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Tumorigenesis and anti-tumor immune responses in *Xenopus*

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1. ABSTRACT

Despite intense study, the role of the immune system in detecting (immunosurveillance), controlling and remodeling (immunoediting) neoplasia remains elusive. We present here a comparative view of the complex interactions between neoplasia and the host immune system. We provide evidence, in the amphibian *Xenopus laevis,* consistent with an evolutionarily conserved and crucial role of the immune system in controlling neoplasia, which involves a striking variety of anti-tumoral immune effectors including conventional CTLs, classical MHC class Ia unrestricted CTLs (CCU-CTLs) that interact with nonclassical MHC class Ib molecules, CD8 NKT-like cells and NK cells. We also review the tumors found in *X. laevis* with an emphasis on thymic lymphoid tumors and a rare ovarian dysgerminoma. Finally, we consider the use of *X. laevis* for *in vivo* study of tumorigenesis. Given our current knowledge, the experimental systems already established in *X. laevis*, and the rapid accumulation of genetic resources for the sister species *Silurana (Xenopus) tropicalis*, it is our conviction that these species provide an ideal alternative to the murine system for studying tumorigenesis and tumor immunity.

2. INTRODUCTION

Neoplasia, or cancers, are defined as abnormal proliferation of genetically altered cells that can be caused by oncogenic viruses or predisposing conditions. Despite some confusion in the literature about the diagnostic distinction between pseudoneoplasia and real neoplasia and other types of deregulated proliferation resulting from wound repair or infection (e.g., granuloma caused by mycobacterial or mucosal infection), there is little doubt that cancerous diseases are widespread in eumetazoa. Reported cases include calicoblastic neoplasms in coral (1), tumorigenic germinal cell mediated by Notch in *C. elegans* (2), leukemia in bivalve mollusks (oysters) (3,4), intestinal and pigmented tumor in sea urchin (5) and hepatoma in lampreys (6). Similarly, species among all jawed vertebrate classes develop tumors and cancer (7) including sharks (8) that have been mistakenly claimed by some (9) to be tumor-free. A teleost platyfish-swordtail tumor system has even been used to study the etiology of cancer and reports have demonstrated that neoplasia in these fishes is due to aberrant regulation of certain genes during the multistep process of cancer formation (10).

Literature pertaining to amphibian neoplasia is scattered, often incomplete, and difficult to interpret due to inconsistencies in diagnosis, nomenclature and misdiagnosis of infectious and inflammatory conditions (11). Besides reports of neoplasia in different Urodele (salamanders) and Anuran (frogs and toads) species in the wild (11), two types of tumors have been studied in more detail. The first is the Lucké tumor. This renal adenocarcinoma found in *Rana pipiens* was the first documented case of a virally-induced tumor (12,13). The second is a series of spontaneous thymic lymphoid tumors that occurred in the African frog *Xenopus laevis* (14) from which stable tumor lymphoid cell lines were derived. The availability of these tumor lines as well as genetically defined MHC compatible inbred strains and clones of *X. laevis* has provided the unique opportunity to study both tumorigenesis and anti-tumor immunity (reviewed in 15). This comparative model, in *Xenopus*, may provide an alternative to the mouse in exploring, within an evolutionary context, the interactions between tumors and their host.

Recent studies in mice and humans have stressed the multiple and complex interactions that occur between malignant tumors or cancers and their hosts (reviewed in 16). On the one hand, immune surveillance maintained by the immune system is crucial in detecting and preventing tumors; on the other hand, the immune system establishes selective pressures that shape and even may generate new variants displaying increased tumorigenicity. Although some evidence is consistent with both immune surveillance and immunoediting concepts, there are still many unanswered questions. *Xenopus* may help this area of research by providing an evolutionary perspective. We review here what is actually known in *Xenopus* concerning tumor occurrence and immunity to tumors.

3. TUMOR OCCURRENCE IN *XENOPUS*

Despite the wide usage of *Xenopus* as an experimental animal model, there are only a few reports of tumor occurrence in this species. In addition, the prevalence, etiology and pathogenesis of these diseases are not well established. We will succinctly review these cases before considering two types of tumors in more details.

Adenoma of the liver appears to be relatively frequently encountered. It is usually detected during dissection as a markedly enlarged liver lobe that can reach twice its natural size (17). The tumor tissue during these late stages usually has large necrotic areas. In most cases, further diagnostics are difficult. In our *Xenopus* colony, we estimate that such hepatomas affect about 5% of our animals per year. Spontaneous thyroid-containing teratomas have also been described recently in *Xenopus laevis* (18). This teratoma was associated with developmental defects in multiple sibling frogs suggesting a genetic basis for this disease. Other neoplasias in *Xenopus* include tumors of the digestive track, melanophoromas, and more rarely, renal carcinoma and nephroblastoma (19-22).

One particular case of a pseudotumor needs to be mentioned, namely an infectious granuloma which was originally reported as a spontaneous malignant lymphosarcoma. In 1962, five new cases of a spontaneous lymphosarcoma were reported in a colony of animals of the genus *Xenopus* (23). The most frequently affected organs were the kidney and liver and the authors determined that the tumor could be successfully transmitted by the tumor tissue itself, as well as cell free extracts, into allogeneic and xenogeneic recipients. Experiments involving the injection of cell-free filtrates or extracts of normal tissues or lymphosarcomas into adult or immature *Xenopus* were later carried out and a viral etiology for this tumor was proposed (24). However, no virus particles have ever been detected by electron microscopy (25). Eventually, through a series of transplantation studies, the *Xenopus* tumor-like growth was found to be a transmissible and not a transplantable disorder (26). Histopathology and transmissibility studies of the disease demonstrated that what was originally reported as a lymphosarcoma in *Xenopus* was, in fact, a bacteria-induced infectious granuloma (27). This case highlights some of the confusion in the literature pertaining to the true diagnosis of neoplasia in amphibians.

3.1. *Xenopus* ovarian tumors

Ovarian tumors are poorly described in amphibians (11). Over the 15 years that we have established our *Xenopus* colony, however, we have observed, on several occasions, the occurrence of spontaneously arising ovarian tumors in adult *Xenopus laevis* as well as in clones of *X. laevis* x *X. gilli* hybrids (LG clones). The etiology of these tumors is not known but they tend to occur in five- year-old or older females for which long-term human chorionic gonatotropin hormone treatment has been administered for breeding purposes. For example, on July 23, 1996 in one tank holding 21 LG-15 cloned adults at the University of Rochester (5-7 years old when they were received from the Basel Institute of Immunology, Basel, Switzerland), 17 frogs were found dead and 4 were moribund. The following day the 4 moribund animals died. Of the 21 total animals, 19 had large abdominal masses; autopsies revealed large tumors, with some hemorrhaging, restricted to the ovaries, as well as inflamed intestines with no obvious metastasis in the gut or liver. Tumors from three animals were harvested and frozen for histopathological analysis while several small pieces of tumor were each implanted sub-cutaneously on the dorsal surface of three animals, two LG-15 and one outbred. Additionally, nine whole carcasses and 2 frozen tumors were sent to Dr. Earl Green at the National Wildlife Health Center in Madison, Wisconsin for pathology reports.

Pathology reports ruled out that this mass was a granuloma; acid fast staining failed to demonstrate any organisms, ruling out mycobacteriosis, one of the most common causes of granulomas in amphibians. Cells within the tumor nodules resembled seminoma cells of the testis but since the sex of the frog was female, the equivalent tumor in females is ovarian dysgerminoma, one of the three main types of ovarian tumors. Until recently (28), this neoplasm had never been reported in amphibians. Slides of

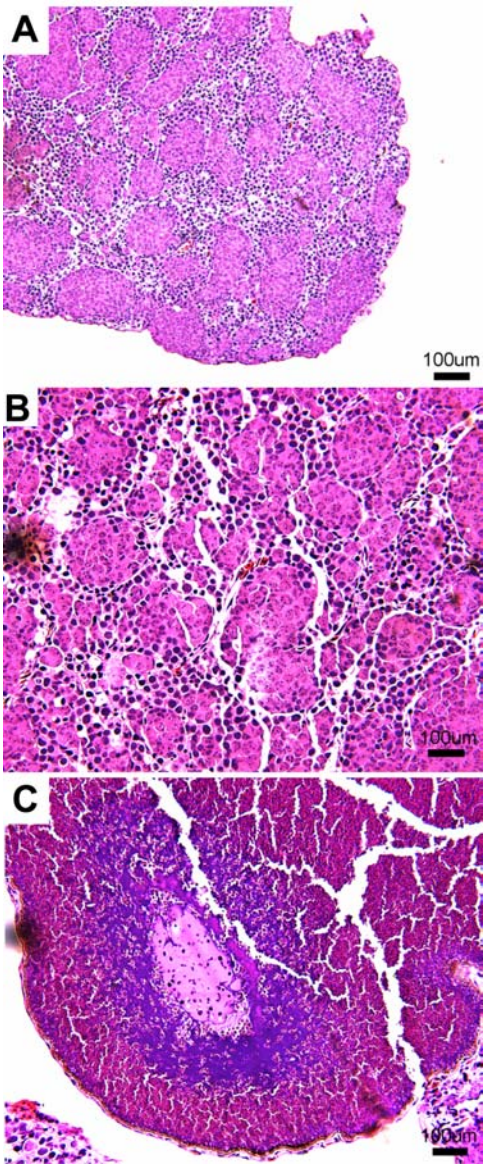

Figure 1. Hematoxylin and eosin (H&E) staining of paraffin sections of spontaneously arising ovarian tumor in LG-15 cloned *Xenopus*. (A) Tumors contain nodules of round and ovoid cells with large pleomorphic nuclei as well as vacuolated roundish cells. (B) Section showing leukocyte infiltration into the ovarian stroma and the disorganized tissue architecture. (C) Higher magnification of a developing follicle with a dense layer of follicular epithelium.

these tumors were submitted to the Registry of Tumors in Lower Animals in May of 2000. Hematoxylin and eosin staining of paraffin sections of these tumors (Figure 1A) demonstrates nodules of round and ovoid cells with large pleomorphic nuclei as well as vacuolated roundish cells. Upon higher magnification (Figure 1B), one can see the obvious cellular infiltrate into the stroma of the ovaries and the disorganized tissue architecture characteristic of tumors. One can also observe a developing follicle with a dense layer of follicular epithelium (Figure 1C). Unfortunately, these tumors never grew in the two LG-15 or in the outbred animal into which they were transplanted.

We have also observed that in *X. laevis*, females of the *ff* partially inbred strain tend to be more susceptible to develop these ovarian tumors. Additionally, a high incidence of these tumors was also observed in LGL triploid hybrids (Du Pasquier, personal communication). In summary, we report the first characterization, in *Xenopus*, of spontaneously arising ovarian dysgerminomas.

3.2. *Xenopus* thymic lymphoid tumors

Although the literature on lymphoma in amphibians is often confusing and contradictory, proven cases of lymphoma are characterized best in *X. laevis* (11). A series of spontaneous T cell lymphomas, reported both in this species and in hybrids with *X. gilli*, involve the thymus and initially present as a unilateral swelling of the caudal cephalic region (29). Similar thymus-derived lymphoid tumors have also been reported by others (30) and cultured *in vitro* to some extent.

During the early 1990's various lymphoid tumor cell lines (named B3B7, 15/0, 15/40 and ff-2) were established from spontaneously occurring thymic tumors (14, 29 and reviewed in 15) in *Xenopus*. The ff-2 and B3B7 cell lines are derived from tumors that arose in the partially inbred frog strain of the *ff* homozygous MHC haplotype while the 15/0 and 15/40 tumor cell lines originated from LG-15 cloned frogs. These cell lines are all non-adherent and grow continuously at 27ºC with a generation time of 18 to 24 hours (31). Although all of the cell lines have a mixed T/B cell phenotype, they differ in terms of their cell surface expression of MHC antigens. The ff-2 and 15/40 tumor cell lines, but not the 15/0 and B3B7 line express cell surface classical MHC class Ia (class Ia) proteins. Additionally, there is no surface expression of MHC class II proteins on the 15/0, ff-2 and B3B7 cell lines, whereas moderate levels of class II are detected at the surface of 15/40 tumors.

The tumor cell lines also differ in terms of their tumorigenicity. For example, the ff-2 tumor does not take in adults of any genetic background and only grows in larvae that share at least one MHC haplotype (*f*) with the tumor. In contrast, the 15/0 tumor is very tumorigenic and grows in adults that share the same MHC alleles (*a/c*) with the tumor and in larvae that share at least one MHC haplotype (*a* or *c*) with the tumor. Therefore, the *Xenopus* model of transplantable tumor cell lines with differential tumorigenic capacities *in vivo* along with clones and inbred strains of frogs with known MHC haplotypes, provides a powerful comparative system where one can study immune surveillance to tumors as well as immunosubversion by the tumors themselves.

A recent case of a spontaneous leukocytic tumor in *Xenopus laevis* is reported in this special issue as an addendum by Du Pasquier et. al. (32). Cells from a splenic tumor were cultured and, although they look very different from the thymic tumors mentioned above, their

morphology suggests some sort of leucocytic tumor, possibly monocytic in origin. It is unique in its intense staining of surface MHC class II and has a very different phenotype from the thymic tumors. Its karyotype suggests a very unstable genome with some cases of aneuploidy. Although these cells grow very slowly in culture, a cDNA library was generated and has yielded valuable information regarding the expression of MHC class II beta loci in *Xenopus laevis*.

4. TUMORIGENESIS

Since natural cases of spontaneous neoplasias in amphibians are rare in comparison with the number of fish and reptile malignancies (33), there have been a few attempts at chemically inducing tumors in these animals to establish experimental model systems. However, known carcinogens in mammals and other vertebrates, such as N-methyl-N-nitrosourea (NMU) and N-dimethylnitrosamine (DMN), tend not to induce malignant tumors in amphibians in general and *X. laevis* in particular (34, 35). Despite these observations, some groups have taken advantage of the fact that the *Xenopus* embryo is very amenable to experimental manipulations, and alternative methods of generating tumor phenotypes have been developed.

4.1. *In vivo* models of tumorigenesis

The *Xenopus* model has been used to study the profound link between cancer and embryonic development (reviewed in 36). Several groups have created tumor phenotypes in developing tadpoles by expressing a variety of tumor suppressors and proto-oncogenes (37-39). The technique involves fertilization of *Xenopus* embryos *in vitro* followed by microinjection of *in vitro*-synthesized mRNA encoding the protein to be tested (40). 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) (38). Similarly, expression of a dominant-negative p53 elicits tumor formation in the brain, spinal cord, muscle, kidney and epidermis (37) while overexpression of Rel mRNA generated tumors that depended on the location of the mRNA injection site (39).

With these tools the *Xenopus* embryo provides a robust model system for analyzing the molecular biology of cancer by performing *in vivo* studies and analyzing initial tumorigenic events. A key question that still remains regarding these induced tumor phenotypes is whether these masses are indeed neoplastic or contain transformed cells that can be transplanted in MHC compatible hosts. *In vitro* cell cultures of the induced tumors also remain to be established and these should aid in the characterization of these tumor-like masses as neoplastic cells.

4.2. Translocation/chromosomal aberration/genomic instability

Neoplastic cells typically possess various genomic mutations and chromosomal aberrations, including point mutations, gene amplifications and deletions, and replication errors (41). Genomic stability ensures cellular homeostasis and genetic continuity in multicellular organisms. Cellular genomes, however, are continually subjected to endogenous and environmentally induced structural damage that can lead to stable mutations that alter the informational integrity of the cellular DNA. The *Xenopus* thymic-derived lymphoid tumor cell lines are no exceptions. Chromosome spreads from the original B3B7 thymic tumor, as well as from the derived tumor cell line after cloning, demonstrate that both the original tumor and the cell lines are aneuploid and in no case was the karyotype stable (14). Even when two individual cells had the same number of chromosomes, the composition of the karyotype differed. Furthermore, expression of c-myc and other oncoproteins have been shown to compromise genomic integrity (42). Interestingly, the class Ia-negative 15/0 and B3B7 tumor cell lines express more c-myc mRNA than the class Ia-positive tumors ff-2 and 15/40 (29). Even though deregulated expression of c-myc has been shown to contribute to the neoplastic phenotype, the precise tumorigenic event causing these spontaneous thymic lymphoid tumors is not known.

Although the 15/0 thymic lymphoid tumor-derived cell line does not express cell surface class Ia, mRNA for β2-microglobulin (β2-m) and nonclassical MHC class Ib (class Ib) is detected (29). During an investigation to address which class Ib genes were expressed by the 15/0 tumor cell line, we performed 5' RACE-PCR using a reverse primer sitting in a consensus region of the α3 domain of class Ib, and class Ia as a control experiment. We sequenced a total of four clones and unexpectedly, this resulted in the amplification of class Ia mRNA consisting of 5' UTR followed by an α1, α2 and α3 domain, all in-frame. When the amino acid sequence of the α1 (Figure 2A) and α2 (Figure 2B) domains of these mRNAs (clones 5-1-1 through 5-1-4) were aligned with known class Ia alleles from LG-15 cloned frogs (the cloned frog from which tumor originated), it became evident that the tumor class Ia sequences were of the LG-a/c-2 allele and thus were *bonafide* class Ia gene products. Since our consensus primer was likely biased towards the *X. laevis* class Ia allele, we cannot attribute this message to either species (*X. laevis* or *X. gilli*). Upon further examination, we noted that although the nucleotide sequences upstream of the class Ia heavy chain were identical in all four clones (Figure 2C) they did not code for *bonafide* class Ia Signal Sequence (SS) (Figure 2D). The four sequences were practically identical in the α1 and α2 domains but their 5' UTRs suggested some kind of translocation event. Translation of these nucleotide sequences in all 6 frames did not result in any SS coding region (data not shown).

To determine whether this aberrant class Ia message from 15/0 tumor was the result of some kind of genomic translocation event, we conducted various BLAST analyses using the mRNA sequence upstream of the 15/0 class Ia α1 domain coding region (73bp total), and asked if this sequence matched to a different gene or area of the genome outside the class Ia locus. We started with an EST analysis. While BLAST of NCBI's Genebank EST database with *X. laevis* class Ia SS nucleotide query results only in hits of *X. laevis* class Ia sequences, 15/0 tumor putative SS query matches one cDNA from a *X. laevis*

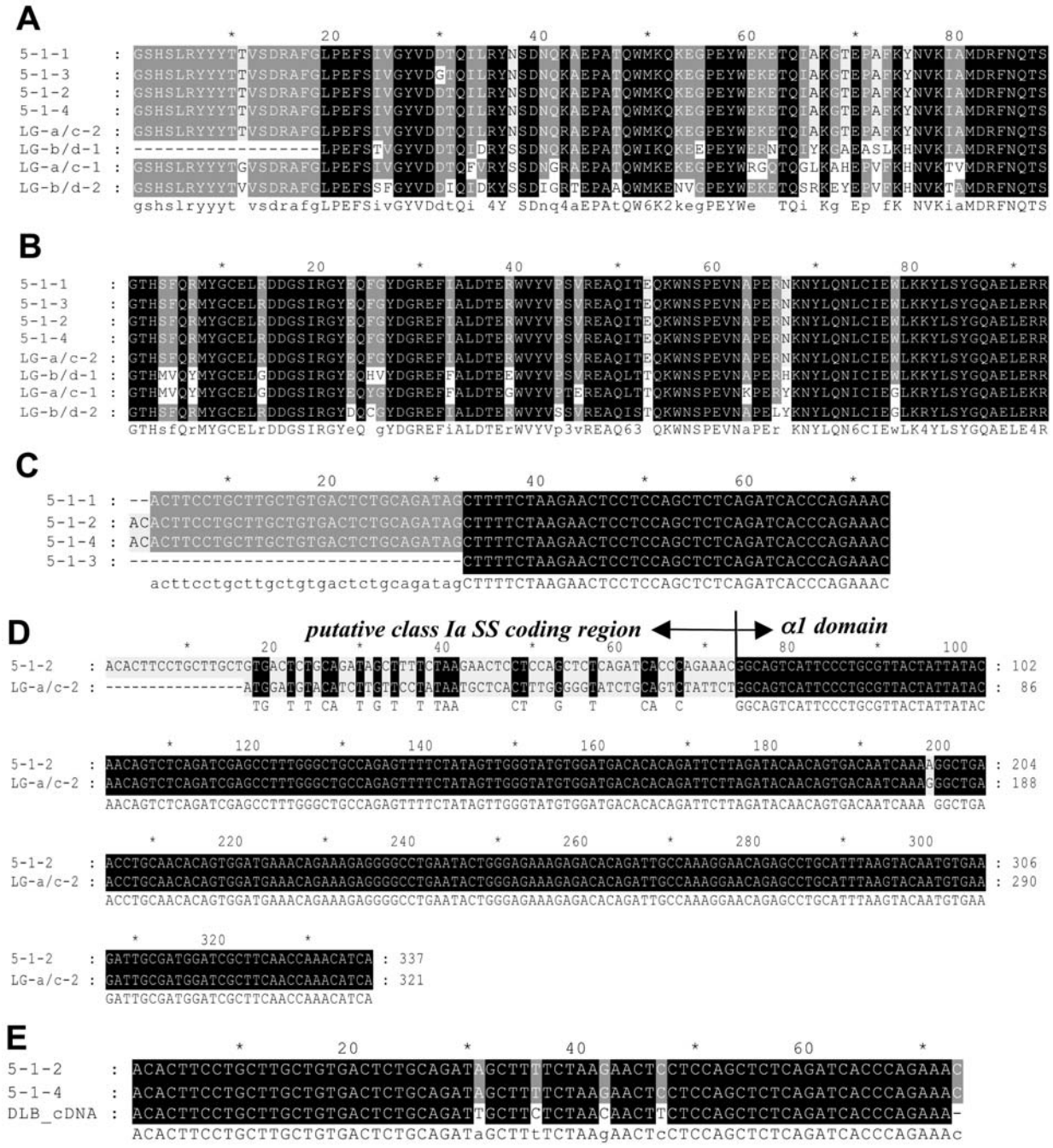

Figure 2. Nucleotide and Amino Acid Alignments of 15/0 Tumor and *X. laevis* class Ia mRNA sequences. (A) Amino acid alignment of 15/0 and *X. laevis* x *X. gilli* LG-15 class Ia α1 domain sequences. Note: Although the LG-b/d-1 sequence is incomplete in the α1 domain it was included in the analysis. (B) Amino acid alignment of 15/0 and LG-15 class Ia α2 domain sequences. (C) Nucleotide alignment of the putative SS coding region (upstream of the α1 domain coding sequence) of the four class Ia mRNA sequences amplified from 15/0 tumor by 5' RACE-PCR. (D) Nucleotide alignment of the putative SS and α1 domain coding regions from 15/0 tumor sequence 5-1-2 and the LG-ac/-2 allele. (E) Nucleotide alignment of the putative SS coding region from 15/0 tumor sequences 5-1-2 and 5-1-4 with a stretch in the reverse orientation of *Xenopus laevis* cDNA amplified from an embryonic dorsal blastopore lip library (Accession #: CO386240).

embryonic dorsal blastopore lip library (Accession #: CO386240). The 15/0 putative SS is 94% identical to a stretch in the reverse orientation of this cDNA (Figure 2E). Thus it is possible that a translocation event generated this aberrant class Ia message. In an attempt to identify the genomic region containing the gene encoding the cDNA

from blastopore lip library, we first screened the fully sequenced genome of *S. tropicalis* without much success. While BLAST of the *S. tropicalis* genome (version 4.1) using *X. laevis* 5' class Ia SS sequence has a match in scaffold 895 that contains the single class Ia gene, no significant hit was found with the 5'UT 15/0 class Ia sequence over the whole *S. tropicalis* genome. When we performed tBLASTx searches with the blastopore lip library cDNA however, 29% of this 5'UT 15/0 class Ia sequence was similar to a small portion of scaffolds 2156 and 602, neither of which contain the *S. tropicalis* class Ia gene. Therefore, the putative *S. tropicalis* homolog of the *X. laevis* gene encoding the blastopore lip cDNA is likely to be in a region outside of the class Ia locus. In any event, this aberrant 15/0 class Ia transcript is interesting considering that Rag1 and Rag2 (Du Pasquier, personal communication) as well as AID (Robert, unpublished results) are still expressed in this tumor. The deregulation of these genes involved in gene rearrangements or gene conversions are likely to contribute to genomic instability. Indeed, other aberrant genes have been found, including a TCR gene rearranged to an Ig gene, as well as a ribosomal gene rearranged to an Ig light chain gene (Du Pasquier, personal communication).

As previously mentioned, the 15/0 tumor does not express any cell surface class Ia protein and by Northern blotting experiments, class Ia heavy chain mRNA has also not been detected (29). Since RACE-PCR is a much more sensitive technique than Northern blotting, it is likely that there is only a low level of class Ia message in the 15/0 tumor cells. Since this is a tumor-derived cell line, it is always possible, however, that the acquisition of aberrant class Ia mRNA expression is a recent event which occurred during maintenance of these cell lines *in vitro*. In any event, since hydrophobic SS are required for the transport of proteins from the cytoplasm to the endoplasmic reticulum in order to enter the secretory pathway (reviewed in 43), perhaps this aberrant class Ia message from the 15/0 tumor, which is missing SS coding region, could help to explain the lack of detection of class Ia protein on the cell surface of 15/0 tumor cells. Although the precise mechanism leading to this aberrant class Ia mRNA expression in this tumor is unknown, this is yet another example of how tumors can manipulate the expression of molecules recognized by the immune system in order avoid their recognition contributing to immunosubversion.

5. CHARACTERIZATION OF ANTI-TUMOR EFFECTOR CELLS

In vivo as well as *in vitro* studies have demonstrated that CD8+ T cells, NK cells, and other less well-characterized cell types are responsible for generating anti-tumor immune responses in *X. laevis*. Strategies to analyze anti-tumor effector cells have included sub-lethal γ-irradiation of adult hosts (31), early thymectomy experiments (44), *in vivo* antibody-mediated inhibition assays (45), heat shock protein immunization strategies (46) and RNA interference techniques (47). In this section we will summarize how these experimental approaches have unveiled a multitude of effector cells that participate in anti-tumor immune responses in *Xenopus*.

5.1. NK-like effectors

As previously mentioned, 15/0 cells are able to grow when transplanted into histocompatible isogeneic LG-15 hosts. To investigate whether NK cell anti-tumor activity is also elicited *in vivo* upon tumor challenge, anti-NK mAb inhibition assays have been performed. *X. laevis* adults were treated with an anti-NK cell antibody (1F8) (48) one day prior to challenge. Although this antibody does not deplete 1F8+ NK cells, this treatment results in accelerated appearance of tumors as well as an increase in the rate of tumor growth (49), thereby impairing the rejection of the transplanted tumor. These experiments establish the critical involvement of NK cells in the *in vivo* anti-tumor immune response.

Potent NK-like anti-tumor activity *X. laevis* has also been demonstrated *in vitro.* When splenocytes from either early thymectomized frogs or control frogs that have been depleted of CD8 T cells by magnetic cell sorting are cultured in growth factor rich medium, significant levels of cytotoxicity are observed against B3B7 allogeneic thymus tumor targets (50). These studies reveal that a non-T/non-B splenocyte population displays natural cytotoxicity towards allothymus tumor cells following culture in T cell-derived growth factor rich medium. This cytotoxic activity, however, requires culture in the presence of T-cell derived growth factors highlighting the importance of T cells and their secretory products for the development of a potent anti-tumor immune response.

As stated earlier, immunization with the heat shock proteins gp96 or hsp70 purified from tumor elicits in *X. laevis*, as well as in mammals, a specific anti-tumor immune response (46). Using this immunization strategy, *Xenopus* NK-like anti-tumor activity has been demonstrated in a syngeneic setting. When adult LG-15 frogs are immunized with gp96 from normal LG-15 liver or 15/0 tumor, the CD8-depleted splenocyte fraction exhibits potent *in vitro* killing activity against the class Ia-negative 15/0 tumor but not against class I+ outbred or LG-15 splenocytes (51). Since neither *in vitro* restimulation of these effectors from naive frogs, nor effectors from LG-15 frogs immunized with total protein lysate were sufficient to generate such potent NK-like killing activity, these cells must be generated or activated *in vivo* by the chaperone gp96.

Mammalian NK cells are activated through the cumulative signals integrated by their cell surface activating and inhibitory receptors (52). Ligands for NK cell receptors include MHC class I molecules and either their absence or dysregulated expression on the surface of cells can lead to the activation of NK cells (53). Evidence for some nonclassical MHC class Ib molecules serving as ligands for NK cell inhibitory receptors in *X. laevis* was recently obtained by using an RNAi approach. Although 15/0 tumor cells do not express class Ia molecules on their surface they do express some class Ib (54). RNAi-mediated

knockdown of class Ib on the surface of 15/0 tumor cells results in their increased killing by splenic NK cells *in vitro* (47). Interestingly, even though there is increased killing of these cells *in vitro* by NK cells, tumor transplantation experiments demonstrate that these class Ib-silenced tumor cells are more tumorigenic *in vivo*.

Taken together, these data suggest that although NK cells are important effectors in potent anti-tumor responses, NK cells by themselves are insufficient to control tumor growth.

5.2. MHC class I-restricted and –unrestricted CD8+ T cell effectors

Although NK cells have the ability to mediate anti-tumor effector responses, T cells exhibit the most robust cytotoxic activity against tumors. Initial characterization of T cell involvement in *in vivo* anti-tumor immune responses has been performed by larval thymectomy experiments (44) in animals of the *ff* partially inbred strain. *Xenopus ff* larvae were thymectomized 5 to 7 days post-fertilization, before the initial colonization of the thymus with embryonic stem cells, and then challenged with a syngeneic ff-2 tumor during adulthood. While control *ff* adults reject transplants of these tumor cells, thymectomy profoundly impaired tumor rejection by adult hosts. These experiments were the first to reveal the involvement of T cells and thymus-dependent effector cells in MHC-restricted *Xenopus* anti-tumor immune responses.

The aforementioned experiments were performed with ff-2 tumor cells that express cell surface class Ia molecules. To address whether the involvement of T cells in the *Xenopus* anti-tumor immune response also extends to class Ia-negative tumor cells, similar experiments were performed with the 15/0 tumor cell line. Outbred larvae were thymectomized at 5 to 7 days post-fertilization and challenged with 15/0 cells during adulthood. Five of six thymectomized outbreds developed tumor after 15/0 injection (Horton and Robert, unpublished results) whereas non-thymectomized siblings did not. This is remarkable considering that 15/0 tumor has only been able to be successfully transplanted into histocompatible hosts. This experiment speaks to the critical role of thymus-dependent effectors in response to tumors, regardless of the status of MHC expression of the tumor.

Further clues as to the nature of anti-tumor effector cells have come from the development of an effective protocol for mAb-mediated *in vivo* depletion of CD8+ T cells in adults (45). *In vivo* depletion of adult CD8+ cells before challenge with MHC class Ia-negative 15/0 tumor cells results in an impaired anti-tumor immune response characterized by accelerated tumor appearance by almost 10 days, compared to controls, and an increase in the rate of tumor growth (45). Moreover, this technique also revealed the involvement of CD8+ T cells in the rejection of MHC-disparate skin allografts. Therefore, CD8+ T cells in *Xenopus* are involved in both MHC class I-dependent and –independent immune responses.

Immunization with 15/0 tumor-derived gp96, but not normal liver-derived gp96, generates CD8+ T cells in adult *Xenopus* that specifically kill the 15/0 tumor (51). Since 15/0 tumor cells do not express any cell surface MHC class Ia molecules, then, by definition, any CD8+ T cell that kills this tumor has to be class Ia-unrestricted. Therefore, we wanted to test the idea that in the absence of class Ia expression on the surface of the tumor, the CD8+ T cells might be interacting with class Ib. To do this, we used RNAi to silence either β2-microglobulin or *Xenopus* nonclassical MHC class Ib (XNC), which resulted in a knockdown of XNC surface protein on the 15/0 tumor (47). This treatment resulted both in an impairment of the CD8+ T cell's ability to recognize and kill this tumor *in vitro* and in the increased tumorigenicity of the XNC-silenced tumor clones *in vivo*. These experiments point to the critical role of CD8+ T cells as anti-tumor effector cells in *Xenopus* and also demonstrate that at least a subset of these effector cells can interact with class Ib molecules to mediate anti-tumor immune responses.

Last but not least, some indirect evidence suggests that NKT-like cells may also be involved in anti-tumor immune responses. As stated earlier, *in vivo* treatment with the mAb 1F8 impairs rejection of transplanted tumor cells. Importantly, this NK cell-associated molecule is also expressed by a minor population of CD8+ T cells in which fully rearranged TCRβ mRNA can be identified (49). Approximately 4% of adult splenocytes co-express CD8 and the NK cell-associated molecule. Therefore, in *Xenopus*, there exists a population of cells that express NK- and T cell-associated markers. So, in addition to inhibiting NK cells, *in vivo* treatment with the 1F8 mAb likely also impairs NKT-like cells in *Xenopus*.

6. CONCLUSIONS AND PERSPECTIVES

Cancer immunosurveillance predicts that the immune system can recognize precursors of cancer and, in most cases, destroy these precursors before they become full blown cancer (55). Although this concept of immunosurveillance dates back more than 30 years ago (56), its importance in the control of cancer still remains controversial (reviewed in 57). Studies in *Xenopus* support the crucial role of the immune system in controlling neoplasia. Functionally impairing components of the immune system, either by mAb-mediated inhibition, sublethal γ-irradiation and/or thymectomy makes individuals more susceptible to tumor formation following transplantation. Conversely, cancer cells can avoid immunosurveillance by the outgrowth of poorly immunogenic tumor-cell variants (immunosubversion or immunoediting). One common strategy to escape a T cell-mediated immune response is the down-regulation or loss of expression of HLA class I molecules (55). In *Xenopus,* the generation of thymic-derived tumor cell lines with differences in their expression of class Ia surface proteins provides one such example. Interestingly, tumorigenicity of these tumor cell lines inversely correlates with the level of surface class Ia expression (29). Therefore, the *Xenopus* system of transplantable tumors into MHC defined isogenetic clones and inbred strains of frogs is a comparative model that can be used to further address the

impact of immunosurveillance on immunoediting and immunosubversion.

Although the *Xenopus* model has also been used to characterize diverse types of anti-tumor effector cells, there is still more work to be done. In all experiments performed to assess the role of CD8+ versus CD8-negative splenocytes in anti-tumor immune responses thus far in our laboratory, the focus has been on addressing these populations as putative classical CTL and NK-like cells, respectively. However, we have shown that the CD8+ splenic population is heterogeneous both in its effector capability, containing classical MHC class I-restricted as well as –unrestricted T cells, as well as in its surface phenotype, with CD8+ cells differing in their surface expression of an NK-cell marker. For this reason, it is possible that NKT-like cells are also involved in anti-tumor responses in *Xenopus* as they are in mammals (58). It will be interesting to determine whether in *Xenopus,* like their mammalian counterparts, there is a sub-population of these cell types that express invariant TCR α chains that interact with conserved moieties of cell surface MHC molecules (59). Additionally, the recent characterization of the arsenal of FcR- and KIR-like activating and inhibitory receptor genes in *Xenopus laevis* (reviewed by 60 in this special issue) provides more tools with which to study immune molecules expressed on lymphocytes and their possible contribution to anti-tumor immunity. Therefore, our model offers the opportunity to examine a heterogeneous pool of anti-tumor effector cells conserved for over 350 million years of evolution, and characterize the conditions under which they exert their functions.

Finally, genetic studies in *X. laevis* are difficult because of its tetraploid genome and its long generation time (i.e., between one and two years to obtain fertile adults). Recently, however, a sister species, *S. tropicalis*, has been developed as an alternative model system for biological and biomedical study. It shares all of the physical characteristics that make *X. laevis* a useful model but has the advantage of a diploid genome and shorter generation time (i.e., fertile adults can be obtained between six months and one year). The rapid accumulation of genetic resources for this species (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html; http://xlaevis.cpsc.ucalgary.ca/common/) and the success of pilot mutagenesis screens have helped propel this model system forward. For these reasons, taking advantage of the current knowledge already established for *X. laevis* and applying it to *X. tropicalis* may provide a platform to identify potential new genes involved in tumorigenesis, as well as in anti-tumor immune responses.

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Abbreviations: AID: activation-induced cytidine deaminase, BLAST: Basic Local Alignment Search Tool, CTX: cortical thymocyte marker of Xenopus, HLA: Human Leukocyte Antigen, Ig: Immunoglobulin, LG: (Xenopus laevis) x (Xenopus gilli) hybrid, MHC: Major Histocompatibility Complex, NK: Natural Killer, RAG: recombination-activating genes, SS: Signal Sequence , TCR: T cell receptor, UTR: untranslated region

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