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The genus *Xenopus* as a multispecies model for evolutionary and comparative immunobiology of the 21st century

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Abstract

The *Xenopus* model for immunological research offers a collection of invaluable research tools including MHC-defined clones, inbred strains, cell lines, and monoclonal antibodies. Further, the annotated full genome sequence of *X. tropicalis* and its remarkable conservation of gene organization with mammals, as well as ongoing genome mapping and mutagenesis studies in *X. tropicalis*, add a new dimension to the study of immunity. In this paper, we review uses of this amphibian model to study: the development of the immune system; vascular and lymphatic regeneration; immune tolerance; tumor immunity; immune responses to important emerging infectious diseases; and the evolution of classical and non-classical MHC class I genes. We also discuss the rich potential of the species with different degrees of polypoidy resulting from whole genome-wide duplication of the *Xenopodinae* subfamily as a model to study regulation at the genome level.

Keywords

Immunogenetics; tumor immunity; nonclassical MHC class I; genome evolution

1. Introduction

Greg Warr has long been an ardent supporter of comparative immunology and science education. This statement is easily validated by his research program, his training of graduate and postdoctoral students, his leadership role as the editor of DCI and by his current position at the NSF. Early in 2002, Greg organized a remarkable workshop1 in Charleston, SC entitled “Evolutionary Immunobiology: New Approaches, New Paradigms” that led to a white paper for the NSF [1]. The objective of this workshop was to: provide an overview of the current knowledge in the field of comparative immunology, reveal areas and problems of future potential high impact, and identify the needs for the continued development of this discipline. As recognized in the white paper [1] and in a meeting report prepared by Louis Du Pasquier and Courtney Smith [2], the richness and complexity of defense systems of plants and animals far exceeds earlier predictions. This realization has

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been amply justified by research during the past few years. Indeed, the diversity of solutions utilized by organisms during evolution to control pathogens and tumors seems boundless. The dilemma recognized during the workshop and one that still remains unresolved is how to best deal with such a diversity and complexity.

A question that most comparative immunologists face soon or later is what is the relevance of studying the immune system of a "funny creature" (in the parlance of Bill Clem who co-edited DCI with Greg) rather than focusing all efforts and resources to studying the immune system of *Homo sapiens* and its immune alter ego, *Mus musculus*. We, as well as Greg, strongly believe that it is in fact of considerable value to explore a variety of nonmammalian species from the whole tree of life not only as models for better understanding the human condition but also because of the intrinsic value of knowing how the splendid diversity of organisms have adapted to their environment and have coped successfully with parasites, microbial pathogens, and malignant cell transformations.

The development of technologies such as genomics and proteomics during the last decade has opened new frontiers. The numerous genome projects associated with these new technologies such as deep-sequencing and bioinformatics provide boundless novel opportunities to explore biological diversity and from our particular perspective, the diversity of immunity. Therefore, since this special edition of DCI represents a tribute to Greg's sustained effort to promote our field, advocate the use of new technologies and to some extent, chart the direction of our journal, we think it is appropriate to illustrate the potential of a new age of comparative immunology with our animal model of choice, species of the *Pipidae*. This amphibian family includes *Xenopus laevis* and its sister species, *X. tropicalis*, whose genome has now been fully sequenced and annotated [3,4]. The *Pipidae* is composed of species with various degrees of polyploidy (2 to 12 n) [5]. Thus, the *Pipidae* family, and especially the *Xenopodinae* subfamily, is a unique group among vertebrates owing to their postulated evolutionary emergence by genome duplication. For a more comprehensive account of the taxonomy, ecology, behavior, genetics, immunology, sensory physiology, and evolution of this amphibian taxa the reader can consult the monograph of reference edited by Tinsley and Kobel [6]. We think that the combination of the long term extensive characterization of the immune system of *X. laevis*, the ongoing genetic and genomic characterization of *X. tropicalis*, and the availability of a set of species with various degrees of polyploidy, in contrast to other models based on a single species, brings the possibilities for investigation to a new level. In this review, we will first present a short overview of the actual potential of the *Xenopus* model. Some of this information has also been discussed in the 2009 *Xenopus* Community White Paper 2009 prepared for the National Institutes of Health (http://xlaevis.cpsc.ucalgary.ca/community/xenopuswhitepaper.do). We will then discuss the future promises of the extended *Xenopodinae* model using as an example our recent work on nonclassical MHC class I genes.

2. Existing potential of the *Xenopus* model for comparative immunology

X. laevis continues to provide a powerful nonmammalian comparative model with which to study many facets of immunity. These include: humoral and cell-mediated immunity in the context of MHC restricted and unrestricted recognition; ontogeny; phylogeny; and defense against tumors, viruses, fungi and bacteria (reviewed in [3,7]). Notably, the *X. laevis* model offers a collection of invaluable research tools including MHC-defined clones, inbred strains, cell lines (including lymphoid tumor, fibroblast and kidney cell lines), and mouse monoclonal antibodies specific for a variety of *Xenopus* cell surface makers (e.g., general leukocytes, pan T cells, CD8, NK, IgM, IgY, IgX, IgL, MHC class I, class II). All these reagents, tools and animals, as well as related information, are available through a *X. laevis*

research resource for immunobiology (http://www.urmc.rochester.edu/mbi/resources/Xenopus/). Additional *Xenopus* resource can be found on Xenbase (http://xlaevis.cpsc.ucalgary.ca/common/).

Finally, the annotated full genome sequence of *X. tropicalis* and its remarkable conservation of gene organization with mammals, as well as ongoing genome mapping and mutagenesis studies in *X. tropicalis*, add a new dimension to the study of immunity. In this paper, we will succinctly review some salient uses of this *Xenopodinae* model.

2.1. Model to study the development of the immune system

One of the earliest (and still important) scientific uses of *X. laevis* has been as a tool to understand embryogenesis and subsequent stages of development (reviewed in [8]). From our comparative perspective, *X. laevis* has taught us much about the early ontogeny of the immune system. *X. laevis* has all the lineages of hematopoietic cells that mammals have. Unlike mammals, however, early developmental stages of *X. laevis* are free from maternal influence, and are easily accessible and amenable to experimentation. This provides an ideal system to study early commitments and fates of myeloid and lymphoid lineages [9,10]. For example, a primitive myeloid cell population arising in the anterior ventral blood island at the end of the neurula stage has been recently characterized in *Xenopus* embryos. During the next 6–8 hours of development (i.e., early tail bud stages), these cells migrate and populate the entire embryo [11]. These migratory cells are the earliest differentiated blood cells described to date and their formation occurs well before both the differentiation of primitive erythrocytes (previously thought to be the earliest blood cells to differentiate), and the formation of a vascular network. Moreover, these primitive myeloid cells, which are the only cells with potential immunological function in the early embryo, are quickly and efficiently recruited to wounds over large distances before the establishment of functional vasculature [12]. The *Xenopus* system has the additional advantage of the accessibility of the thymus early in development. Indeed, thymectomy can be efficiently performed in *Xenopus* at early developmental stages (before the migration of stem cells) to generate T cell-deficient animals (reviewed in [13]). Similar to the use of nude or RAG knockout mice [14], T cell-deficient *Xenopus* are critical for studying the role of T cells in transplantation and tumor immunity. Combined with MHC-defined stains and clones, further *in vivo* characterization of T cell effector subsets (e.g., cytotoxic CD8 T cells) by adoptive transfer is possible.

Although other animal models (e.g., zebrafish) also make it possible to access immune tissues early in development free of maternal influence, *Xenopus* with its second developmental period during metamorphosis provides a truly unique experimental model to study immune differentiation, regulation, and self-tolerance. During metamorphosis, the larval thymus loses most of its lymphocytes and a new differentiation occurs from a second wave of stem cell immigration; this results in completely distinct adult immune system [15–17]. Notably, autoimmunity against the many new adult-specific proteins needs to be prevented by a new balance of self-tolerance through T cell education [18].

In summary, *Xenopus* has been, and still is, frequently used to study T cell ontogeny, and with the advent of genomic and genetic technologies, it offers new ways to analyze genes and function in a complementary manner.

2.2. Model to study vascular and lymphatic regeneration

The *Xenopus (X. laevis* and *X. tropicalis)* tadpole has recently emerged as a very powerful system for tissue and vasculature regeneration research [19]. Within 7–10 days following tail amputation, a completely new functional tail with all its tissue types (including muscles,

spinal cord, etc) regenerates in this system. Formation, maintenance and regeneration of lymphatics and blood vessels has become a major area of investigation in its own right [20–22]. The transparency of *Xenopus* tadpoles coupled with new transgenic techniques that permit one to trace cells expressing particular fluorescent markers and to induce the expression of important regulatory factors (e.g., bone morphogenetic protein), are likely to increase the attractiveness of this system [23,24]. In addition, several efficient transgenic techniques are now currently used both in *X. laevis* and *X. tropicalis*, including the original Restriction Enzyme Mediated Integration (REMI) technique [25], as well as the more recently developed PhiC31 integrase, the Sleeping Beauty transposase and the I-Sce meganuclease techniques [26] that efficiently mediates the insertion of plasmid DNA into the *Xenopus* genome at low copy number. Besides allowing expression of particular transgenes, these techniques now permit the generation of transgenic lines with leukocyte or lymphocyte subsets specifically labeled by fluorescent gene reporter expression. The availability of the *X. tropicalis* genome sequence and an extensive Fosmid genomic library based on the bacterial F-plasmid, will facilitate the identification and cloning of putative promoters and regulatory regions of gene of interest (e.g., *CD4* as a T cell marker, or *spib* as a myeloid cell marker) for driving the expression of fluorescence gene reporters. The optimization of a lab diet and water temperature for *Xenopus* has considerably shortened their generation time (sexually mature adult obtained in 8–10 months for *X. laevis* and 6–8 months for *X. tropicalis*), which is important for obtaining transgenic progeny from founders in a reasonable time period.

The *Xenopus* larval tail is fascinating in an immunological context. The regenerative ability of *Xenopus* tadpoles is transiently impaired at early larval stages (stages 45–47). During this so-called “refractory period,” tadpoles fail to generate a regeneration bud (blastema) and thus fail to regenerate their lost tails [27]. The regeneration capacity recovers after stage 47. Interestingly, the refractory period appears to be immune dependent since immunosuppression induced by either pharmacologic immunosuppression or immune cell depletion by morpholino knockdown of PU.1, significantly restores regenerative ability during this refractory period [28]. The role of the immune system in tail physiology becomes even more complex at pre-metamorphic and metamorphic stages when the tail is resorbed. Although tail degeneration is usually thought to be controlled mainly by a cell-autonomous mechanism of programmed cell death triggered by thyroid hormone, a critical involvement of the immune system has been shown [29]. Two keratin proteins, Ouro1 and Ouro2 proteins, specifically expressed in the tail skin, are necessary and sufficient to promote T cell-mediated tail regression. The mechanisms that lead to the education and antigen recognition of these T cells are currently unknown. It’s important to note that very early thymectomy that renders *Xenopus* T-cell function deficient, does not appear to affect metamorphic processes including tail resorption. Whether this reveals a non thymus-dependent population involved in metamorphosis, some compensatory pathway, or some heretofore unknown process are intriguing points to ponder, It’s noteworthy that some studies with such early thymectomized animals have hinted at the possibility of an extra thymic pathway of alloreactive T cell differentiation in this species [30]. These results underscore the potential role of immune system in remodeling and other morphogenetic processes, and the usefulness of the *Xenopus* model to investigate these processes.

2.3. Model to study immune tolerance

X. laevis serves as an exciting novel model to explore self-tolerance because of the ease with which allotolerance to minor minor histocompatibilty (H) antigens (Ags) on adult skin grafts can be induced just prior or during metamorphosis, a key transitional period when *Xenopus* undergoes a temporary period of altered immunoregulation [18]. During this period, one can experimentally induce long-lasting specific non-deletional (“split”) anergic-like tolerance to

minor H-Ags that persists long after metamorphosis is completed [31–33]. MHC genes are also differentially regulated in larvae and adults. MHC class I Ags are first detected on erythrocytes and a minor splenocyte population at the beginning of metamorphosis [34,35]. In fact, neither classical class I, non-classical class I, nor LMP7 mRNAs can be detected in the thymus until metamorphosis [36]; this strongly suggests an absence of class I education during larval life. MHC class II Ag expression during larval life is restricted to the thymic epithelium centrally and to B-cells and accessory cells in the periphery, whereas after metamorphosis, class II Ags are expressed constitutively on virtually all thymocytes and mature peripheral T- as well as B-cells [37,38]. Whereas larval $CD8^+$ splenocytes express fully rearranged TCRβ transcripts (Robert unpublished results), their MHC restriction and cytotoxic capacity is presently unknown. The change in MHC gene regulation during metamorphosis, the new histogenesis in the thymus, and the ease with which one can experimentally manipulate larvae (e.g., thymectomy, blocking or accelerating metamorphosis) allows one to address questions about MHC restriction, autoimmunity, and the development of self-tolerance that can not be easily studied in other animal models.

2.4. Model to study tumor immunity

Given its genetic distance from mammals, *Xenopus* offers a convenient model to explore the complex interactions that occur between malignant tumors or cancers and their hosts. To date, *X. laevis* is the only amphibian species where a series of true lymphoid tumors have been discovered and cell lines have been obtained, thereby opening new avenues for tumor biology and the isolation and characterization of membrane proteins. Involvement of innate anti-tumor NK cell responses was revealed *in vivo* by anti-NK antibody treatment followed by tumor transplantation assays, and *in vitro* by cytotoxic assays [13,39,40]. More importantly, transplantation studies with these lymphoid tumors have shown that as mammals, tumor immune responses are thymus-dependent and that CD8 T cells are crucial to control malignancy [41–44]. However, the absence of MHC class Ia expression by the thymic 15/0 lymphoid tumor has revealed an unsuspected role of nonclassical MHC class Ib (XNCs) molecules that are expressed by this tumor, in *Xenopus* anti-tumor responses mediated by unconventional cytotoxic CD8 T cells [45]. Notably, stable 15/0 tumor transfectant cells that were rendered deficient for XNC expression by RNA interference targeting either β2-microglobulin or a XNC consensus sequence, were more tumorigenic when transplanted in MHC compatible LG-15 cloned frogs than non-transfectants. Further, these tumor transfectants were more resistant to killing *in vitro* by unconventional CTLs. These results suggest that some class Ib gene products are involved in immune surveillance and adaptive CD8 T cell responses to tumors. The biological function of the multiple class Ib molecules in mammals, and their respective role in tumor immunity is still poorly understood. As such, *Xenopus* can provide a useful model to unravel some of these complexities.

Xenopus and its thymic lymphoid tumors have also been influential in demonstrating the biological significance of heat shock proteins (hsps) in immune responses against tumors (reviewed in [44,46]). In particular, *Xenopus* hsp gp96 is as efficient as human or mouse gp96 in chaperoning and promoting cross-presentation of tumor antigens as well as in generating potent anti-tumor responses including reactivity against tumors that have down-regulated MHC class Ia but still express class Ib molecules [40,47,48]. Similar anti-tumor immune responses were shown for Hsp70. Gp96 in *Xenopus* as in mammals is able to elicit Ag-specific conventional CTL response by interacting with the endocytic receptor CD91 expressed by antigen presenting cells and through the channeling of chaperoned Ags to the class Ia presentation pathway [49]. So far, however, it has only been demonstrated in *Xenopus*, that gp96 is also able to stimulate unconventional CD8 T cell effectors that can kill class Ia-negative 15/0 tumor and likely interact with class Ib molecules. Different innate and

unconventional CD8 T cells have been described in mice, but the possibility that hsps can stimulate their response remains to be investigated [50,51]. Owing to the overall conservation of hsp function in immunity among vertebrates, this novel connection between hsps, class Ib and tumor immune surveillance would merit investigation in other mammalian species including man.

The *Xenopus* model has also been used to study the profound link between cancer and embryonic development (reviewed in [52]). Several groups have created tumor phenotypes in developing tadpoles by expressing a variety of tumor suppressors and proto-oncogenes [52–54]. The technique involves fertilization of *Xenopus* eggs *in vitro* followed by microinjection of *in vitro* synthesized mRNA encoding the protein to be tested [55]. For example, this technique has led to the identification of the zinc-finger transcription factor Gli1 as a major player in the molecular biology of basal cell carcinoma (BCC) [53]. Similarly, expression of a dominant-negative p53 elicits tumor formation in the brain, spinal cord, muscle, kidney and epidermis [52] whereas overexpression of Rel mRNA generated tumors that depended on the location of the mRNA injection site [54].

As a final note, it's worth mentioning that the distinct immune systems of larvae and adults, together with the ease of manipulating their maturation during metamorphosis, provides a unique opportunity to investigate, *in vivo*, the possible influence of the immune system on the selection of more aggressive tumors. Recent studies in mice and humans have indeed suggested that while the immune system maintains a surveillance that is crucial for detecting (immune surveillance) and controlling tumors, the same immune system also establishes selective pressures that remodel and even promote (i.e., immunoediting) new tumor variants that escape immune surveillance and display increased tumorigenicity and metastatic potential (reviewed in [56]). In summary, *Xenopus* can provide both a developmental and evolutionary perspective that is useful for better understanding the complex role of the immune system in tumorigenesis and tumor immunity.

2.5. Model to study immune responses to important emerging infectious diseases, and parasites

Xenopus provides a powerful laboratory model to study immunity to important emerging infectious diseases in amphibians that are caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd; reviewed in [57]) and by ranaviruses (*Iridoviridae*; reviewed in [58]). The recognized threat of these emerging wildlife diseases on global biodiversity, which ultimately impact human health, makes it urgent to better understand host-pathogen interactions in vertebrates other than mammals. Thanks to our extensive knowledge of the *Xenopus* immune response and the biology of both Bd and ranaviruses, as well as the availability of *Xenopus* microarrays and the complete genome sequence of *X. tropicalis*, *Xenopus* is an ideal model for such studies. For example, comparison between susceptible tadpoles and resistant adults to ranaviral infection, and between susceptible *X. tropicalis* and resistant *X. laevis* to Bd infection, provides ways to elucidate virulence and immune escape mechanisms that are of high fundamental relevance [59,60]. More importantly, the use of the *X. laevis* model with its immunological tools has allowed one to demystify (better than microarrays), the apparent lack of host immune responses, especially the adaptive immune responses, in the etiology of Bd infection. Indeed, experimentation in *X. laevis* has shown unambiguously that both innate (antimicrobial peptides) and adaptive (antibodies and T cell) responses are critically involved in controlling Bd infection [61]. Now that we have a better idea of the immune defense capability of an amphibian host to these pathogens, it will be possible to determine whether, and if so, how Bd overcomes these defenses. In short, the *Xenopus* model is likely to be critical for better understanding the epidemiology of this infectious fungal disease.

The unique antimicrobial peptides in skin secretions produced by *Xenopus* are very potent against HIV and many human gram negative and positive bacteria, and, therefore, are of significant biomedical interest. Available genomic information will provide further insight about the regulation and evolution of the genes encoding these proteins [62,63].

Environmental chemicals (e.g., pesticides, dioxin, endocrine disruptors), physical factors (e.g., UV light), and behavioral stressors have well-documented inhibitory effects on the vertebrate immune system. In this regard, the global decline of so many amphibian species is considered by some to be the proverbial canary in the coal mine [64].

A last aspect worthy of mentioning concerns the use of *Xenopus* to study host-parasite interactions [6]. The different species of *Xenopus* hosts and interactions with a complex variety of parasite species (e.g., flat worms) that offers a unique system for investigating ecology, population dynamic, genetics and evolution of symbiosis [65,66]. Notably, further studies with natural and experimentally-generated interspecies hybrids could be extremely useful owing to the remarkable degree of species-specificity showed by different parasites. Besides parasite-specific factors, the specificity of the interaction suggests the critical involvement of the host immune system.

2.6. Model to study immunogenetics

The *X. tropicalis* genome has provided compelling evidence for the similarity of gene repertoire in both the adaptive and innate immune systems [4,67]. More importantly, it has unveiled the amazing degree of conservation of gene clustering or synteny with mammals, which is far better preserved with *Xenopus* than with any of the fish species whose genomes have undergone extensive diversification during evolution [67–69]. Gene synteny is helpful for identifying diverged genes such as immune genes. For example, in *Xenopus* as in mammals CD8 beta retains proximity to CD8 alpha, whereas CD4 is closely linked to Lag3 and B genes (Chida et al., submitted). Ongoing whole genome mutagenesis will allow one to search for genes critically involved in immune functions.

Xenopus can also be cloned using gynogenetic development of diploid eggs coming from interspecies hybrids [7,70]. These clones, which are easily maintained and propagated in the laboratory, constitute a unique *in vivo* way to study genome regulation. Clones with identical MHC combinations but differences at minor H gene loci provide an excellent biological system to study immune responses *in vivo*. *X. laevis* is the only species where aneuploid animals can be generated for studying the segregation of immune functions linked to a specific chromosome [71]. *In situ* hybridization techniques are now available both for chromosome and for whole mounts embryos [72,73].

In the 1952, nuclear transplantation, now called SCNT, was pioneered in the leopard frog by Briggs and King [74]. In the mid 1970s, Du Pasquier and colleagues determined, in *Xenopus*, that nuclei from differentiated lymphocytes remain genetically equivalent to zygotic nuclei in promoting development [75]. More recently, SCNT has enjoyed a resurgence of activity by immunologists interested in further understanding of the molecular basis of and differences in reprogramming stem cells and more differentiated cells such as lymphocytes [76–78]. This technique has also shown promise for exploring the contribution of epigenetic and genetic changes to cellular differentiation and transformation. Given the large number and size of their eggs, SNCT is relatively easy and inexpensive to perform in *Xenopus*. Together with the rapid progress in transgenesis and genetics, *Xenopus* could prove to be a powerful model for future SCNT studies.

2.7. Model for education

Adult frogs and tadpoles are inoffensive, easy to manipulate, simple to maintain, and fun to watch. The rapid development of *Xenopus* from egg through embryonic stages, larval life, metamorphosis and young adulthood provides a rich source for illustrating anatomy, physiology, embryogenesis, and evolution as well as more specific biological processes (e.g., transplantation, heart physiology). The transparency of tadpoles permits observation of the beating heart and circulating red blood cells. As such, *Xenopus* is an excellent educative model for students from middle school level up to university (see examples at http://www.urmc.rochester.edu/mbi/resources/Xenopus/outreach.cfm)

3. Future potential of the amphibian *Xenopodinae* taxon as a model to study regulation at the genome level

Evidence has accumulated to support Susumu Ono's [79,80] hypothesis that at least two rounds of genome duplication occurred in a short evolutionary time period in early vertebrate evolution ~550 MYA. For example, the human genome sequence has revealed that there are more than 1000 regions in which homologous genes are linked in a similar order [81]. These so-called "paralogous" regions can be explained by genome duplication. The MHC and HOX regions are the best examples of such duplication [81]. As mentioned previously, *Xenopus* is the only genus where polyploid as well as diploid species exist naturally and also can be artificially produced with various degrees of polyploidy (2N to 12N), The polyploid *Xenopus* species found in nature arose by whole genome-wide duplication with no major reorganization of the chromosomes, which provide investigators with a series of relatively recently derived taxa (1–30 MYA) (reviewed in [70]). These natural species and the possibility of generating in the laboratory viable polyploid animals offer a powerful, but still under-exploited, experimental approach to studying the consequences of whole genome duplication. This is a subject of major interest nowadays for understanding the origin of the vertebrate genome, as well as the effects of gene dose on host resistance or defense against pathogens.

Study of genomic organization and regulation is of particular relevance in the context of immunity since the immune system is based on genetic diversification both at the germinal and somatic level. Indeed, although the immune system of vertebrate is characterized by a system of somatic diversification of lymphocyte receptors (e.g., Ig and TCR) by gene rearrangement, other immunologically-important cell surface receptor including NK, FcR-like, and MHC class I and II molecules are encoded by polymorphic multigenic families. The diversity in these gene families is not only due to allelic polymorphism, but also to gene polymorphism (e.g., presence or absence of a particular gene). To date, the mechanisms involved in the regulation of gene numbers are poorly understood.

One remarkable feature revealed by studying polyploid *Xenopus* species is the active and relatively fast (1 to 30 million years) diploidization of certain immune genes including MHC [71,82–84], RAG [85] and the Ig heavy chain [72]. Comparison of the fully sequenced genome of *X. tropicalis*, the only diploid *Xenopus* species possessing 20 chromosomes, with available information from gene databases of *X. laevis* that has 36 chromosomes (likely due to an allotetraploidization event from its diploid ancestor), suggests that 25–50% of genes are retained as a double-copy in *X. laevis* after whole genome duplication [4,86]. One proposition from these studies is that slowly evolving genes are more likely to be retained as paralogs with slightly different functions (subfunctionalization) or regulation, whereas fast evolving genes are more likely subjected to purifying selection (i.e. gene loss of one of the duplicates; [4,86]). Several recent reviews have discussed diploidization of MHC in detail [71,87].

3.1. Evolution of classical and non-classical MHC class I genes

As an example of the potential of using the *Xenopus* genus as a model for genome evolution and regulation, we will now consider nonclassical MHC class Ib (class Ib) genes. These are heterogeneous genes encoding molecules structurally similar to classical MHC class Ia (class Ia) but with a more limited tissue distribution and polymorphism. Mammalian class Ib genes have diverse and often uncharacterized functions, and because of their rapid rate of evolution, class Ib phylogeny is difficult to establish. Class Ib genes have been identified in all taxa of jawed vertebrates [87].

In *X. laevis*, there are as many as 20 *X. laevis* class Ib (*XNC*) genes per haplotype that are grouped in 11 subfamilies based on sequence similarity [88,89]. They are distant from the MHC locus but are located at the telomeric extremity of the same chromosome [72]. Taking advantage of the *X. tropicalis* fully sequenced genome, we have recently annotated and compared the class Ib gene of this species with those of *X. laevis* (Goyos et al., submitted). This study has revealed the unusual conservation of a large number of these genes; this contrasts with the difficulty in finding class Ib gene orthologs in mammals. We identified 29 *Xenopus (Silurana) tropicalis* class Ib (SNC) genes and pseudogenes, 12 of which have been supported by expression data. These have been grouped in 14 *SNC* subfamilies. Remarkably 9 of these 14 SNC subfamilies are conserved with *X. laevis*, including a unique monogenic lineage represented by the divergent XNC10 gene and its unequivocal SNC10 ortholog. Using degenerate primers to amplify α1 or α2 domains, we have been able to identify putative XNC10/SNC10 in most species of the *Pipidae* family including polyploidy species such as *X. clivii* (8N) and *X. ruwenzoriensis* (12N), as well as *Hymenochirus curtipes* and *Pipa carvalhoi* (Taran, Goyos, and Robert, unpublished). Although further investigation is needed, these preliminary data suggests an evolutionary conservation of the XNC10/SNC10 over at least 120 million years. Interestingly, both XNC10 and SNC10 are preferentially expressed by thymocytes themselves from the onset of thymic organogenesis. In the case of XNC10, we now know (thanks to of the availability of a *X. laevis*-specific anti-CD8 monoclonal antibody) that the expression pattern is mainly associated with the CD8 lineage in the thymus and in the periphery [89]. This pattern of expression is reminiscent of some mammalian class Ib. For example, the class Ib molecules TL and CD1d are expressed on murine cortical DP thymocytes and seem to be the key to the development of a subset of γ/δ as well as NKT cells, respectively [90]. It is tempting, therefore, to speculate that the XNC10/SNC10 lineage has been selected and conserved for a specialized role in the development a particular T cell subset. One intriguing feature of the *XNC10*/*SNC10* genes is that in contrast other class Ib genes, they appear to be under pressure to remain monogenic. Both Southern blot analysis ([89]; Goyos et al., submitted) and preliminary cloning data indicate that the number of genes remains minimal even in *Xenopus* polyploidy species. Therefore, as in the case of Ig, MHC class II and class Ia, there seems to be an evolutionary pressure for an optimal number of genes involved in adaptive immunity.

The collection of species with different ploidy in the *Xenopus* genus offers a unique opportunity to explore, in a comparative model system, how gene number is regulated. This is of fundamental relevance owing the variation in gene numbers of various families of immune genes including KIR, NKp, FcR-like gene family. These genes families vary in numbers between species and sometimes among individuals.

4. Perspective and challenges

In this mini-review (and hopefully as a tribute to Greg Warr's interest in applying newer technologies and a multidisciplinary approach to address fundamental evolutionary questions), we have attempted to highlight why and how *Xenopus* continues to provides a unique and versatile non-mammalian model with which to investigate many facets of the

immune system. Among these areas of interest are: the ontogeny of immunity, self-tolerance, autoimmunity, tumor immunity, and the adaptation of host immune defenses to emerging pathogens.

It seems ironic that several major recent breakthroughs in the field of comparative immunology with potential to have a major conceptual impact on fundamental immunology have occurred coincident with the flattening/stagnation of federal and other funding opportunities. Given competition for limited monetary resources, it has become increasingly difficult to convince the biomedical community of the relevance of nonmammalian animal models such as *Xenopus* (or most other organisms), for immunological studies. In our view, this shortsightedness impacts the education and training of a future generation of scientists, since bright and creative students, as well as more senior investigators, are forced to follow the money rather than pursue interesting and evolutionarily-relevant questions that may shed light on the origins and functions of the immune system.

Training scientists as well as educating the public about the complexity of immunity and the evolutionary basis of this complexity is, in our view, of major importance for a variety of reasons, one of which relates to the seemingly relentless attacks on the theory of evolution by so-called creationists. Immunology is a subject used by believers in intelligent design to bolster their arguments against natural selection [91,92]. Comparative immunologists are among those experts who can counter assertions of creationists and properly educate the public and perhaps more importantly, young students. Now more than ever, our discipline needs to be defended and publicized by our society and our journal.

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