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Effective RNAi-mediated β2-microglobulin loss of function by transgenesis in *Xenopus laevis*

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Summary

To impair MHC class I (class I) function *in vivo* in the amphibian *Xenopus*, we developed an effective reverse genetic loss of function approach by combining I-SceI meganuclease-mediated transgenesis with RNAi technology. We generated transgenic outbred *X. laevis* and isogenic *laevis/gilli* cloned lines with stably silenced expression of β2-microglobulin (β2m) critical for class I function. Transgenic F1 frogs exhibited decreased surface class I expression on erythrocytes and lymphocytes, decreased frequency of peripheral CD8 T cells and impaired CD8 T cell-mediated skin allograft rejection. Additionally, β2m knockdown increased susceptibility to viral infection of F0 transgenic larvae. This loss of function strategy offers new avenues for studying ontogeny of immunity and other developmental processes in *Xenopus*.

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Key words: Amphibian, Immunology, I-SceI meganuclease, RNA interference, MHC class I, T cell development

Introduction

The amphibian *Xenopus laevis* has long been the ectothermic vertebrate species of choice for studying the ontogeny and phylogeny of the complex immune system. In particular, the evolutionary distance of *X. laevis* from mammals permits distinguishing species-specific adaptations from more conserved features of the immune system. Recent progress in genomics and reverse genetics of this species and its sister species *X. tropicalis* has further empowered this animal model. In particular, several new efficient transgenesis techniques have opened the way to develop reverse genetic approaches to assess gene function *in vivo* (Chesneau et al., 2008). Owing to our previous success in stably silencing immunologically relevant genes in *Xenopus* cell lines (Goyos et al., 2007), we thought it would be valuable to expand the potential of the loss of function approach in *Xenopus* by combining RNA interference with I-SceI meganuclease-mediated transgenesis. We have recently adapted this method to isogenic *laevis/gilli* (LG) clones of *Xenopus* (Nedelkovska and Robert, 2012). These LG clones are interspecies hybrids between *X. laevis* and *X. gilli*, whose progenies are generated by gynogenesis using UV irradiated spermatozoids that activate diploid LG eggs without contributing any genetic material (Kobel and Du Pasquier, 1975).

LG clones such as LG-6 and LG-15 that share the same MHC haplotype (a/c) but differ at multiple minor H loci have been instrumental to establish the antigen-specificity and MHC class I restriction of CD8 T cells in *Xenopus*, as well as to establish the critical involvement of CD8 T cells in viral immunity and skin graft rejection (Robert et al., 2002). These and other studies have demonstrated the high degree of structural and functional conservation of the immune system between amphibians and mammals (Robert and Ohta, 2009). In contrast to mammals, however, the *Xenopus* immune system undergoes striking developmental changes twice during its life: first during embryogenesis, and then again during the transition from larva to adult (Flajnik and Kasahara, 2001). Specifically, the *Xenopus* thymus is initially colonized by embryonic stem cells a few days after fertilization (Flajnik et al., 1984; Kau and Turpen, 1983). During metamorphosis, the thymus loses more than 90% of its lymphocytes (Du Pasquier and Weiss, 1973). This loss is followed by a second wave of stem cell immigration (Bechtold et al., 1992). Additionally, the MHC class I gene is differentially regulated during metamorphosis. In fact, the larval stage of *Xenopus* presents an intriguing immunological enigma, since despite the lack of classical MHC class Ia (class Ia) protein expression until metamorphosis, the tadpole is immunocompetent and possesses CD8 T cells. Comparatively in humans, MHC class Ia deficiency results in severe autoimmunity and/or death. While it is still unclear which ligands are involved in the education and restriction of viral CD8 T cells, certain non-classical MHC class Ib (class Ib) genes are expressed very early in tadpoles, thus representing good candidates for this process. Class Ib genes encode proteins structurally similar to class Ia but usually displaying limited tissue distribution, low polymorphism, and relatively lower levels of cell surface expression (Gleimer and Parham, 2003; Hofstetter et al., 2011). An additional common thread between class Ia and class Ib molecules is their critical interaction with the β2-microglobulin (β2-m) molecule for efficient cell surface expression (Hofstetter et al., 2011; Ulbrecht et al., 1999). Accordingly, to investigate the respective role of class Ia and class Ib molecules in CD8 T cell development, and concomitantly optimize a suitable reverse genetic methodology for *Xenopus*, we focused on the role of β2-m.

As alluded to, in mammals β2-m is a critical component of the MHC class I molecule (Solheim, 1999). Efficient cell surface
expression of class Ia molecules require binding of 8–10 amino acid peptides as well as the non-covalent association with β2-m in the endoplasmic reticulum (Das and Janeway, 1999; Vugmeyster et al., 1998). β2-m also associates with several class Ib molecules (e.g. CD1, Qa, HLA E). The function of β2-m has been investigated in mice models deficient for b2m. In the absence of β2-m molecule, the class I peptide binding groove is unstable and very limited amount of class I molecules, mostly empty of peptides, is expressed at the cell surface. Such impairment of class I surface expression and function in turn result in severe defects in intrathymic CD8 T cell differentiation and in peripheral immune function (Koller et al., 1990; Zijlstra et al., 1990).

The b2m, class Ia and class Ib genes have been identified in all classes of jawed vertebrates. In X. laevis, besides a single class Ia gene and two b2m genes, there are at least 20 class Ib genes per genome (Flajnik et al., 1991a; Flajnik et al., 1993; Shum et al., 1993). Taking advantage of a transplantable thymic lymphoid tumor (15/0 tumor) that expresses several class Ib but no class Ia genes, we report the tumorigenicity of this tumor (15/0 tumor) that expresses several class Ib but no class Ia genes and two b2m genes has been identified in all classes of jawed vertebrates. In X. laevis, besides a single class Ia gene and two b2m genes, there are at least 20 class Ib genes per genome (Flajnik et al., 1991a; Flajnik et al., 1993; Shum et al., 1993). Taking advantage of a transplantable thymic lymphoid tumor (15/0 tumor) that expresses several class Ib but no class Ia genes, we report the tumorigenicity of this tumor (15/0 tumor) that expresses several class Ib but no class Ia genes and two b2m genes. The double expression cassette vector (I-SceI-b2m) was subsequently cloned into the GFP reporter cassette both flanked by the I-SceI 18 bp recognition sites (Fig. 1A).

Plasmid construction

The double expression cassette vector (I-SceI-b2mRNA-GFP) was constructed by cloning a 2.2 Kb GFP reporter cassette into the SacI and PstI sites of the I-SceI-PBSISiK (provided by Dr. Graninger, University of Virginia) vector flanked by the I-SceI 18 bp recognition sites. The anti-b2m or scrambled shRNA cassettes were first cloned into the BbsI and Xhol sites of the pBS-βU6-1 vector (Goyo et al., 2007). The resulting 400 bp shRNA cassette consisting of the βU6 Pol III promoter and the shRNA was subsequently cloned into the EcoRI and KpnI sites of the I-SceI-GFP vector. The final construct for transgenesis consisted of both the shRNA cassette and a GFP reporter cassette both flanked by the I-SceI 18 bp recognition sites (Fig. 1A).

Microinjection of Xenopus eggs

OB, LG-6 and LG-15 females were primed with 10–20 IU and boosted with 20–40 IU of human chorionic gonadotrophin (hCG, Sigma) one day before egg collection. An additional injection of 100 IU of hCG may be necessary the morning of the day of transgenic injection. Testis from outbred Xenopus laevis males were homogenized in 2 ml Deboer’s solution (100 mM NaCl, 1.3 mM KCl, 0.4 mM CaCl₂) and for gonosperms were UV irradiated (UV light source at 253.7 nm, Gelman Instrument) for 6 minutes before use. Eggs were squeezed into a Petri dish and fertilized in vitro with 500 µl of normal (for OB) or irradiated (for LGs) sperm. 10 minutes later the eggs were dejellied with 2% cysteine HCl 8.0 (made in 0.1x MBS), washed with 0.1x MBS (13˚C) several times, and placed into injection medium (4% Ficoll 400 in 0.3x MBS, 13˚C). Eggs were injected using a microinjector (PLI-100, Harvard Apparatus) with 10 nl total volume into the animal pole close to the site of sperm entry.

The I-SceI vectors were digested with the I-SceI meganuclease (New England Biolabs) for 40 minutes at 37˚C and 80 pg DNA along with 1 x 10⁻⁵ U I-SceI were injected per egg. The digests were used within an hour. Note that it is critical that the I-SceI meganuclease is stored at ~80˚C at all times (storage at ~20˚C leads to inefficient or no transgene incorporation into the genome). Furthermore, freeze thawing of both the enzyme and the buffer significantly reduce the transgenesis efficiency; therefore, single use aliquots must be made after purchase. After injection all embryos were incubated at 13˚C for 4 hours in order to delay cell division providing extended time for transgene integration. The embryos were then transferred into 0.3× MBS with 50 µg/ml gentamycin and reared at 18˚C until hatching. Larvae were then raised in dechlorinated water at room temperature.

Fluorescence microscopy

Transgenic larvae (stage 56–58) were screened for GFP expression using SMZ1500 Nikon stereomicroscope equipped with a DS-Qi1 Monochrome Cooled Digital Camera (Nikon). Tadpoles that presented positive were raised to adults and bred to produce F1 progeny.

RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) following manufacturer’s directions. 500 ng of total RNA was used to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) following manufacturer’s directions. As a control for genomic DNA contamination ~RT controls were synthesized for each cDNA sample. RT-PCR was then performed using the 2X Master Mix (Genescript Corp.). 1 µl cDNA or ~RT was used as a template in each RT-PCR reaction. Each RT-PCR also had a non-template control to test for possible contamination of the primers and used following the specific primers were used: GFP (F: 5’-ACGGCCCAAGTTAGCGGTGAC-3’; R: 5’-GTCGATCTCGAGAGTGATCG-3’; Tm: 64˚C), b2m (F: 5’-CCCGGTGGCTCAAGGGTTTACG-3’; R: 5’-GAGATCTAGGTGTTGTAGTA-3’; Tm: 58˚C), MHC class 1a (F: 5’-GGCCGTCATTCCCTCCTGGCTATTAT-3’; R: 5’-CTGGTGAAGGGCCTACATTGC-3’; Tm: 61˚C) and GAPDH (F: 5’-ACCCCTTCCACGTACCGAC-3’; R: 5’-GGGACCGACAGTTGGTATG-3’; Tm: 55˚C).

Collection of blood leukocytes

Six-month-old adults were anesthetized by immersion in a 0.1% aqueous solution of tricaine methane sulfonate (TMS, MS-222) buffered with sodium bicarbonate and were bled from the dorsal tarsus vein using a pulled glass needle (Nedelkovska et al., 2010). The blood was collected in a 10 ml solution of ice cold Amphibian PBS (APBS) and 500 units of heparin in order to prevent clotting, washed once with 10 ml APBS and resuspended at 1×10⁶ cells in 500 µl of staining buffer (APBS+1% BSA+0.01% NaN₃).

Flow cytometry

Blood cells or single cell suspensions harvested from spleen and thymus were stained at a cell density of 1×10⁶/ml in APBS containing 1% BSA and 0.01% NaN₃, with 0.5 mg/ml of fluorescein-specific phenotypic Suba 2X DB (AM22), or undiluted hybridoma supernatants anti-CDS (pan T cell marker, 2B1) and class Ia (TB17) followed by PE or APC labeled secondary anti-mouse Abs or streptavidin. All these mAbs are described and can be obtained from the X laevis Research Resource for Immunobiology at the University of Rochester Medical Center (http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm). For each sample a negative control with only secondary Ab staining was included. GFP expression was also recorded using the FITC channel. 50,000 events were collected on a FACScanlii (BD Biociences). Analysis of gated live leukocytes was performed using the FlowJo software (Tree Star Inc.).

Skin grafting

A piece of ventral skin (abdominal skin that appears silvery due to the presence of irridophores on the grafted skin). Grafts were checked every 2 days under a dissecting microscope.

FV3 infections

Frog virus 3 (FV3, Iridoviridae) was grown in and purified from Baby Hamster Kidney (BHK-21) cells line incubated at 30˚C during the infection as previously described (Chen et al., 2011). Viral titers were determined by plaque assay. Tadpole at stage 49–50 were infected by immersion in a total volume of 2 ml containing 5×10⁶ plaque forming unit for 1 hour. Subsequently, tadpoles were transferred into 1-liter containers, and cumulative mortality was monitored daily over a 30-day period. FV3 infections were verified as the cause of tadpole deaths by performing PCR using primers specific for VDNA polymerase (F: 5’-AGGAGGCCTGGAAGACCTACA-3’; R: 5’-TGTTGTTCCTCAGCCTCCC-TTGG-3’; Tm: 56˚C).
Results
Effects of b2m silencing on transgenic F0 larvae
We have previously used shRNA to successfully and stably silence b2m gene expression in the 15/0 tumor cell line (Goyos et al., 2007). Silencing of b2m should result in downregulation of surface expression of class Ia as well as class Ib molecules, since they require association with the β2-m molecule for efficient cell surface expression. The b2m shRNA was designed in a consensus b2m region that is conserved between the two different Xenopus genes (Flajnik et al., 1993).

To produce transgenic Xenopus with KD of b2m expression, we engineered a double expression cassette vector (I-SceI-b2mshRNA-GFP) flanked by I-SceI meganuclease recognition sequences that contains the anti-b2m shRNA under the control of the human U6 RNA polymerase III promoter (hU6) and a GFP reporter gene driven by the human EF-1α promoter (Fig. 1A). This I-SceI-b2mshRNA-GFP vector was used to generate transgenic LG cloned and outbred frogs. Briefly, we microinjected 80 pg of vector digested with 1 × 10⁻³ unit of I-SceI meganuclease into one cell stage eggs. The injected embryos were reared to stage 56 and then screened for GFP expression by fluorescence microscopy. For most tadpoles that screened positive for GFP reporter expression (>80%), the use of I-SceI meganuclease resulted in strong and uniform non-mosaic expression of GFP. These results are in agreement with those we and others have previously reported (Chesneau et al., 2008; Nedelkovska and Robert, 2012). We postulated that larvae exhibiting GFP expression should additionally display silenced expression of b2m. Accordingly, we performed RT-PCR analysis on stage 56 (one-month-old) transgenic larvae that screened positive for GFP expression as well as control tadpoles from the same batch, lacking GFP expression (Fig. 1B). This RT-PCR analysis confirmed that transgenic animals were GFP positive, but more importantly they revealed that the anti-b2m shRNA induced potent silencing of both b2m genes at the mRNA level (Fig. 1B). Notably, in this experiment 6 of the 14 tested transgenic LG tadpoles had >50% KD of both b2m genes when compared to control larvae. To test for the specificity of the KD we checked for expression of class Ia genes in these animals (i.e. although no class I protein is detected until metamorphosis, class I mRNA is expressed, (Goyos et al., 2011)). Our RT-PCR results confirmed that class Ia mRNA expression remained unaltered in both the control and transgenic larvae (Fig. 1B).

Together, our results indicated that the KD specifically targeted b2m. Importantly, the shRNA expression did not cause any obvious lethal effects on the developing larvae or on metamorphic frogs.

b2m loss of function in F1 transgenic LG and outbred frogs
We have previously demonstrated using the GFP reporter that our construct had a germline transmission of 100% in LG cloned embryos as a result of gynogenesis (Nedelkovska and Robert, 2012). To understand transmission of the shRNA-mediated KD of b2m and the resulting effects on immune function we raised F1 progenies from both LG (gynogenetic) and OB (crosses between Tg parents) transgenic animals to adulthood. We first determined the level of b2m gene expression in skin samples by RT-PCR (Fig. 2). Both OB and LG progenies showed significant b2m KD in most individuals tested, as compared to age matched controls. However, OB transgenic animals showed a wider individual variation than LG clones. This could be due to the fact that the OB F1 transgenic progeny were produced from male and female transgenic F0 frogs having different levels of β2-m shRNA integration. Additionally, it is possible that the β2-m shRNA has been inactivated in some individuals. Nevertheless, these data provide strong evidence that the β2-m shRNA was transmitted to the F1 progeny and remained effective in interfering with b2m expression.

We next determined whether class Ia surface expression was affected in Tg animals. For this purpose we bled animals and stained peripheral blood leukocytes (PBLs) with the Xenopus-specific anti-class Ia monoclonal antibody TB17 that recognizes most MHC class Ia haplotypes including the LG (a/c) haplotype (Flajnik et al., 1999; Flajnik et al., 1991b). For both OB and LG Tg, consistent reduction in class Ia surface expression (2 to 4 fold) on PBLs was detected by flow cytometry compared to age matched controls (Fig. 3). We confirmed that OB frogs did not express a class Ia haplotype not recognized by the TB17 mAb by determining their genotype using PCR and sequencing (supplementary material Fig. S1).

To further examine the effect of b2m KD on CD8 T cells differentiation and abundance in the periphery, we analyzed thymocytes and splenocytes from young Tg LG adults (2 months of age) by flow cytometry. As expected, thymocytes class Ia surface expression was barely detectable in Tg animals and was not significantly different from age-matched controls, despite

Fig. 1. In vivo b2m silencing in transgenic F0 Xenopus. (A) A map of the I-SceI-anti-b2mshRNA-GFP construct used to generate transgenic LG clones. (B) LG eggs were injected (Experimental) with 1 × 10⁻³ I-SceI units and 80 pg of I-SceI-b2mshRNA-GFP in a total volume of 10 nl per egg. RNA extracted from 7-day-old (stage 48) tadpoles was used to synthesize cDNA. Shown is RT-PCR analysis of experimental and control non-transgenic dejellied animals from the same batch (Dejellied controls) using primers specific for Xenopus b2m, class Ia, GAPDH and GFP. % KD is the percent silencing of b2m (β2-m) in transgenic larvae compared to the average expression in the control animals. RT controls for all samples were negative. One representative experiment out of two is shown.
GFP expression (supplementary material Fig. S2A). Furthermore, two color flow cytometry using Xenopus-specific anti-CD8 mAb AM22 and the pan T cell marker CD5, recognized by the 2B1 mAb, did not show any consistent difference between Tg and control thymocytes (supplementary material Fig. S2B). Unfortunately, to date there are no Xenopus-specific anti-CD4 Abs available, thus preventing us from examining whether h2m KD induced more subtle defects on thymocyte differentiation.

In contrast, flow cytometry analysis revealed a 50% decrease in the fraction of CD8 T cells (CD8+/CD5−) in the spleen (Fig. 4). There was also an increase in the fraction of double negative cells (CD5−/CD8−) in the Tg tested. Whether this is also an effect of the h2m KD is difficult to determine, since this cell fraction includes a variety of cell types, from B cells to macrophages and other leukocytes, and can vary considerably from one individual to another, presumably because in Xenopus the spleen functions both as primary and secondary lymphoid organ. Nevertheless, a similar 50% decrease of CD8 T cells (CD8+/CD5−) was found in the blood circulation of Tg frogs (Fig. 4). Moreover, flow cytometry analysis of blood leukocytes from several LG-6 controls and Tg animals provided consistent evidence that h2m KD resulted in a statistically significant reduction of the frequency of circulating CD8 T cells (Table 1). Furthermore, there were no significant differences in the CD8+/CD5− population (Fig. 4; Table 1), which further suggests that not all T cells were affected by the h2m KD but rather only CD8 T cells.

To evaluate the effect of h2m silencing on CD8 T cell function we used an in vivo skin graft rejection assay, which is well characterized in Xenopus and has the further advantage of keeping our Tg animals alive. As in mammals, skin graft rejection in Xenopus critically involves Ag-specific MHC-restricted CD8 T cells (Rau et al., 2001). We grafted Tg LG-6 and aged-matched control LG-6 recipients with ventral skin from a MHC disparate skin donors of the MHC homozygous inbred J strain. The rejection kinetics and the time for complete rejection were determined by monitoring the destruction of white iridophore pigments (i.e. complete rejection was considered when 100% of these iridophore pigments were destroyed). The rejection capacity of Tg LG-6 was considerably impaired, as compared to LG-6 controls, which acutely rejected MHC mismatched skin from J donors transplant within 20 days (23±1 days; Fig. 5). Furthermore, the rejection kinetics of Tg LG clones were slower with delays in the complete rejection (32±1.5 days, P≤0.0009; Fig. 6A). Significant differences were also notable when the median survival of the transplanted skin was calculated (Table 2). To extend our findings, we performed the skin grafting assay with OB h2m KD transgenic frogs. These data strongly supported the LG-6 findings. The outbred transgenic animals carrying the h2m shRNA fully rejected the MHC disparate grafts in 32±3 days, in contrast to the scrambled shRNA transgenic outbred controls (20±1.5, P=0.02; Fig. 6B).

With respect to the lower frequencies of circulating and splenic CD8 T cell in Tg animals and the overall slower rejection kinetics, it is possible that the delays in complete rejection were at least in part due to lower numbers of CD8 T cells infiltrating the skin transplants. To obtain more direct evidence of this, we monitored CD8 T cell infiltration by a whole-mount...
immunohistology procedure, which we have previously adapted for *Xenopus* (Ramanayake et al., 2007). Skin grafts were surgically removed 8 days post-transplantation and directly stained with anti-CD8 mAb without fixation. The number of cells stained by the anti-CD8 mAb was markedly reduced in skin tissue transplanted onto Tg recipients, when compared to those transplanted onto control animals (Fig. 7). These results are consistent with a diminished infiltration of CD8 T cells in Tg animals, although they do not exclude the possibility that CD8 T cells in Tg animals are also functionally impaired.

**Effect of b2m knockdown on viral immune immunity in F0 tadpoles**

Given the high fraction of non-mosaic transgene expression that can be obtained using the I-SceI meganuclease technique, we were interested to determine if F0 transgenic tadpoles could be used directly for *in vivo* KD experiments at late larval stages, which cannot usually be achieved by using morpholinos antisense oligomers. To determine if *in vivo* downregulation of b2m also leads to an immunological loss of function in naturally class Ia-deficient F0 tadpoles, we took advantage of the ranavirus FV3, a pathogen infecting numerous amphibian species including *Xenopus* (Chinchar et al., 2009). Although *Xenopus* tadpoles are more susceptible to FV3 when compared to adults, they do mount anti-FV3 immune responses and it takes several weeks for the majority of tadpoles to die from FV3 infection (De Jesús Andino et al., 2012; Gantress et al., 2003). Since outbred females produce as many as 200–400 eggs, which is 2 to 3 times more than LG clones, we used transgenic outbred larvae for these experiments.

In two different experiments using outbred animals of different genetic backgrounds, we generated F0 outbred transgenic larvae harboring either the anti-b2m shRNA or the scrambled shRNA. The resulting stage 45–50 (2–3 weeks of age) larvae were screened for GFP expression and only the GFP+ tadpoles were used for subsequent experiments. GFP+ tadpoles were then infected via waterborne exposure by adding $5 \times 10^6$ plaque forming units of FV3 in 2 ml of water for 1 hour before being placed into clean water for the remainder of the experiments. The results of the first experiment revealed that tadpoles with silenced expression of b2m were significantly more susceptible to FV3 infection in comparison to control non-transgenic larvae (Fig. 8A). Their median survival time was twofold less than the control animals (19.5 versus 38.5 days, $P<0.00008$; Table 3). In the second experiment, b2m KD animals died significantly faster than scrambled shRNA transgenic controls, which demonstrates that the defect is b2m specific (Fig. 8B; Table 3).

### Table 1. Percent of CD8$^+$ T cells and total CD8$^-$ (CD5$^+$) T cells in blood leukocytes of age-matched controls and transgenic LG-6 animals. Statistical significance was determined by a Student’s t-test.

|                  | % CD8$^+$ T cells | % CD8$^-$ T cells |
|------------------|-------------------|-------------------|
| Control LG-6 ($n=6$) | 13.5±2.6          | 10.4±2.9          |
| LG-6 Tg-b2m ($n=9$) | 12.3±1.4          | 6.0±1.4           |
| Student’s t-test  | $P<0.14$         | $P<0.0012$        |

**Fig. 5. Effects of b2m knockdown on allograft rejection.** OB and LG-6 anti-b2m shRNA transgenic and aged-matched controls adults were grafted with a MHC disparate J skin. At 8 days post-transplantation, pigment destruction is easily notable in control animals (*), whereas little rejection is visible in both transgenic recipients.
Thus, silencing of b2m leads to decreased host immune defenses and increased susceptibility to the FV3 virus. Notably, these data show that the I-SceI meganuclease technique can provide a convenient alternative to morpholinos antisense oligomers for loss of function studies at late larval stage onward without the need to generate and screen F1 progenies.

Discussion

The present study provides for the first time in ectothermic vertebrates direct in vivo evidence that the b2-m molecule is critical for an efficient class I-mediated CD8 T cell function. Additionally, our work establishes the feasibility of a reverse genetic approach, which combines RNA interference and transgenesis in Xenopus. Indeed, here we show that the I-SceI meganuclease transgenesis technique is ideally suited to generating transgenic isogenetic clones that are maintained by gynogenesis. Aside from a 100% transgene transmission, our data demonstrate a higher penetrance of RNA interference in F1 LG cloned progeny than in outbred progenies. Furthermore, RNA interference established by the I-SceI meganuclease-mediated transgenesis is applicable for experimentation on the F0

Table 2. Median skin graft survival in days ± s.d. of age-matched controls and transgenic outbred and LG-6 animals. Statistical significance to age-matched controls was determined by a Student’s t-test.

|         | OB   | LG-6 |
|---------|------|------|
| Controls: 16.0 ± 0.6 (n=6) | Controls: 19.5 ± 2.5 (n=6) |
| Tg-Scrambled: 17.0 ± 1.0 (n=3), P<0.2 | Tg-b2m: 23.0 ± 0.75 (n=6), P<0.003 |
| Tg-b2m: 22.4 ± 0.75 (n=6), P<0.002 |

Fig. 6. In vivo b2m silencing results in delayed MHC disparate skin graft rejection. (A) Adult anti-b2m shRNA transgenic LG-6 clones (LG-6 Tg) and control naïve LG-6 animals (LG-6 C) grafted with a MHC disparate J skin. Skin graft rejection was monitored daily at 21˚C until complete rejection. (B) Adult outbred frogs carrying either anti-b2m shRNA (OB β2-m shRNA) or scram shRNA (OB scram shRNA) transgenes were grafted with a MHC disparate J skin. Skin graft rejection was monitored at 21˚C until complete rejection. Each group consisted of 3 animals and the graphs show the average skin graft rejection of the group. P values were determined using the Student’s t-test.

Fig. 7. Decreased CD8 T cell infiltration in skin grafts from transgenic OB frogs with silenced β2-m. Transgenic outbred anti-b2m shRNA adults (B,D) or aged-matched control OB frogs (A,C) were grafted with a MHC disparate J skin. Eight days later grafts were harvested and were stained without fixation with a Xenopus specific anti-CD8 antibody (AM22). Scale bar: 20 µm.

Fig. 8. Transgenic OB tadpoles carrying anti-b2m shRNA are more susceptible to FV3 infections. (A) F0 transgenic tadpoles (stage 56, GFP+) carrying either a shRNA targeting b2m (Tg, 23 larvae) or WT (Control, 10 larvae) were infected with 5 × 10⁶ PFU FV3 via the water route for 1 hour. (B) F0 transgenic tadpoles (stage 56, GFP+) carrying either a shRNA targeting b2m (Tg β2-m shRNA, 36 larvae) or a scrambled shRNA (Tg scram shRNA, 22 larvae) were infected with 5 × 10⁶ PFU FV3 via the water route for 1 hour. Survival was monitored daily. P values were determined using the Student’s t-test.
Table 3. Median survival in days ± s.d. following FV3 infection of transgenic (GFP+) tadpoles at stage 49–50 compared to dejellied controls (Exp. 1) or Tg injected with a scrambled shRNA construct controls (Exp. 2). Statistical significance was determined by a Student’s t-test.

| Exp. 1 | Exp. 2 |
|--------|--------|
| Controls: 38.5 (n=25) | Tg Scrambled: 28.0 (n=30) |
| Tg-b2m: 19.5 (n=25) | Tg-b2m: 13.5 (n=30) |
| P<8×10^{-3} | P<0.002 |

above observations are corroborated by our findings using the transgenic frogs described here. This suggests that animals with downregulated b2m have ineffective CD8 responses, while the complete graft rejection may actually be mediated by other cell effectors such as CD4 T cells. As we generate more adult transgenic animals, it will be interesting to further assess the cytotoxic function of transgenic CD8 T cells since the Xenopus CD8 T cell response against skin antigens has been shown to be class Ia restricted (Robert et al., 2002).

Although we successfully demonstrated both cell surface and functional class Ia-mediated impairment in transgenic animals carrying the anti-b2m shRNA, we do not know the effects of the KD on class Ib responses in these animals. Notably, our data revealed that early developmental stage tadpoles with silenced b2m gene expression were more susceptible to FV3 infection in comparison to both scrambled shRNA transgenic as well as WT OB age matched larvae. As previously mentioned, Xenopus larvae are naturally class Ia deficient until metamorphosis; however, they do express several class Ib genes. Since there are no class Ia molecule to mediate anti-viral immune responses at early larval stages, this suggests that class Ib molecules may be involved. Perhaps the central and most enigmatic topic of Xenopus immunology, currently warranting elucidation are the specifics of the biological mechanisms governing Xenopus larval immunocompetence and their possession of functional CD8 T cells despite the absence of functional class Ia. Our findings, presented here, are amongst the first to lend insight into these complex larval immune regulation pathways and highlight the possible roles of class Ib gene products as the key tadpole elements for regulating immune surveillance. Further work, utilizing transgenic technology such as that described here, will be instrumental to gaining insight into these processes in Xenopus immune ontogeny and bridging the gap in our understanding of vertebrate immune development and organization.

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Competing Interests

The authors have no competing interests to declare.

References

Apasov, S. G. and Sitkovsky, M. V. (1994). Development and antigen specificity of CD8+ cytotoxic T lymphocytes in beta 2-microglobulin-negative, MHC class I-deficient mice in response to immunization with tumor cells. *J. Immunol.* 152, 2087-2097.

Barlow, E. H. and Cohen, N. (1983). The thymus dependency of transplantation allotolerance in the metamorphosing frog Xenopus laevis. *Transplantation* 35, 612-619.

Bechtold, T. E., Smith, P. B. and Turpen, J. B. (1992). Differential stem cell contributions to thymocyte succession during development of Xenopus laevis. *J. Immunol.* 148, 2975-2982.

Chen, G., Ward, B. M., Yu, K. H., Chinchar, V. G. and Robert, J. (2011). Improved knockout methodology reveals that frog virus 3 mutants lacking either the 18K immediate-early gene or the truncated vEF-2a gene are defective for replication and growth in vivo. *J. Virol.* 85, 11131-11138.

Chenoua, A., Sachs, I. M., Chai, N., Chen, Y., Du Pasquier, L., Loeber, J., Pollet, N., Relly, M., Weekes, D. L. and Bronchais, O. J. (2008). Transgenesis procedures in Xenopus. *Biol. Cell* 100, 503-529.

Chinchar, V. G., Hyatt, A., Miyazaki, T. and Williams, T. (2009). Family *Iridoviridae*: poor viral relations no longer. *Curr. Top. Microbiol. Immunol.* 328, 123-170.
Cook, J. R., Solheim, J. C., Connolly, J. M. and Hansen, T. H. (1995). Induction of peptide-specific CD8+ CTL clones in beta 2-microglobulin-deficient mice. J. Immunol. 154, 47-57.

Das, G. and Janeway, C. A., Jr. (1999). Development of CD8α/α and CD8ββ T cells in major histocompatibility complex class I-deficient mice. J. Exp. Med. 190, 881-884.

De Jesús Andino, F., Chen, G., Li, Z., Grayfer, L. and Robert, J. (2012). Susceptibility of Xenopus laevis tadpoles to infection by the ranavirus Frog-Virus 3 correlates with a reduced and delayed innate immune response in comparison with adult frogs. Virology 432, 435-443.

Du Pasquier, L. and Weiss, N. (1973). The thymus during the ontogeny of the toad Xenopus laevis: growth, membrane-bound immunoglobulins and mixed lymphocyte reaction. Eur. J. Immunol. 3, 773-777.

Du Pasquier, L., Chardonnens, X. and Miggiano, V. C. (1995). Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. Immunity 15, 351-362.

Flajnik, M. F., Canel, C., Kramer, J. and Kasahara, M. (2001). Identification of class I major histocompatibility complex encoded molecules in the amphibian Xenopus. Immunogenetics 20, 433-442.

Flajnik, M. F., Canel, C., Kramer, J. and Kasahara, M. (1991a). Evolution of the major histocompatibility complex: molecular cloning of major histocompatibility complex class I from the amphibian Xenopus. Proc. Natl. Acad. Sci. USA 88, 537-541.

Flajnik, M. F., Taylor, E., Canel, C., Grossberger, D. and Du Pasquier, L. (1991b). Reagents specific for MHC class I antigens of Xenopus. Amer. Zool. 31, 580-591.

Flajnik, M. F., Kasahara, M., Shum, B. P., Salter-Cid, L., Taylor, E. and Du Pasquier, L. (1993). A novel type of class I gene organization in vertebrates: a large family of non-MHC-linked class I genes is expressed at the RNA level in the amphibian Xenopus. EMBO J. 12, 4385-4396.

Flajnik, M. F., Ohta, Y., Greenberg, A. S., Salter-Cid, L., Carrizosa, A., Du Pasquier, L. and Kasahara, M. (1999). Two ancient allelic lineages at the single classical class I locus in the Xenopus MHC. Immunity 13, 3826-3833.

Freland, S. and Ljunggren, H. G. (2000). β2-microglobulin/CD8−/− mice reveal significant role for CD8+ T cells in graft rejection responses in β2-microglobulin−/− mice. Scand. J. Immunol. 51, 219-223.

Gantress, J., Maniero, G. D., Cohen, N. and Robert, J. (2003). Development and characterization of a model system to study amphibian immune responses to iridoviruses. Virology 311, 254-262.

Gleimer, M. and Parham, P. (2003). Stress management: MHC class I and class I-like molecules as reporters of cellular stress. Immunity 19, 469-477.

Goyos, A., Guselnikov, S., Chida, A. S., Sniderhan, L. F., Maggirwar, S. B., Nedelkovska, H. and Robert, J. (2007). Involvement of nonclassical MHC class Ib molecules in heat shock protein-mediated anti-tumor responses. Eur. J. Immunol. 37, 1494-1501.

Goyos, A., Sowa, J., Ohta, Y. and Robert, J. (2011). Remarkable conservation of distinct nonclassical MHC class I lineages in divergent amphibian species. J. Immunol. 186, 372-381.

Hofstetter, A. R., Sullivan, L. C., Lukacher, A. E. and Brooks, A. G. (2011). Diverse roles of non-diverse molecules: MHC class Ib molecules in host defense and control of autoimmune. Curr. Opin. Immunol. 23, 104-110.

Kau, C. L. and Turpen, J. B. (1983). Dual contribution of embryonic ventral blood island and dorsal lateral plate mesoderm during ontogeny of hemopoietic cells in Xenopus laevis. J. Immunol. 131, 2262-2266.

Kobel, H. R. and Du Pasquier, L. (1975). Production of large clones of histocompatible, fully identical clawed toads (Xenopus). Immunogenetics 2, 87-91.

Koller, B. H., Marrack, P., Kappler, J. W. and Smithies, O. (1990). Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. Science 248, 1227-1230.

Lamoussé-Smith, E., Clements, V. K. and Ostrand-Rosenberg, S. (1993). Beta 2M−/− knockout mice contain low levels of CD8+ T lymphocytes that mediate specific tumor rejection. J. Immunol. 151, 6283-6290.

Nedelkovska, H., Cruz-Luna, T., McPherson, P. and Robert, J. (2010). Comparative in vivo study of gp96 adjuvanticity in the frog Xenopus laevis. J. Vis. Exp. 43, 2026.

Nedelkovska, H. and Robert, J. (2012). Optimized transgenesis in Xenopus laevis/galli isogenic clones for immunological studies. Genesis 50, 300-306.

Ramanayake, T., Simon, D. A., Frelinger, J. G., Lord, E. M. and Robert, J. (2007). In vivo study of T-cell responses to skin allografts in Xenopus using a novel whole-mount immunohistology method. Transplantation 83, 159-166.

Rau, L., Cohen, N. and Robert, J. (2003). MHC-restricted and -unrestricted CD8 T cells: an evolutionary perspective. Transplantation 72, 1830-1835.

Robert, J. and Ohta, Y. (2009). Comparative and developmental study of the immune system in Xenopus. Dev. Dyn. 238, 1249-1270.

Robert, J., Gantress, J., Rau, L., Bell, A. and Cohen, N. (2002). Minor histocompatibility antigen-specific MHC-restricted CD8 T cell responses elicited by heat shock proteins. J. Immunol. 168, 1697-1703.

Shum, B. F., Avila, D., Du Pasquier, L., Kasahara, M. and Flajnik, M. F. (1993). Isolation of a classical MHC class I cDNA from an amphibian. Evidence for only one class I locus in the Xenopus MHC. J. Immunol. 151, 5376-5386.

Solheim, J. C. (1999). Class 1 MHC molecules: assembly and antigen presentation. Immunol. Rev. 172, 11-19.

Ulbrecht, M., Couturier, A., Martinozzi, S., Pla, M., Srivastava, R., Peterson, P. A. and Weiss, E. H. (1999). Cell surface expression of HLA-E: interaction with human β2-microglobulin and allelic differences. Eur. J. Immunol. 29, 537-547.

Vugmeyster, Y., Glas, R., Péirano, B., Lemonnier, F. A., Eisen, H. and Ploegh, H. (1998). Major histocompatibility complex (MHC) class I Δ15Tβ−/− deficient mice possess functional CD8+ T cells and natural killer cells. Proc. Natl. Acad. Sci. USA 95, 12492-12497.

Zijlstra, M., Bix, M., Sinister, N. E., Loring, J. M., Raulet, D. H. and Jaenisch, R. (1990). β2-Microglobulin deficient mice lack CD4+ 8+ cytolytic T cells. Nature 344, 742-746.
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Fig. S1. Amino acid alignment of MHC class Ia alpha 1 and alpha 2 regions from four different OB Tg animals that displayed reduced anti-MHC class Ia surface staining, and one OB scram control. Residues identical to the X. laevis a/c-1 haplotype (GenBank accession number: AF185583) sequence are shown as (.). The different haplotypes are indicated in the sequence name. The mAb TB17 epitope (GRDEPATQWMKQKEGPEY) is boxed and the invariant proline (P47) essential for antibody binding is in bold (Flajnik et al., 1999).

Fig. S2. Comparison of Class Ia, CD5 and CD8 surface expression by transgenic and control LG-6 frogs. Thymocytes from two transgenic LG-6 frogs with silenced β2-m expression and from an age-matched LG-6 control were stained with the anti-class I mAb TB17 and APC-conjugated goat anti-mouse Ab and analyzed by two color flow cytometry for GFP and class I expression (upper panel). Thymocytes were also stained with anti-CD5 (2B1), biotinylated anti-CD8 (AM22) mAbs, and APC-conjugated goat anti-mouse Ab and PE-conjugated-streptavidin and analyzed by flow cytometry (lower panel). In each case 10,000 events were collected and gated on live lymphocytes. Analysis was performed using the FlowJo software.