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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 isogenetic *laevis/gilli* cloned lines with stably silenced expression of β2-microglobulin (*b2m*) critical for class I function. Transgenic F_1 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, *b2m* knockdown increased susceptibility to viral infection of F_0 transgenic larvae. This loss of function strategy offers new avenues for studying ontogeny of immunity and other developmental processes in *Xenopus*.

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 intereference with I-SceI meganuclease-mediated transgenesis. We have recently adapted this method to isogenetic *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 larval 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 molecules, we obtained stable and effective silencing of *b2m* that impaired class Ib surface expression and resulted in increased tumorigenicity of this tumor *in vivo* (Goyos et al., 2007). To expand on these findings, we have adopted this RNAi approach, now at the organism level, by transgenesis. Here, we report the successful knockdown (KD) of *b2m* both in F_0 and F_1 progenies of *Xenopus* by transgenesis. Our results indicate that *b2m* KD results in impairment of class Ia surface expression and CD8 T cell function in adult *Xenopus*, as well as decreased antiviral immune defense in naturally class Ia-deficient, but class Ib-expressing tadpoles.

Materials and Methods

Animals

Outbred (OB) *Xenopus laevis* as well as LG-6 and LG-15 animals were obtained from our *Xenopus laevis* Research Resource for Immunology at the University of Rochester (http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm). All animals were handled under strict laboratory and UCAR regulations (Approval number 100577/2003-151), minimizing discomfort at all times.

Plasmid construction

The double expression cassette vector (I-SceI-*b2m*shRNA-GFP) was constructed by cloning a 2.2 Kb GFP reporter cassette into the SacI and *Pst*I sites of the I-SceIpBSIISk+ (provided by Dr Grainger, 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 XhoI sites of the pBS-hU6-1 vector (Goyos et al., 2007). The resulting 400 bp shRNA cassette consisting of the hU6 Pol III promoter and the shRNA was subsequently cloned into the *Eco*RI and *Kpn*I 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 $CaCl_2$) and for gynogenesis 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 pH 8.0 (made in 0.1× MBS), washed with 0.1× MBS (13℃) several times, and placed into injection medium (4% Ficoll 400 in 0.3× MBS, 13℃). 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℃ and 80 pg DNA along with 1×10^{-3} 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℃ at all times (storage at −20℃ 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℃ for 4 hours in order to delay cell division providing extended time for transgene integration. The embryos were then transferred in 0.3× MBS with 50 µg/ml gentamycin and reared at 18℃ 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 screened positive were raised to adults and bred to produce F_1 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 performed using the 2X Taq Master Mix (Genescript Crop.). 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 reagents and primers used. The following specific primers were used: GFP (F: 5′-ACGGCCACAAGTTCAGCGTG-3′, R: 5′-GTCCATGCCGAG-AGTGATCC-3′, Tm: 64℃); b2-m (F: 5′-CCGGTGGTCAAGGTTTACACTG-3′, R: 5′-TAGAGATCAGTGATTGGATGA-3′, Tm: 58℃); MHC class 1a (F: 5′-GGCAGTCACTCCCTGCGCTATTAT-3′, R: 5′-CTGGTTGAAGCGATCCATT-GC-3′, Tm: 61℃) and GAPDH (F: 5′-ACCCCTTCATCGACTTGGAC-3′, R: 5′-GGAGCCAGACAGTTTGTAGTG-3′, Tm: 55℃).

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 1×10^6 cells in 500 µl of staining buffer (APBS+1% BSA+0.01% NaN_3).

Flow cytometry

Blood cells or single cell suspensions harvested from spleen and thymus were stained at a cell density of 1×10^6/ml in APBS containing 1% BSA and 0.01% NaN_3 with 0.5 mg/ml of *Xenopus*-specific biotinylated anti-CD8 (AM22), or undiluted hybridoma supernatants anti-CD5 (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 FACSCantoII (BD Biosciences). 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 irridophore pigmented cells, 20 mm×5 mm) was removed from anesthetized J donor frogs, cut in smaller pieces (5 mm×5 mm) and placed on ice in a Petri dish containing sterile APBS. A small incision was made on the dorsal (back) skin of anesthetized recipient frogs, and a graft was inserted under the recipient skin with the silvery side up. Twenty four hours later the overlaying host skin was removed so that the graft could be freely visualized. Scoring of the graft started immediately. Skin graft rejection was determined by the percent destruction of the 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) cell lines incubated at 30℃ 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 5×10^6 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′-ACGAGCCCGACGAAGACTACATAG-3′, R: 5′-TGGTGGTCCTCAGCATCC-TTTG-3′, Tm: 56℃).

Results

Effects of *b2m* silencing on transgenic F_0 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-*b2m*shRNA-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-*b2m*shRNA-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 froglets.

b2m loss of function in F_1 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). In order to assess the transmission of the shRNA-mediated KD of *b2m* and the resulting effects on immune function we raised F_1 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 F_1 transgenic progeny were produced from male and female transgenic F_0 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 F_1 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

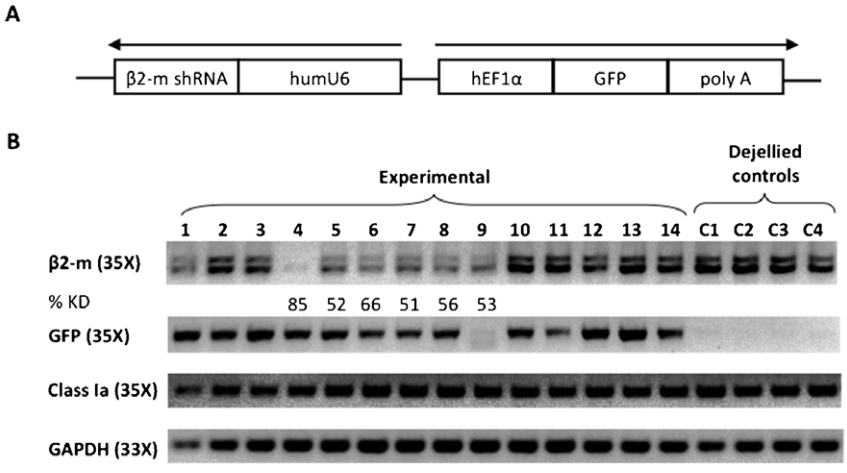

Fig. 1. *In vivo b2m* silencing in transgenic F_0 *Xenopus*. (**A**) A map of the I-SceI- anti-*b2m*siRNA-GFP construct used to generate transgenic LG clones. (**B**) LG eggs were injected (**Experimental**) with 1×10^{-3} I-SceI units and 80 pg of I-SceI-*b2m*siRNA-GFP in a total volume of 10 nl per 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.

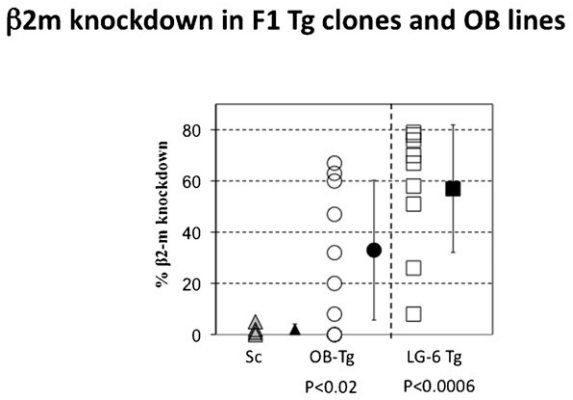

Fig. 2. Effective *b2m* silencing *in vivo* in transgenic F_1 OB and LG *Xenopus* clones. RT-PCR was performed on RNA from the skin of young adults (2 months of age) OB F_1 transgenic (GFP^+) carrying either the anti-*b2m* shRNA (OB-Tg, 9 individuals), the scram shRNA transgene (Sc, 6 individuals) and RNA from skin of LG-6 anti-*b2m* shRNA transgenic (LG6-Tg, 9 individuals). RT-PCR analysis was performed using primers specific for *Xenopus* GAPDH and *b2m*. −RT controls for all samples were negative. The percent of *b2m* KD was determined by densitometry after normalization to GAPDH by comparing *b2m* expression of each transgenic to the average expression in the respective WT controls. Average ± s.d. of *b2m* is also indicated by filled symbols for each group of transgenic. Statistical significance between controls and transgenic animals was determined with a *t*-test.

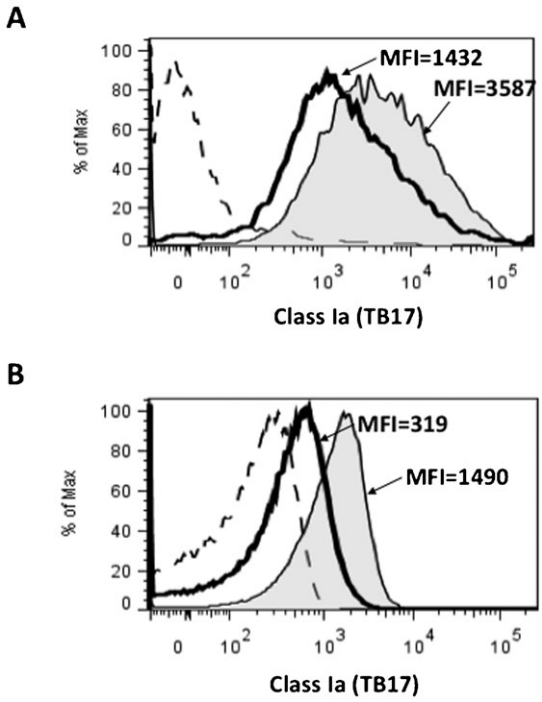

Fig. 3. *Xenopus* LG and OB F_1 progeny have downregulated cell surface expression of MHC class Ia molecules. F_1 transgenic progeny were reared to adulthood and screened for GFP fluorescence. Frogs were bled and the blood (including both red blood cells and lymphocytes) was analyzed by flow cytometry for surface MHC class Ia using the *Xenopus*-specific anti-class Ia mAb TB17. 50,000 events were collected, gated on live cells (includes both red blood cells and lymphocytes) and analyzed using the FlowJo software. (**A**) LG transgenic F_1 frog carrying the β2-m shRNA (thick black histogram) and LG age matched control (gray tinted histogram) are shown. (**B**) OB F_1 transgenic anti-*b2m* shRNA (thick black histogram) and a control F_1 transgenic scram shRNA frog (gray tinted histogram) are shown. Secondary antibody controls are shown as dashed histograms. The median fluorescence intensities (MFI) of both the transgenic and control frog are indicated. One representative individual of each group 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 *b2m* 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 *b2m* 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 *b2m* 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 *b2m* KD but rather only CD8 T cells.

To evaluate the effect of *b2m* 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, $P \leq 0.0009$; 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 *b2m* KD transgenic frogs. These data strongly supported the LG-6 findings. The outbred transgenic animals carrying the *b2m* shRNA fully rejected the MHC disparate grafts in 32±3 days, in contrast to the scrambled shRNA transgenic outbred controls (20±1.5, $P \leq 0.02$; 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

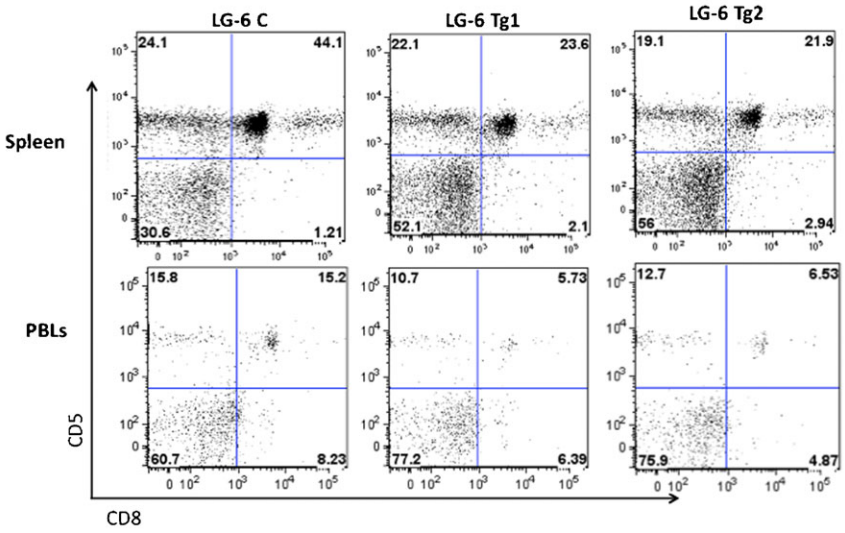

Fig. 4. LG-6 clones with silenced *b2m* expression have reduced frequency of circulating CD5/CD8 T cells in the spleen and the blood. Spleen and blood were harvested from two transgenic LG-6 froglets with silenced *b2m* expression as well as an age matched LG-6 naïve animal. Cells were stained for CD5 and CD8 and were analyzed by flow cytometry. 10,000 events were collected and were gated on live leukocytes. Analysis was performed using the FlowJo software. Shown are dot plots of CD5 and CD8 expression with % of each population labeled.

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 recipient, 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 F_0 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 F_0 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 F_0 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 F_0 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 5×10^6 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	$P<0.14$	$P<0.0012$

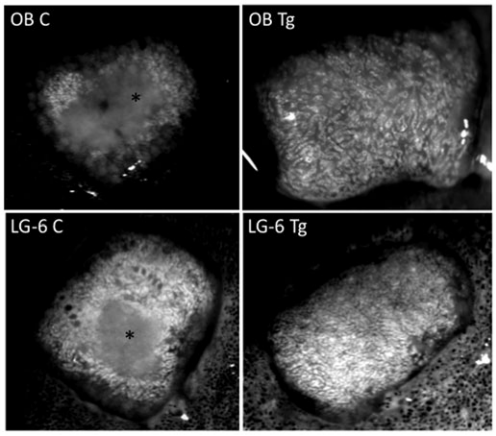

Fig. 5. Effects of *b2m* knockdown on allograft rejection. OB and LG-6 anti-*b2m* shRNA transgenic and aged-matched controls adults were grafted with a MHC disparate J skin. At 8 days post-transplantation, pigment destruction is easily notable in control animals (*), whereas little rejection is visible in both transgenic recipients.

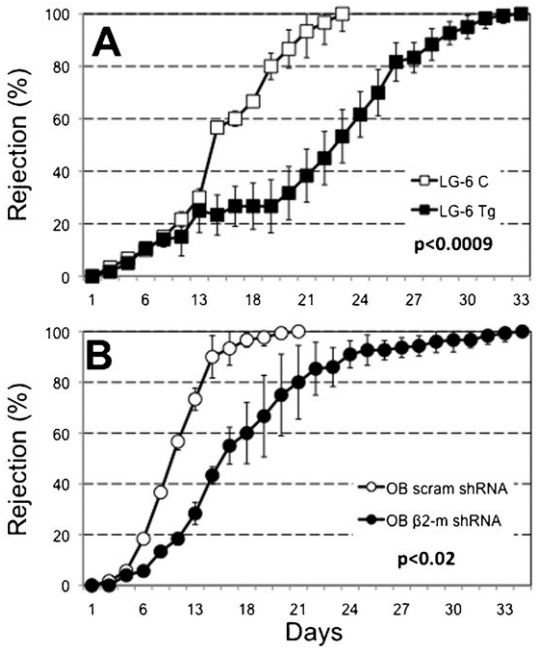

Fig. 6. *In vivo b2m* silencing results in delayed MHC disparate skin graft rejection. (**A**) Adult anti-*b2m* shRNA transgenic LG-6 clones (LG-6 Tg) and control naïve LG-6 animals (LG-6 C) grafted with a MHC disparate J skin. Skin graft rejection was monitored daily at 21℃ until complete rejection. (**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℃ 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.

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 F_1 progenies.

Discussion

The present study provides for the first time in ectothermic vertebrates direct *in vivo* evidence that the β2-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 F_1 LG cloned progeny than in outbred progenies. Furthermore, RNA interference established by the I-SceI meganuclease-mediated transgenesis is applicable for experimentation on the F_0

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	

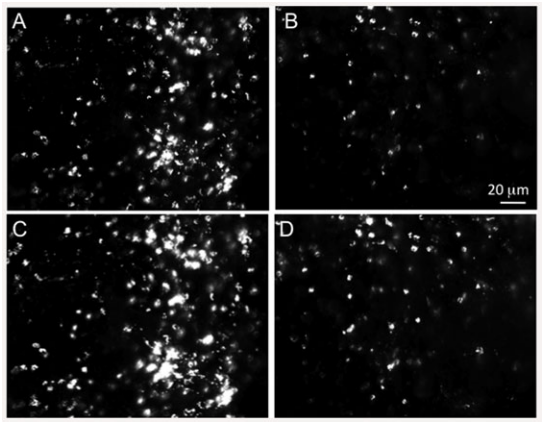

Fig. 7. Decreased CD8 T cell infiltration in skin grafts from transgenic OB frogs with silenced β2-m. Transgenic outbred anti-*b2m* shRNA adults (**B**,**D**) or aged-matched control OB frogs (**A**,**C**) were grafted with a MHC disparate J skin. Eight days later grafts were harvested and were stained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

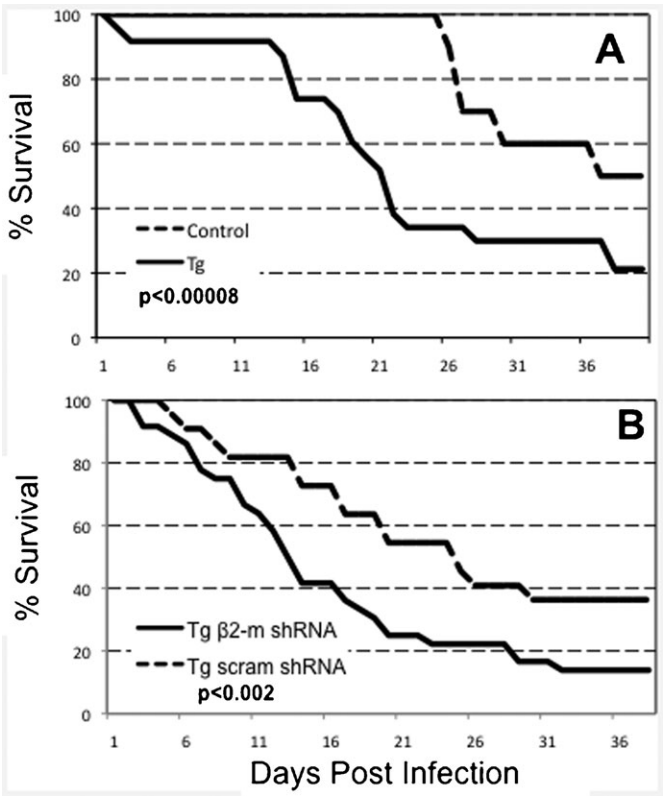

Fig. 8. Transgenic OB tadpoles carrying anti-*b2m* shRNA are more susceptible to FV3 infections. (**A**) F_0 transgenic tadpoles (stage 56, GFP^+) carrying either a shRNA targeting *b2m* (Tg, 23 larvae) or WT (Control, 10 larvae) were infected with 5×10^6 PFU FV3 via the water route for 1 hour. (**B**) F_0 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 5×10^6 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)
$P<8\times10^{-5}$	$P<0.002$

generation. This feature could provide an appreciable advantage for loss of function studies at late larval stages or even adult stages that cannot be easily achieved with morpholinos. The use of a fluorescence gene reporter such as GFP in the construction containing the shRNA provides a convenient and rapid way to screen transgenic animals. Although the correlation between GFP expression and the potency of *b2m* KD was not as robust in F_0 as in F_1, we were able to obtain significant and reproducible effects on the immune function as shown by the increased susceptibility of Tg tadpoles to viral infection. Together, these techniques represent a powerful and reliable loss of function approach for the investigation of developmental processes and immune regulations during late larval, metamorphic and adult stages.

The original report of homozygous mice with a *b2m* gene disruption ($b2m^{-/-}$ mice) demonstrated that these animals expressed very low to no class Ia molecules and were deficient for CD8 T cells (Zijlstra et al., 1990). Furthermore, these animals exhibited defects in CD8 T cell mediated cytotoxicity. However, later studies showed that $b2m^{-/-}$ mice do possess a population of Ag-specific CD8 T cells that are highly functional, but may have delayed responses (Apasov and Sitkovsky, 1994; Cook et al., 1995; Freland and Ljunggren, 2000; Lamousé-Smith et al., 1993). The results of the present study pertaining to transgenic adult *Xenopus* with *b2m* KD are consistent with the murine data and provide further evidence of the degree of conservation of the immune system and immune function between mammals and amphibians.

Interestingly, even though the observed *b2m* KD was incomplete, it resulted in substantial decreases of class Ia expression at the surface of blood leukocytes. With the tools currently available in *Xenopus* (e.g. Abs, *in vitro* assays), it is difficult to determine the respective impact of lower surface class Ia expression in the differentiation of CD8 T cells in the thymus and their activation in periphery by antigen presenting cells. Although we did not observe any major defects in thymocyte development in either tadpoles or adult frogs, the lower frequency of CD8 T cells in the spleen and the blood of adults suggest a less efficient thymic education for CD8 T cells when *b2m* is downregulated. Importantly, the partial downregulation of *b2m*, in both outbred and LG cloned transgenics was sufficient to significantly impair CD8 T cell functions as evidenced by a delayed MHC-mismatched skin graft rejections and decreased CD8 T cell infiltration in the allografts. Previous, thymectomy experiments have shown that skin allograft rejection is strictly T cell-mediated in *Xenopus* (Barlow and Cohen, 1983; Du Pasquier et al., 1975). In addition, CD8 T cell depletion by mAb treatment significantly delays allograft rejection by 6–7 days, and CD8 T cell infiltration in the allograft tightly correlates with the kinetics of its rejection (Ramanayake et al., 2007; Rau et al., 2001). The 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.

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Supplementary Material

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                          < Alpha 1 domain
a/c-1                     GSHSLRYYYTGVSDRAFGLPEFSIVGYVDDTQFVRYSSDNGRAEPATQWMKEKEGPEYWR
OB scram       a/c-1      ............................................................
Tg:2003-151A   a/c-1      ............................................................

Tg:2003-151-1 j/j         ..............T.....C.......EA.I.......QKF........Q.......E
Tg:2003-151-2 b/d-2       ......................SF.....I.IDK....I..T...A.....NV.....E

Tg:2003-151B-1 j/j        ..............T.....C.......EA.I.......QKF........Q.......E
Tg:2003-151B-2 b/d-2      ......................SF.....I.IDK....I..T...A.....NV.....E

Tg#3-1 j/j                ..............T.....C.......EA.I.......QKF........Q.......E
Tg#3-2 b/d-2              ......................SF.....I.IDK....I..T...A.....NV.....E


                                                  < Alpha 2 domain
a/c-1                     GQTQGLKAHEPVFKHNVKTVMDRFNQTSGTHMVQYMYGCELGDDGSIRGYEQYGYDGREF
OB scram       a/c-1      ........Q...................................................
Tg:2003-151A a/c-1        ........Q...................................................

Tg:2003-151-1 j/j         RE..KA.GE.AW......VA......S....SL.M.C....RE.N.....N......K..
Tg:2003-151-2 b/d-2       RE..SR.GS.LVY......A......T....SF.R......RE.N.....N.........

Tg:2003-151B-1 j/j        RE..KA.GE.AW......VA......S....SL.M.C.............N......K..
Tg:2003-151B-2 b/d-2      RE..SR.GS.L.Y......A...........SF.R..............N.........

Tg#3-1 j/j                RE..KA.GE.AW......VA......S....SL...C.............N......K..
Tg#3-2 b/d-2              RE..SR.GS.L.Y......A...........SF.R..............N.........


a/c-1                     FALDTEGWVYVPTEREAQLTTQKWNSPEVNKPERYKNYLQNICIEGLKRYLSYGQAELEK
OB scram       a/c-1      ...............................V............................R
Tg:2003-151A   a/c-1      ...............................V............................R

Tg:2003-151-1 j/j         I.....RS.....V......E.............D..........W..K..........R
Tg:2003-151-2 b/d-2       I..G..R....HSV......E.........L...N..........W..K..........R

Tg:2003-151B-1 j/j        I.....RS.....V......E.............D..........W..K..........R
Tg:2003-151B-2 b/d-2      I.....R....HSV....LAE.........L...N..........W..K..........R

Tg#3-1 j/j                I.....RS...P.V......E.............D..........W..K..........R
Tg#3-2 b/d-2              I.....R....HSV......E.........L...N..........W..K..........R
```

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).

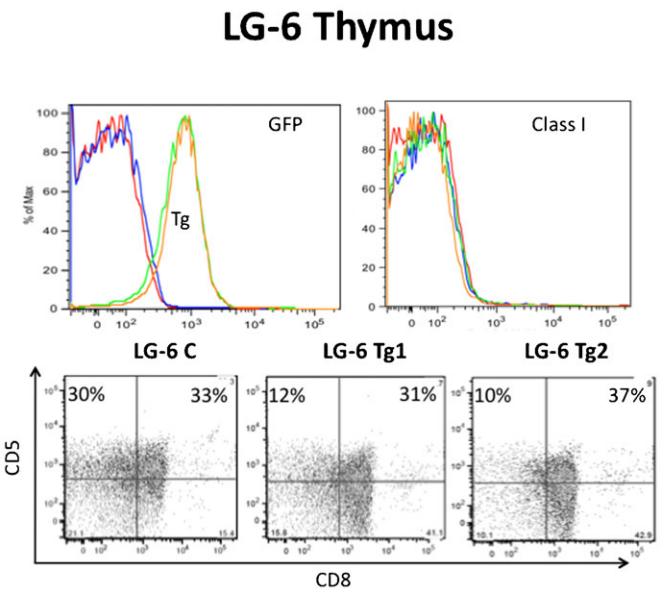

Fig. S2. Comparison of Class Ia, CD5 and CD8 surface expression by transgenic and control LG-6 frogs. Thymocytes from two transgenic LG-6 froglets with silenced β2-m expression and from an age-matched LG-6 control were stained with the anti-class I mAb TB17 and APC-conjugated goat anti-mouse Ab and analyzed by two color flow cytometery 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.