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Video Article

Comparative *in vivo* Study of gp96 Adjuvanticity in the Frog *Xenopus laevis*

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Abstract

We have developed in the amphibian *Xenopus laevis* a unique non-mammalian model to study the ability of certain heat shock proteins (hsps) such as gp96 to facilitate cross-presentation of chaperoned antigens and elicit innate and adaptive T cell responses. *Xenopus* skin graft rejection provides an excellent platform to study the ability of gp96 to elicit classical MHC class Ia (class Ia) restricted T cell responses. Additionally, the *Xenopus* model system also provides an attractive alternative to mice for exploring the ability of gp96 to generate responses against tumors that have down-regulated their class Ia molecules thereby escaping immune surveillance. Recently, we have developed an adoptive cell transfer assay in *Xenopus* clones using peritoneal leukocytes as antigen presenting cells (APCs), and shown that gp96 can prime CD8 T cell responses *in vivo* against minor histocompatibility skin antigens as well as against the *Xenopus* thymic tumor 15/0 that does not express class Ia molecules. We describe here the methodology involved to perform these assays including the elicitation, pulsing and adoptive transfer of peritoneal leukocytes, as well as the skin graft and tumor transplantation assays. Additionally we are also describing the harvesting and separation of peripheral blood leukocytes used for flow cytometry and proliferation assays which allow for further characterization of the effector populations involved in skin rejection and anti-tumor responses.

Protocol

1. Animals

X. laevis x *X. gilli* hybrids LG-6 and LG-15 isogenetic clones [1] are from our breeding colony at the University of Rochester (http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm). LG-6 and LG-15 share the same heterozygous MHC haplotype (a/c) but differ at minor histocompatibility (H) loci. Progeny from these clones are produced by gynogenesis, in which diploid eggs produced by the female are activated by UV-irradiated sperm (no DNA contribution to the progeny).

The use of gloves is facultative. Some people prefer to not wear them because it is more difficult to handle the frogs (slippery) and in fact it appears to make the frogs uncomfortable.

2. Purification of gp96 from 15/0 Tumor (Expresses Both Tumor and Minor H-Ags)

Gp96 purification has been previously described [2, 3]. Briefly, gp96 is purified by 50-70% ammonium sulfate fractionation, followed by conA-sepharose and DEAE chromatography. About 20-50 µg of protein can be obtained per 1 mL of tumor tissue. Purity of the preparation is determined by SDS-PAGE and sliver staining.

3. Elicitation and Harvest of Peritoneal Leukocytes (PLs) from Minor H-Ag-disparate LG-6 Frogs

1. Grow a 25 mL overnight *E. Coli* culture in a 50 mL conical tube at 37°C with shaking.
2. The next day heat kill the bacteria by boiling it for 1 hour.
3. Centrifuge the heat killed *E. Coli* for 15 min at 2,000 rpm (1,500 g) at 4°C.
4. Remove the supernatant and resuspend the bacterial pellet in 1/10th the original culture volume (2.5 mL) in APBS. At this point the bacterial culture is ready for use and must be used within 24 hrs.
5. Inject intraperitoneally (i.p.) 200 (for a 2 inch frog) or 300 µL (for a 3 inch frog) of the heat-killed bacterial preparation per frog using a 25 gauge 5/8 needle.
6. Three days after injection PLs are harvested by intraperitoneal lavage.
7. Before PL harvesting, adult frogs are anesthetized by immersion in a 0.1% aqueous solution of tricaine methane sulfonate (TMS, MS-222) buffered with sodium bicarbonate for up to 5 min until all movement ceases (duration depends on size and age). Animals wake within 10-20 min after treatment.
8. Disinfect the abdomen of the frog with a small amount of 70% ethanol.
9. Inject 5 mL (for a 2 inch frog) or 10 mL (for a 3 inch frog) of sterile APBS pre-warmed at room temperature into the peritoneal cavity using a 18 gauge 1 ½ needle. Remove the needle and gently massage the frog for a minute to insure that the injected buffer equilibrates with the fluid in the body cavity.
10. Use a new 18 gauge 1 ½ needle without a syringe to collect the peritoneal fluid that will drip from the back of the needle into a clean 50 mL conical tube. Make sure to retrieve as much of the initial injected volume as possible. Be careful to avoid blood vessels in the central area of the abdominal region.
11. Once PLs are harvested put the frog in a container with shallow water until it is awake at which point it can be placed back in its cage.

4. Pulsing and Adoptive Transfer of Lg-6 PIs Into Naíve Lg-6 Recipients

1. Wash the PLs once with cold APBS and centrifuge them at 1,000 rpm (750 g) for 10 min at 4°C.
2. Remove the supernatant and resuspend the PLs in APBS.
3. Pulse the PLs with gp96 at a concentration of 1 µg gp96 per 5 x 10^5 PLs.
4. Once the appropriate amount of gp96 is added to the PLs, mix them by pipeting and incubate on ice for 1 hour.
5. Centrifuge the cells either at 1,000 rpm for 10 min at 4°C or at 14,000 rpm for 1 min to remove any unbound gp96.
6. Wash the PLs 3X with cold APBS to ensure there is no residual gp96 left.
7. Resuspend the pulsed PLs at a concentration of 5 x 10^5 PLs per 300 µL.
8. Adoptively transfer PLs by i.p. injection using 25 gauge 5/8 needle. Inject 300 µL of pulsed PLs (5 x 10^5 cells) per animal.
9. Frogs need to be primed at least 3 days before continuing with skin graft or tumor challenge experiments.

5. Skin Graft Assay

Skin graft rejection is a well-established assay in *Xenopus* [4, 5, 6]. In *Xenopus*, a recipient rejects donor skin displaying 1 or 2 MHC haplotype mismatches within 18-22 days at 21-22°C. In contrast, skin grafts between LG-6 and LG-15 clones that differ only by minor H-Ags are rejected more slowly (more that 30 days). However, this rejection is accelerated in recipients that have been primed against donor minor H-Ags either by a previous skin graft or by immunization with gp96 purified from the donor. No rejection at all occurs when the donor and the recipient are genetically identical (e.g., cloned or fully inbred animals). Therefore, this simple technique is very powerful for characterizing *in vivo* immune responses elicited by gp96.

1. Anesthetize donor frog as described in 3.7.
   Note: Only minimal precautions need to be taken since *Xenopus* produce potent anti-microbial peptides (e.g., magainin) in the skin that obviates the need for total aseptic procedures. In fact, treatment of the frog with any disinfectant would be detrimental for the skin. In addition, *Xenopus* do not have any pathogens that can be transferred to humans. Therefore, the use of gloves is optional. However, all the dissection instruments used need to be autoclaved and all solutions need to be sterile.
2. Cut and remove a small (20 mm X 5 mm) piece of ventral skin (abdominal skin which appears silvery due to the presence of irridophore pigmented cells) from a LG-15 donor frog using scissors.
3. Place the skin in a Petridish containing APBS and keep it on ice. Manipulate the skin tissue very gently; avoid holding it in between forceps.
4. Using a razor or scissors cut individual grafts into 5 mm X 5 mm pieces. Keep the fragments in APBS on ice.
5. At this point the donor frog is placed in a container with shallow water until it is awake and then it is placed in water containing antibiotics (Penstrep at concentration of 5 mg/L). The frog is kept in water with antibiotics for two days at which point it is returned in normal water. The small wound at the site where the skin was removed does not need to be sewed and heals within a week.
6. Anesthetize the recipient LG-6 frog.
7. Make a small incision on the dorsal (back) skin of the recipient and insert the 5 mm X 5 mm graft under the skin with the silvery side up. Be careful not to introduce large air bubbles under the skin because that may lead to displacement or even loss of the graft.
8. Make sure you note the scissor and forceps markings on the grafts because those are not counted as graft rejection once the scoring starts.
9. 24 hours later a part of the overlaying host skin needs to be removed from the graft.
10. Anesthetize the recipient LG-6 frog. Handle the animal as described in 5.1.
11. Using autoclaved scissors cut out a window around the graft so that the graft can be freely visualized. Be careful not to touch the donor skin or to induce bleeding. Let the frog recover as described in 5.5.
12. Start scoring the graft right away by taking a sketch. Skin graft rejection is determined by the percent of destruction of the irridophores on the grafted skin.
13. The grafts need to be checked every 2-3 days but the animals do not need to be anesthetized for this. To visualize the grafts, the frogs need to be placed in a petridish under a dissecting microscope.

6. Whole-mount Immunohistology of Transplanted Skin

Frog whole-mount immunohistology has been previously described [6]. Briefly, the frog is anesthetized and placed under the microscope on a sterile paper towel pre-wet with frog water (the working area is also aseptically prepared). The transplanted skin is then harvested together with a small amount of surrounding host skin and it is stained with antibodies similarly to cell staining for flow cytometry analysis [6]. Autoclaved instruments and sterile buffers need to be used. After staining the skin is placed on a microscope slide and gently pressed with a cover slip. At this point the skin can be visualized using a fluorescent microscope for different cell populations that have infiltrated the graft. After the procedure the frog is kept in water with antibiotics as described in section 5.5. and returned in normal water. The small wound left where the skin was removed heals within a week.

7. Characterization of Blood Leukocytes

1. Before removing blood from the frog, one needs to prepare "glass needles" by pulling sterile Pasteur pipettes over flame. The fine extremity of the pipette is sharpened under the stereomicroscope. The pipette is then connected to an aspiration plastic tube.
   All instruments such as the forceps and scissors need to be autoclaved before use.
2. Also prepare ice cold 10 mL of APBS and heparin solution by adding 50 units heparin per 1 mL of APBS. Keep solution on ice. All solutions used need to be sterile.
3. Anesthetize frog as described in 3.7, and follow the same animal handling procedures as described in 5.1. .
4. Cut the skin above the posterior foot to expose the dorsal tarsus vein.
5. Fill the Pasteur pipette with 1-2 mL of APBS+heparin solution.
6. Insert the "glass needle" into the vein and start collecting the blood by slowly aspirating with the mouth. It is important to have APBS+heparin solution in the "glass needle" since this will prevent the blood from clotting and clogging the tip of the needle.
7. 1 to 2 mL of blood can be obtained from one average sized frog.
8. The small incision on the frog's leg does not need to be sewed and the wound heals within one week.
9. Centrifuge the blood at 1,000 rpm for 10 min at 4°C.
10. Remove the supernatant and wash the cells 1X with 10 mL of cold APBS.
11. Overlay 2 mL of blood (1-5 x 10^6 cells) onto 1 mL of ficoll Histopaque 1.077 (Sigma) pre-warmed at room temperature in order to separate the blood leukocytes from the red blood cells.
12. Centrifuge 20 min at 1,000 rpm at room temperature with no brake, and then collect the leukocyte band.
13. Wash the cells 2X with APBS by centrifugation at 1,400 rpm for 10 min at 4°C to remove the residual ficoll.

14. At this point the cells are ready to be stained with antibodies for flow cytometry analysis, or to be use for *in vitro* culture assays.

8. Tumor Transplantation Assay

1. About a week before the experiment thaw out a fresh batch of 15/0 tumor cells and make sure that the cells start growing well. Culture medium is succinctly described in the next section and in more detail in [3].
2. Expand the 15/0 tumor cells.
3. On the day of the experiment, count the cells and determine cell death by Trypan blue exclusion. Cell death should be less than 5%. Wash cells 1X in cold APBS, and centrifuge at 1,000 rpm at 4°C for 10 min.
4. Resuspend the cells in tumor culture medium at a density of 5 x 10^5 15/0 cells per 300 µL.
5. Transplant 5 x 10^5 cells in 300 µL volume per frog by subcutaneous injection using a 25 gauge 5/8 needle on one side of the dorsal (back) side of the animal.
6. Tumor growth will start within 2-3 weeks after tumor challenge. The initial tumor appearance must be noted and the tumor volume needs to be recorded every 2-3 days. Tumor volume (height X length X width) is measured using calipers.
7. Once the tumor grows to 3,500 mm^3 or the frog starts looking lethargic, it needs to be euthanized to prevent discomfort.

9. Reagents Needed:

- Amphibian Phosphate Buffered Saline (APBS): 6.6 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO. pH to 7.5 using 10N NaOH and filter sterilized through 0.2 µm filter.
- Tricaine Methane Sulfonate (TMS, MS-222) (Crescent Research Chemicals CAS#886-86-2).
- Sodium bicarbonate (Fisher Scientific S-233-500).
- Histopaque-1077 (Sigma-Aldrich 10771-100 mL)
- Heparin Sodium Salt (Sigma-Aldrich H3149-50KU)
- Culture medium for *Xenopus* 15/0 tumors [see 3 for more details]: 1 L of Iscove DMEM basal medium (Gibco-Invitrogen 11965) with 10 mL Insulin, 10 mL non-essential amino acids, 10 mL penicillin-streptomycin; 10 µg/ mL of Kanamycin; 3 mL of primatone (Sheffield Products Division), 1 mL of β2-mercaptoethanol, and 3.02 g $NaHCO_3$ in water (pH 7.0). This medium is diluted to amphibian osmolarity by adding 30% double distilled water, and supplemented with 5% fetal bovine serum, 20% superantant from a *Xenopus* kidney cell line A6, and 0.25% of normal *Xenopus* serum.

10. Representative Results

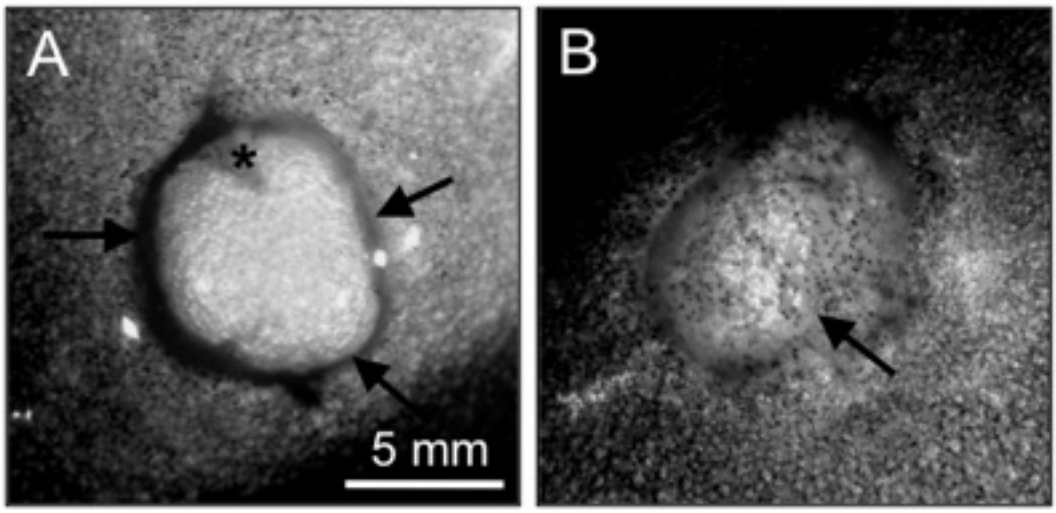

Figure 1. Stereomicroscopic analysis of skin graft rejection 12 days post-transplantation. LG-6 cloned frog received a skin graft from either (A) a MHC-identical LG-6 (shows no rejection) or (B) a MHC-disparate outbred donor (80% rejection). Arrows shows silvery iridophore pigmented cells marking healthy (non-rejected) grafted tissue. (*) Mark of forceps not due to rejection.

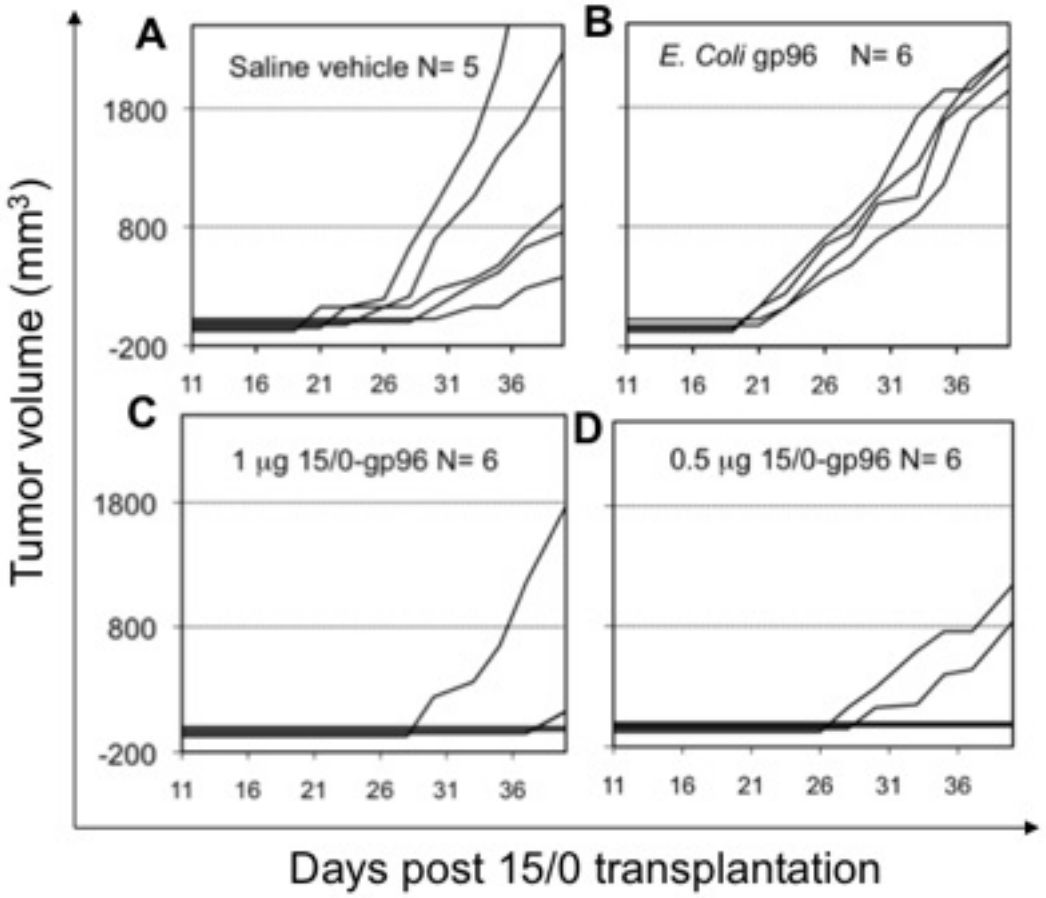

Figure 2. Gp96 facilitates cross-presentation of tumor antigens in *Xenopus*. LG-15 PLs (5 x 10^5) were pulsed for 1 hr on ice either with APBS (negative control), 1 µg of recombinant gp96 purified from an *E. Coli* culture, or 1 or 0.5 µg of gp96 purified from 15/0 tumor tissue. After 3 washes, cells were adoptively transferred into LG-15 adult recipients (1 x 10^6/individual). Three days later, live 15/0 tumor cells (5 x 10^5) were transplanted by s.c. injection. Each curve represents the kinetics of tumor growth in one frog. Days post challenge when tumors first appeared were monitored, and tumor size was determined periodically with a caliper (length x width x thickness).

Discussion

The amphibian *Xenopus* is a unique versatile non-mammalian model to study immunity. Its extensive use in biomedical and immunological research has yielded in many important research tools such as the MHC defined clones LG-6 and LG-15 as well as different cell lines and monoclonal antibodies. Using these tools we have established different *in vitro* and *in vivo* assays to study the ability of heat shock proteins such as gp96 to mediate potent Ag-specific anti-minor H-Ag and anti-tumor T cell responses [7]. This model system allows us to further investigate the immunological properties gp96 during the priming and effector cell phases.

Concerning the priming phase, initial studies of these responses used subcutaneous immunization with purified gp96 that required two injections of 10 µg gp96 at two week intervals before *in vivo* assays such as skin grafting or tumor transplantation [2, 8]. In comparison, the cross-presentation method we have developed [7] and are currently using is more convenient and efficient. The multiple advantages of this priming strategy include time and amount of protein needed for each experiment. For instance in order to immunize an animal it takes 4 weeks and 20 µg of gp96, while with PL priming we need only 3 days and 0.5 to 1 µg of protein. Additionally there is less variability because the priming consists of only one injection of PLs pulsed with gp96. This is a critical step because if the needle is pulled out too quickly during one of the immunization by s.c. injection, a significant fraction of protein may be lost therefore causing a greater individual variability. Importantly, this cross-presentation assay provides a way to further investigate the mechanisms of gp96-mediated immune responses. For example, we can also modulate the expression of certain molecules such as class Ia on the surface of PLs before pulsing with gp96 to investigate their role in gp96 mediated immune responses and T cell priming. The process of gp96 internalization can also be studied by pre-incubating PLs with antibodies or competitors interfering with endocytic receptors [7].

Concerning the effector phase, the *Xenopus* model is not only suitable to characterize immune cell effectors *in vitro* by flow cytometry, killing and proliferation assays [8, 9], but also provides powerful *in vivo* assays such as minor H-Ag-disparate skin grafting and tumor transplantation assays. Both of these assays are well established in the *Xenopus* model however there are a few critical steps that must be followed. For instance in the skin grafting assay special attention must be paid when handling the graft especially when cutting out the window of overlaying host skin. The window has to be slightly smaller than the graft itself so that the graft will not fall out. Furthermore, during tumor transplantation it is important to first inject half of the control animals followed by the experimental ones and than finish with the second half of the control frogs. This will ensure that the tumor is viable during the injection process and will produce more consistent data. The fact that this model system relies on cloned animals further allows to extend studies of effector cells stimulated by hsps using adoptive cell transfer [10].

In summary, the methods presented here highlight the frog *Xenopus* as an exceptional non-mammalian model system to study hsps such as gp96 role in immune surveillance and immune responses.

Disclosures

No conflicts of interest declared.

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