmodel species are explored, and with the growing recognition that natural selection has found a plethora of ways to build antigen receptor repertoires in the last half billion years. There are two reasons shark lymphocyte

receptor systems particularly deserve a careful study: i) they may be indicative of the ancestral state of all jawed-vertebrate lymphocytes and teach us what is evolutionarily fundamental in the system; and ii) we may find mechanisms in them that are the product of hundreds of millions of years more battle testing (at the phylogenetic level of Class and Order) than in a relative evolutionary newcomer such as a primate or rodent. Rational drug design would be irrational to ignore the structures, domains and diversification mechanisms that have served vertebrates well for so long.

Declaration of interest

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Affiliation

Michael F Criscitiello[1,2]
[1]Texas A&M University, College of Veterinary Medicine and Biomedical Sciences, Texas A&M Health Science Center, Comparative Immunogenetics Laboratory, Department of Veterinary Pathobiology, Mailstop 4467, College Station, TX 77843, USA
Tel: +1 979 845 4207;
Fax: +1 979 862 1088;
E-mail: mcriscitiello@cvm.tamu.edu
[2]Assistant Professor,
Texas A&M University, Texas A&M Health Science Center, College of Medicine, Comparative Immunogenetics Laboratory, Department of Microbial Pathogenesis and Immunology, Mailstop 4467, College Station, TX 77843, USA