of the molecule in the genomic DNA and the number of cells that have incorporated it. Because BrdU is intracellular, antibodies specific to surface molecules can also be used to identify the cell types incorporating BrdU in a given population. The use of a specific antibody to nascent DNA makes it a useful technique for immunohistochemical, cytometric and microscopic analyses (9).

An alternative to BrdU incorporation assays, and not based on DNA synthesis, is the use of CFSE, a membrane permeable non-toxic fluorescent dye also widely used for the detection of immune cell proliferation *in vitro* (10). CFSE incorporates at similar levels into all cells but dilutes two fold at each cell division. This permits the detection of up to 10 cellular divisions and therefore provides a more robust quantification of the proliferation by a cell population than BrdU incorporation (5).

Whereas activation and expansion of T cells during antiviral

RESULTS AND DISCUSSION

Anti-viral lymphocyte proliferative responses monitored in vivo by BrdU incorporation in *Xenopus*

To assess the *in vivo* proliferative response of *Xenopus* splenocytes during viral infection, we adapted the BrdU incorporation assay used in the mouse model (5). Adult frogs were injected intraperitoneally with the iridovirus FV3 and treated with 1mg/ml of BrdU directly added in the water 2 days before sacrifice. A representative experiment is depicted in Fig. 1A. Forward scatter and side scatter profiles of *Xenopus* splenocytes are comparable to that of mammals, indicative of small cells with little to no granularity. The slight increase in the size of the lymphocytes (indicated by an arrow in Fig. 1 forward scatter and side scatter plots) in infected frogs compared to the uninfected controls suggests activation and consequent blasting of lymphocytes in the spleen as observed in mammals and ectothermic vertebrates (21). Similarly, the total number of splenocytes, as well as Brd+ cells, obtained from infected frogs was pare frogs

proliferative response of primed T cells, we have developed an *in vitro* proliferation method using CFSE, which is a fluorescent

combination with multiple conditions to test antigen presentation and pathogen specificity of which little is known in ectothermic vertebrates.

To our knowledge, this is the first account of a measurable proliferative response of lymphocytes against a viral pathogen in an ectothermic vertebrate. We have shown that commonly used techniques to determine lymphocyte proliferation in mammals can also be applied to assess cellular immune responses in amphibians. We have previously shown that CD8 T cells involved in anti-ranaviral response in *Xenopus* proliferate (e.g., incorporate BrdU) at detectable levels in the spleen, in both primary and secondary infections. Furthermore, T cell priming resulting from FV3 infection can be determined *in vitro* by co-culture of primed splenocytes with antigen presenting cells infected with FV3. The use of CFSE in this instance allows a rigorous analysis of the level of proliferation of various *Xenopus* lymphocyte populations. It is noteworthy that in both BrdU and CFSE based assays, proliferation of activated lymphocytes was minimal compared to that observed in mammals (4,7). We have suggested that the relatively small response of *Xenopus* lymphocytes may be due to the lack of secondary lymphoid organs such as the lymph nodes, which facilitate intimate contact with the antigen presenting cells (22,23). However, since we also observe this effect *in vitro,* it may be due to intrinsic characteristics of the cellular potential in this ectothermic vertebrate. We speculate, for example, that the effect of cytokine secretion in *Xenopus* may be limited by either a relatively lower amount of secreted cytokine, diminished receptor expression in the cell surface or a lack of those cytokines specifically involved in boosting T cell proliferation in mammals (i.e. IL-2, IL-17 and IL-15).

Some technical issues concerning the use of BrdU and CFSE ines was0ved rec avmae0i inYnew@h,thaWcFSI"ng eaebrFGIBfNFÄGtö

models, further bridging the gap to a complete understanding of cellular immune responses throughout vertebrate phyla.

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PROTOCOLS

Protocol I: *In vivo* BrdU incorporation assay

Reagents:

- Amphibian grade PBS- PBS + 30% distilled $H_2 0$
- 5g/L Tricaine methane sulfonate (TMS222; Crescent Research Chemical, Phoenix AZ, cat. # 886-86-2) buffered with sodium bicarbonate 0.5 g/L.
- Staining Buffer 1: APBS containing 1% BSA and 0.01%NaN_3
- DNAse buffer: 40mM Tris HCl, pH8.0 with 10mM NaCl and 6mM $MgCl_2$
- Permeabilization buffer: APBS containing 1% Paraformaldehyde (Sigma) and 0.05% Tween 20 (Sigma)
- Staining Buffer 2: APBS containing 0.01% Tween 20

FV3 infection and Incubation with BrdU:

In vivo BrdU incubation works best with outbred frogs of about 2 years of age or 2-3 inches long. Outbred frogs are injected intraperitoneally with 1-5x10^6 pfus of FV3 in sterile APBS. The animals are kept in our facility away from uninfected animals for 4 days. Each frog is placed separately in a 500ml beaker with 100ml declorinated water containing 1mg/ml BrdU 2 days before sacrifice.

Splenocyte harvest:

1. At time of harvest, euthanize the animals in 5g/L TMS222.
2. Remove the spleen and keep in cold APBS on ice.
3. Scrape the spleen (sterile technique not necessary) with a nylon mesh and wash the cells 2 times with APBS and 1 time with Staining Buffer 1.
4. Count the cells and distribute them at 500,000 ± 1x10^6 cells per sample

Cell staining:

1. After washing, resuspend the cells in 100 ml mAb diluted at 1mg/ml (final concentration) with Staining buffer 1, incubate for 30 min-1 hr on ice.
2. Wash 2 times with Staining Buffer 1.
3. Resuspend cells in 100 ml secondary Ab (if needed) diluted in staining buffer 1, and incubate in the dark on ice for 30 min. Keep cells in the dark from now on.
4. Wash 2 times with staining buffer 1.
5. Resuspend in 50-100 l of permeabilization buffer at room temperature for 30 min in the dark.
6. Wash 2 times with DNase buffer.
7. Resuspend cells in 100 ml DNase buffer + 3 ml DNase I at 37°C for 30min.
8. Wash 2 times in staining buffer 2.
9. Incubate in 3 ml anti-BrdU-FITC in 25 ml of staining buffer 2 on ice for 30min-1hr.
10. Wash 2 times with Staining buffer 2 and resuspend in at least 100 ml staining buffer.
11. Analyze by FACS.

Protocol II: CFSE proliferation assay

Reagents:

- CFSE: Dissolve CFSE powder in DMSO to a 5mM stock. Aliquot 100 ml in eppendorf tube. Keep in -20°C in a dark and dry area.

- ASF-A6-MSF: Iscove DMEM basal medium diluted at the amphibian osmolarity and containing 5% FBS, 10 mg/ml of Kanamycin and 20% supernatant from the A6 kidney fibroblast cell line (ATCC: CCL 102; see ref. 17 for more details).
- Staining Buffer 1: APBS with1% BSA and 0.01%NaN_3.

Priming:

Outbred frogs are primed by intraperitoneal injection of 1-5x10^6 pfus FV3 in sterile APBS. The animals are kept in our facility away from uninfected animals for 3-4 weeks.

Peritoneal leukocyte isolation and in vitro infection:

Peritoneal leukocytes (PLs) from infected and uninfected animals are isolated by peritoneal lavage as described in detailed elsewhere (2). Anesthetized frogs (a 0.1% aqueous solution buffered with sodium bicarbonate of TMS222) are injected intraperitoneally (sterile syringe with a 22 gauge 1½ inch needle) with 5 to 10 ml (depending of the size of animal) of APBS prewarmed at room temperature. The frog is then gently massaged then the solution injected is recovered into a sterile tube by puncture with a 22G 1½ gauge needle. Cells are washed twice with cold APBS, counted with a hemacytometer and seeded in a 24-well plate at 100,000-500,000 cells per well in 1ml ASF-A6 medium. Cells are then infected with FV3 at 1 MOI (or 100,000-500,000 pfu per well), and incubated overnight at 27°C in a CO_2 chamber.

Day 1 Co-culture Splenocytes:

1. Euthanize frogs in 5g/L TMS222 and isolate the spleen using sterile technique.
2. Scrape the spleen and wash with cold sterile APBS. Ensure proper suspension.
3. Incubate with 5-20mM* CFSE at 50x10^6 cells/ml at 27°C in the dark for 20 min.
   **Ex vivo* splenocytes stain well at 5mM concentration, however, a CFSE titration should be done to determine best labeling concentration for cells of interest.
4. Wash cells well 2-3x with cold APBS.
5. Distribute splenocytes with corresponding PLs at a one to one ratio (different stimulator to primed splenocyte ratios may also be done).
6. Incubate the plates at 27°C in a CO_2 chamber for 48- 96 hours.
7. 3Total cFeuUprimasu&dfodc&hdsurfacelabèling.for20 mir

Day 2-Day 6 Cell surface labemsp0p0p0p0p6snsàFgBg0nsPntpensia&Se#VÒ wash wios ð-Ðe2wð-ÐQ wiosð-Ðe0taiin7V fer0.ng.