Characterization of Primary and Memory CD8 T-Cell Responses against Ranavirus (FV3) in *Xenopus laevis*[▽]

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In mammals, resistance to primary and secondary viral infections critically involves major histocompatibility complex class I-restricted cytotoxic CD8⁺ T lymphocytes (CTLs). Although many gene homologues involved in CTL function have been identified in all vertebrate classes, antiviral CTL responses have been poorly characterized for ectothermic vertebrates. Because of the threat of emerging wildlife viral diseases to global biodiversity, fundamental research on comparative viral immunity has become crucial. Ranaviruses (family *Iridoviridae*) are double-stranded DNA viruses possibly implicated in the worldwide decline of amphibian populations. We used the frog *Xenopus laevis* as a model to evaluate adaptive immune responses to the ranavirus frog virus 3 (FV3). FV3 infects the kidneys of adults but is cleared within 4 weeks, with faster clearance upon secondary infections. In vivo depletion of CD8⁺ T cells markedly decreases the survival of adults after viral infection. To further investigate the involvement of anti-FV3 CD8⁺ T-cell effectors in host resistance in vivo, we determined the proliferation kinetics of CD8⁺ T cells in the spleen by bromodeoxyuridine incorporation and their infiltration of kidneys by immunohistology. Upon primary infection, CD8⁺ T cells significantly proliferate in the spleen and accumulate in infected kidneys from day 6 onward, in parallel with virus clearance. Earlier proliferation and infiltration associated with faster viral clearance were observed during a secondary infection. These results provide in vivo evidence of protective antigen-dependent CD8⁺ T-cell proliferation, recognition, and memory in fighting a natural pathogen in *Xenopus*.

Although in mammals the innate immune system effectively controls viruses upon initial encounter, it is the specific adaptive immune response that mediates the clearance of viral pathogens (reviewed in references 12 and 36). This is largely due to the activation of antigen-specific CD8⁺ cytotoxic T lymphocytes (CTLs) that recognize and lyse infected cells that express surface major histocompatibility complex (MHC) class I molecules complexed to viral antigenic peptides (12). The generation of mature CTLs in turn depends on an interaction with antigen-presenting cells (APCs), which, in addition to the class I molecule-antigen complex, express high levels of costimulatory molecules required for T-cell activation (reviewed in reference 26). Typically, the kinetics of antiviral responses involve initial CD8 T-cell activation in primary lymphoid organs, characterized by exponential proliferation at 3 to 6 days postinfection (p.i.). At 6 to 8 days, expanded CD8 T cells exit the lymphoid tissues and move to the site of infection to specifically kill infected cells (12). By days 8 to 10 p.i., infected cells are eliminated and viral clearance is achieved. In parallel, the effector CD8⁺ T-cell pool contracts and a small number of cells remain, constituting the memory T-cell pool (3). In the event of a second infection, these cells are capable of quickly expanding and clearing infected cells within the first 6 days of reinfection.

CTL activation by viral pathogens and specific killing have been demonstrated for mammals (12) and for birds (17). However, not a lot is known about specific antiviral T-cell responses

and their involvement in viral clearance and immunological memory in the lower vertebrates. For fish, T-cell receptors and MHC class I molecules have been characterized (19, 23, 32, 34) and antiviral immune responses were shown to involve cytotoxicity mediated by peripheral blood lymphocytes of virus-infected cells (31, 35). However, in the absence of MHC-defined strains, the evidence of the antigen specificity and MHC restriction of these lymphocytes is missing. In the frog *Xenopus laevis*, CTL activity has been characterized in vitro against alloantigens (5, 21, 33) and shown to involve antigen-specific MHC-restricted CD8⁺ T cells (29). CTL responses to allo- and tumor antigens have also been characterized to some extent in vivo (24, 28), but little is known about their involvement in antiviral responses.

Ranaviruses, which are of the family *Iridoviridae*, are double-stranded DNA viruses possibly involved in the global amphibian population decline (10, 11). Using frog virus 3 (FV3), the best-characterized member and type species of the *Ranavirus* genus, we have established *Xenopus* as an important model with which to study antirranavirus immunity in amphibians (16) and have shown some evidence of the critical role of CD8⁺ T cells in host resistance to FV3 infection (30). Viral resistance in adults and susceptibility in MHC class I molecule-deficient larvae suggest that an efficient MHC-restricted CD8⁺ T-cell response may be responsible for effective antiviral responses. Furthermore, faster recovery observed in adults upon secondary infections could be the effects of primed memory CD8⁺ T cells maintained after the primary infection, as has been observed in mammals. In this study, we have characterized, in vivo, the responses of CD8⁺ T cells upon primary and secondary FV3 infection and have defined the kinetics of their proliferation and infiltration in infected tissues. Our results

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FIG. 1. Proliferation of *Xenopus* splenocyte populations upon primary FV3 infection. Representative two-color flow cytometry analysis of splenocytes from uninfected control frogs and frogs infected for 3 days and 6 days (last column). Cells were surface stained with biotinylated anti-class II (AM20), anti-CD8 (AM22), or anti-CD5 (2B1) MAb followed by APC-conjugated streptavidin (or APC-conjugated goat anti-mouse Ig for 2B1 MAb). Cells were then permeabilized, treated with DNase, and stained with FITC-conjugated anti-BrdU MAb. Twenty thousand events were collected and gated for lymphocyte populations. Percentages of CD8⁺ BrdU⁺ cells of total cells are shown in upper right quadrant.

substantiate our previous observations that, as in mammals, *Xenopus* CD8⁺ T cells are critically involved in FV3 clearance and provide the first evidence for amphibians of the establishment of CD8⁺ memory T cells following a primary viral infection.

MATERIALS AND METHODS

Frogs and FV3 stocks. Two-year-old outbred *Xenopus* frogs were obtained from our breeding colony (<http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm>). Randomized groups of frogs were inoculated by intraperitoneal (i.p.) injection of 5×10^6 PFU of FV3 in 300 μ l of phosphate-buffered saline (PBS) modified to amphibian osmolarity (APBS). FV3 was grown in and purified from A6 *Xenopus* kidney cells as previously described (16, 25). Viral titers were determined using A6 cells by the 50% endpoint dilution method, as already described in reference 25.

In vivo BrdU incorporation assay. Outbred adult frogs were injected intraperitoneally with 3×10^6 PFU of FV3 for 3, 6, 9, and 14 days. In secondary-infection experiments, frogs were first boosted with an i.p. injection of 3×10^6 PFU of FV3 and then reinfected 1 month later 14, 9, 6, and 3 days before sacrifice. Frogs were incubated in 100 ml of water containing 1 mg/ml bromodeoxyuridine (BrdU; Sigma) 2 days before sacrifice. Upon sacrifice, the spleen was removed and scraped using a nylon mesh. Isolated splenocytes were washed twice with APBS, counted, and stained for the surface markers CD8 (AM22) (15), CD5 (2B1) (20), and MHC class II (AM20) (15) immunoglobulin M (IgM; 6.16) (6). Cells were permeabilized in APBS–0.05% Tween 20 and treated with

DNase I (Promega), followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (MAb) (Sigma). Cells were then washed and analyzed with a FACSCalibur (Beckton-Dickinson).

PCR. DNA was extracted from the kidneys of FV3-infected frogs using the QIAGEN DNAeasy DNA extraction kit and quantified by spectrophotometry. PCR was done for FV3 major capsid protein (forward primer, 5'-ATGCTCTC TGTAACCTGGTTCAGG-3'; reverse primer, 5'-GTCTCTGGAGAGAAGA A-3') with an annealing temperature of 52°C for 40 cycles. Primers amplifying the cellular gene β 2-M (forward primer, 5'-CCCTTGTTGGTGTAAC GTGCT C-3'; reverse primer, 5'-GCACACCAATCAGAAAAAGGAC-3') were used as controls.

Immunohistology. Kidneys from infected frogs were embedded in OCT Tissue Tek (Miles Inc.). Frozen sections (5 μ m) were fixed for 1 min in 100% acetone, dried, and stained with MAbs for CD8 or class II molecules (mentioned above) for 12 h and for FITC-conjugated goat anti-mouse Ab (Southern Biotech) that had been adsorbed twice on *Xenopus* erythrocytes (2, 30) for 30 min. Slides were mounted in antifade mountant (component A; Molecular Probes) and visualized with a fluorescence microscope. Positively stained cells were counted in 10 different randomly chosen areas.

RESULTS

In vivo CD8⁺ T-cell proliferation upon primary FV3 infection. CD8⁺ cell depletion experiments (30) suggested that in *Xenopus* adults, CD8⁺ T-cell effectors are critically involved in

FIG. 2. CD8⁺ T cells proliferate but do not accumulate in *Xenopus* spleens upon primary FV3 infection. Percentages of total CD8⁺ T cells (A) and BrdU⁺ CD8⁺ T cells (B) from five different experiments determined by flow cytometry and corresponding logarithmic plots of total CD8⁺ T-cell (C) and BrdU⁺ CD8⁺ T-cell (D) numbers calculated from these percentages. Each symbol represents results for one animal. Control values (C) are from uninfected frogs. Total T-cell numbers in the spleen were determined by adding the percentage of cells in the upper right quadrant and that in the upper left quadrant and multiplying the sum by the total number of cells counted. Individual cell counts and averages $\pm$ standard deviations are indicated for each group. Statistical significance was determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$. D3, D6, and D9, days 3, 6, and 9, respectively.

FV3 clearance. To further characterize CD8⁺ T-cell responses in the spleen, we conducted *in vivo* cell proliferation assays with BrdU incorporation to monitor the kinetics of the activation-induced proliferation of CD8⁺ T cells upon FV3 infection over several days. Flow cytometry results of a representative of five different experiments are shown in Fig. 1, while an overall analysis of the results is presented in Fig. 2 and Table 1. Whereas there was no or only a slight increase in the proliferation of both class II molecule-positive cells (a total leukocyte marker) and CD5⁺ cells (a pan T-cell marker in *Xenopus*) in the spleen (Table 1, BrdU⁺ cell fraction), active CD8⁺ T-cell proliferation was significant 6 days p.i., as revealed by the increase in both the percentage and the number of BrdU⁺ cells (about 10 times more on average) (Fig. 2B and D). The higher percentage and number of proliferating CD8⁺ T cells persisted on day 9 p.i. and were back to the basal levels of uninfected controls by day 15 (data not shown). Contrasting with active CD8⁺ T-cell proliferation, the percentage and number of total CD8⁺ T cells did not significantly increase until day 9 following

FV3 infection ($P < 0.05$) (Fig. 2A and C). On the other hand, there was an accumulation of the splenic leukocyte population identified by the surface expression of MHC class II molecules and the whole T-cell population identified by the CD5 marker that was already detectable at day 3 p.i., which was statistically significant from day 6 to day 9 p.i. only (Table 1, total cells). Interestingly, the average number of proliferating CD8⁺ T cells at day 9 p.i. was higher than that of class II molecule-positive cells. Since in *Xenopus*, adult circulating T cells are usually class II antigen positive, this suggests a down-regulation of class II antigen surface expression by proliferating CD8⁺ T cells.

Given that the kidney is the main site of FV3 infection in *Xenopus* (30), we next determined whether the infiltration of effector cells in the kidney would correlate with CD8⁺ T-cell proliferation kinetics observed in the spleen and with viral clearance. Infiltration of CD8⁺ T cells, monitored by immunofluorescence using *Xenopus*-specific anti-CD8 MAb, became noticeable from days 6 to 9 postinfection, whereas an increased number of class II molecule-positive cells was already detected at day 3 p.i. (Fig. 3A). Compared to that of CD8⁺ T cells, the morphology of class II antigen-positive cells was heterogeneous (Fig. 3A, inset), including small round leukocyte-like cells and other larger cells with dendrite-like extensions. The class II antigen-positive cells that accumulated in kidney from day 3 p.i. onward were mainly of leukocyte-like morphology. While a fraction of CD8⁺ T cells were located in areas close to the blood vessels, especially at days 3 and 6 p.i., single CD8⁺ T cells were increasingly found throughout the tissue at day 6, decreasing substantially by day 9 (Fig. 3A) and disappearing by day 15 (data not shown) p.i. In particular, many CD8⁺ T cells were found in the vicinity of renal proximal tubules, where epithelial cells infected by FV3 have been observed by immunohistology using the anti-FV3 MAb BG11 (28). The average cell count of 10 randomly chosen areas of infected kidney tissues of several animals support a statistically significant increase in the number of infiltrating CD8⁺ T cells from day 3 to

TABLE 1. Numbers of proliferating and total class II molecule-positive, CD5⁺, and CD8⁺ cells in frog spleens 3, 6, and 9 days after a primary FV3 infection

Cells and status	Mean no. of cells $\pm$ SD		
	MHC class II antigen positive	CD5 ⁺	CD8 ⁺
Total cells (10^6) ^a			
Control	7.5 $\pm$ 2.9	3.1 $\pm$ 2.3	4.8 $\pm$ 2.2
Day 3 p.i.	21.8 $\pm$ 18.8	14.1 $\pm$ 12.8 [#]	9.5 $\pm$ 8.0
Day 6 p.i.	22 $\pm$ 7.5**	14.1 $\pm$ 12.8**	10.8 $\pm$ 5.1
Day 9 p.i.	32.8 $\pm$ 18.2*	20.1 $\pm$ 10.8**	20.6 $\pm$ 12.4*
BrdU ⁺ cells (10^5) ^b			
Control	0.9 $\pm$ 0.06	1.6 $\pm$ 0.10	1.01 $\pm$ 0.06
Day 3 p.i.	6.4 $\pm$ 0.62	2.1 $\pm$ 0.09	5.3 $\pm$ 0.73
Day 6 p.i.	8.4 $\pm$ 0.72 [#]	3.8 $\pm$ 0.19 [#]	7.23 $\pm$ 0.11***
Day 9 p.i.	3.6 $\pm$ 0.08**	2.8 $\pm$ 0.18	9.81 $\pm$ 0.56**

^a Numbers of MHC class II antigen-positive, CD5⁺, and CD8⁺ (both BrdU⁺ and BrdU[−]) cells among total cells based on fluorescence-activated cell sorter analysis of spleens after primary infection.

^b Numbers of MHC class II antigen-positive, CD5⁺, and CD8⁺ cells that were also positive for BrdU. Values are averages from five representative experiments. [#], $P = 0.09$ to 0.06 ; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test).

FIG. 3. CD8⁺ T cells and other class II molecule-positive leukocytes infiltrate the kidney upon primary infection. (A) Immunofluorescence microscopy of frozen kidney sections from frogs that were uninfected (control [C]) or from frogs 3 and 6 days (D3 and D6, respectively) after their infection by i.p. injection of 10⁶ PFU of FV3; the tissue was stained either for class II molecule or for CD8 expression. Insets at day 6 depict a class II molecule-positive interdigitating cell (left) and a lymphocyte-like cell (right). White asterisks indicate renal proximal tubules. IC, interdigitating MHC class II antigen-positive APC-like cells; L, class II molecule-positive leukocyte-like cells. Bar = 50 μ m. (B) Averages $\pm$ standard deviations of the numbers of positively stained class II molecule-positive and CD8⁺ T cells counted in 10 randomly chosen areas. Asterisks indicate statistical significance as determined by Student's *t* test of results from uninfected and infected kidneys. **, *P* < 0.01. C, control; D3, D6, D9, and D15, days 3, 6, 9, and 15, respectively. (C, upper panel) PCR of total DNA of kidneys from animals that were uninfected or infected for 3, 6, 9, and 15 days using primers specific for the major capsid protein of FV3 or β 2-M as a positive control. (Lower panel) Semiquantitative PCR of the same DNA samples but which were serially diluted 10-fold before amplification.

day 6 p.i. (Fig. 3B). Furthermore, the time of CD8⁺ T-cell infiltration in kidneys (day 6 p.i.) correlates with the highest viral DNA load determined by PCR (Fig. 3C) and to the significant increase in T-cell proliferation in the spleen (Fig. 2 and Table 1). No marked BrdU incorporation was detected in cells recovered from the kidneys 3 and 6 days p.i. (data not shown). Finally, the amount of viral DNA decreased by day 9 and was undetectable by day 15 (Fig. 3C), in agreement with previously published results (16, 30) and with the kinetics of CD8⁺ T-cell proliferation in the spleen and leukocyte infiltration in the kidneys.

In vivo CD8⁺ T-cell proliferation upon secondary FV3 infection. Outbred *Xenopus* adults recover from FV3 infection faster upon secondary challenge (16). This suggested the development of immunological memory, possibly by CD8⁺ memory T cells. In order to identify and better characterize FV3-

specific memory CD8⁺ T cells and to define the kinetics of a memory antiviral response in *Xenopus*, we conducted in vivo proliferation experiments on frogs infected 1 month earlier and analyzed 3, 6, and 9 days following challenge with a second FV3 inoculum. Flow cytometry plots of a representative experiment are shown in Fig. 4, and an analysis of five different experiments is presented in Fig. 5 and Table 2. Upon secondary infection, a statistically significant proliferative response of splenic CD8⁺ T cells occurred earlier (day 3 p.i.) than during primary infection (day 6 p.i.) and correlated with an increase in total splenic CD8⁺ T-cell numbers at days 6 and 9 postinfection (Fig. 5). Despite their increase in number, the percentage of splenic CD8⁺ T cells did not change significantly. In addition, the levels of both total CD5⁺ T cells and class II molecule-positive leukocytes increased significantly from day 3 to day 9 following secondary infection (Table 2). These results

FIG. 4. Proliferation of *Xenopus* splenocyte populations upon secondary FV3 infection. Representative two-color flow cytometry analysis of splenocytes from uninfected control frogs (C) and frogs infected 1 month prior to the experiment and then reinfected for 3 days and 6 days. Cells were stained as described for Fig. 1 for surface class II, CD5, and CD8 (AM22) molecules and for BrdU. Twenty thousand events were collected and gated for lymphocyte populations. Percentages of CD8⁺ BrdU⁺ cells of total cells are shown in upper right quadrants.

indicate an accelerated kinetic response of CD8⁺ T cells, and possibly of other effector cell types, upon secondary infection.

A small but significant number of CD8⁺ T cells appear in the kidney at the third day after the secondary infection, in parallel with the observed proliferation of CD8⁺ T cells in the spleen (Fig. 6). The number of these infiltrating CD8⁺ T cells remained significantly higher than in uninfected controls at days 6 and 9 p.i. but did not increase as much as during primary infection. On average, only 2 CD8⁺ T cells per area were counted at day 6 after the secondary infection versus 10 cells at 6 days after the primary infection. The number of MHC class II antigen-positive cells in the kidney also increased as early as day 3 after the secondary infection and peaked at day 6, as observed in primary responses, but their numbers were also lower than during the primary infection (on average 18 cells per area in the primary versus 6 cells per area in the secondary infection). Noticeable amounts of viral DNA were detected in the kidney 3 days after the secondary infection at the time that active splenic CD8⁺ T-cell proliferation first occurred (Fig. 6C). However, as previously reported (16, 30), FV3 viral DNA became rapidly undetectable on day 6 after the secondary infection and beyond.

DISCUSSION

The data presented here provide the first in vivo evidence of protective antigen-dependent CD8⁺ T-cell proliferation, recognition, and memory against a viral natural pathogen in an ectothermic vertebrate.

Generation by *Xenopus* adults of an effective antiviral primary CD8⁺ T-cell response. In mammals, antigen-dependent proliferation in lymph nodes is a landmark of CD8⁺ T-cell activation (9, 12). Interactions with antigen-presenting cells expressing antigenic peptides in the context of MHC class I and costimulatory (B7) molecules result in the maturation and expansion of naïve CD8⁺ T cells into cytotoxic CD8⁺ T-cell effectors (CTLs). These effectors can then travel to the site of infection, where they are able to recognize and kill infected cell targets (1, 4, 8). In the absence of lymph nodes, the spleen is the main site for T-cell activation in *Xenopus* (13). The marked increase of BrdU⁺ incorporation by splenic CD8⁺ T cells from day 6 to day 9 after FV3 infection clearly establishes that these cells actively proliferate in response to viral antigens. The kinetics of this proliferation (i.e., no significant BrdU incorporation at early time points) exclude a significant contribution of

FIG. 5. CD8⁺ T cells proliferate but do not accumulate in the *Xenopus* spleen upon secondary FV3 infection. Percentages of total CD8⁺ T cells (A) and BrdU⁺ CD8⁺ T cells (B) from five different experiments as determined by flow cytometry and corresponding logarithmic plots of total CD8⁺ T-cell (C) and BrdU⁺ CD8⁺ T-cell (D) numbers calculated from these percentages. Each symbol represents the result for one animal. Control (C) values are from uninfected frogs. Individual cell counts and averages $\pm$ standard deviations are indicated for each group. Asterisks indicate statistical significance determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. D3, D6, and D9, days 3, 6, and 9, respectively.

other cell types expressing CD8 molecules, such as CD8⁺ NK/T cells and dendritic cells that are unlikely to proliferate to this extent and as late as 6 to 9 days postinfection. Despite the substantial increase in CD8⁺ T-cell proliferation (e.g., an increase in the number of CD8⁺ BrdU⁺ cells), their total number in the spleen does not markedly change until day 9 postinfection, suggesting that anti-FV3 CD8⁺ T cells egress from the spleen to infected tissues at day 6 p.i. On the other hand, the overall accumulation without concomitant proliferation of both total CD5⁺ T cells and class II molecule-positive leukocytes, which includes B and T cells starting as early as day 3 p.i., is consistent with an inflammatory response and increased drainage by the spleen.

While FV3 infection can be transmitted through water (30), the natural route of infection in *Xenopus* or other amphibian species is still unknown. In *Xenopus*, FV3 infection is usually mildly pathogenic, unless the host's immune system is compromised or immature (as seen in larvae). In most cases, FV3 infection results in only temporary symptoms, such as loss of appetite, cutaneous erythema of the legs, and skin shedding. These symptoms appear during the first week following infec-

tion and disappear within 2 to 3 weeks, in parallel with viral clearance as measured by the detection of FV3 DNA (16). Mortality, which is low even when frogs are infected with a high dose of FV3 (less than 20% of adults infected by i.p. injection with 10^7 PFU die within a month), is associated with a typical systemic infection. Animals exhibit both edema and hemorrhages, and FV3 is found widespread in most tissues and in blood. We have previously shown that in *Xenopus*, FV3 has a strong tropism for the kidney (16, 30). In immunocompetent *Xenopus* adults, immunohistology has revealed that early after infection (3 to 9 days), FV3 is confined in discrete areas of the renal proximal tubules, where tissue damage (e.g., necrosis) is observed. The size of these infected areas decreases rapidly and the virus becomes undetectable within 20 days, as determined by both immunohistology and PCR (30). The occurrence of an increasing number of infiltrating CD8⁺ T cells that peaks at day 6 postinfection and the preferential distribution of these CD8⁺ T cells around the renal proximal tubules correlates well with the course of FV3 infection and the postulated egress of splenic CD8⁺ T cells. This further substantiates the involvement of CD8⁺ T cells in primary viral clearance. However, immunohistology studies with anti-class II MAb indicates that cell types aside from CD8⁺ T cells also infiltrate the kidney during infection. Whereas only a small number of class II molecule-positive cells are present in the kidneys of uninfected frogs, they accumulate as early as 3 days following FV3 infection. In adult *Xenopus*, class II molecules are expressed at the surfaces of leukocytes (e.g., granulocytes, monocytes, and macrophages) as well as B and T lymphocytes (13). The fact that the number of class II molecule-positive cells found in infected kidneys increases earlier than (day 3 postinfection) and exceeds the number of CD8⁺ T cells implies that other cell types infiltrate the tissue with different kinetics. Characterization of these infiltrating cells remains to be done.

Ability of *Xenopus* adults to develop an antiviral secondary and memory CD8⁺ T-cell response. In mammals, virus-specific memory CD8⁺ T-cell precursors remain at higher frequencies than those of naïve cells in the host after the clearance of a

TABLE 2. Average numbers of proliferating and total cells in the spleens upon secondary infection

Cells and status	Mean no. of cells $\pm$ SD		
	MHC class II antigen positive	CD5 ⁺	CD8 ⁺
Total cells (10^6) ^a			
Control	7.5 $\pm$ 2.9	3.1 $\pm$ 2.3	4.8 $\pm$ 2.2
Day 3 p.i.	24.1 $\pm$ 9.2**	12.4 $\pm$ 2.8***	8.9 $\pm$ 1.2
Day 6 p.i.	25.8 $\pm$ 11.7**	10.7 $\pm$ 4.2**	15.3 $\pm$ 3.1***
Day 9 p.i.	25.7 $\pm$ 8.5**	10.9 $\pm$ 2.6**	12.3 $\pm$ 1.31**
BrdU ⁺ cells (10^5) ^b			
Control	0.9 $\pm$ 0.06	1.6 $\pm$ 0.10	1.01 $\pm$ 0.06
Day 3 p.i.	3.8 $\pm$ 0.18**	7.2 $\pm$ 0.58#	3.6 $\pm$ 0.11**
Day 6 p.i.	8.2 $\pm$ 0.54*	8.6 $\pm$ 0.95	8.3 $\pm$ 0.57*
Day 9 p.i.	5.3 $\pm$ 0.31*	5.4 $\pm$ 0.49#	10.0 $\pm$ 1.2

^a Numbers of MHC class II antigen-positive, CD5⁺, and CD8⁺ (both BrdU⁺ and BrdU⁻) cells among total cells based on fluorescence-activated cell sorter analysis of spleens after a secondary infection.

^b Numbers of MHC class II antigen-positive, CD5⁺, and CD8⁺ cells that were also positive for BrdU. Values are averages from five representative experiments. #, $P = 0.09$ to 0.06 ; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test).

FIG. 6. CD8⁺ T cells and other class II molecule-positive leukocytes infiltrate the kidney upon secondary infection. (A) Immunofluorescence microscopy of frozen kidney sections from frogs that were either uninfected (control [C]) or infected 1 month earlier and then reinfected for 3 and 6 days by i.p. injection of 10⁶ PFU of FV3 and stained either for class II or CD8 molecule expression. Insets at day 3 depict CD8⁺ T cells near proximal tubules and a class II molecule-positive lymphocyte-like cell in between proximal tubules. White asterisks indicate renal proximal tubules. IC, interdigitating MHC class II antigen-positive APC-like cells; L, class II molecule-positive leukocyte-like cells. Bar = 50 μ m. (B) Averages $\pm$ standard deviations of the numbers of positively stained class II molecule-positive and CD8⁺ T cells counted in 10 randomly chosen areas. Asterisks indicate statistical significance as determined using Student's *t* test on results from uninfected and infected kidneys. **, *P* < 0.01. (C, upper panel) PCR of total DNA of kidneys from animals that were not infected or infected for 3, 6, 9, and 15 days using primers specific for the major capsid protein of FV3 or β 2-M as a positive control. (Lower panel) Semiquantitative PCR of the same DNA samples serially diluted 10-fold before amplification.

primary infection. Upon a secondary infection, the more efficient and rapid response observed is thought to be the result of the expansion from this pool of memory CD8⁺ T cells (12). Whether the faster response kinetics is due solely to the initial higher frequency of precursors or whether additional specific properties of memory CD8⁺ T cells, such as a faster and lower threshold of activation, are also involved is still an active area of research (22).

In *Xenopus*, the faster disappearance of symptoms, the more rapid clearance of FV3, and the generation of thymus-dependent IgY anti-FV3 antibodies during a secondary infection are all indicative of the involvement of T-cell memory. In the present study, we have examined more specifically the CD8⁺ T-cell response during a secondary FV3 infection. Compared

to the results of the primary infection, our data clearly indicate that the significant proliferation of CD8⁺ T cells in the spleen and concomitant CD8⁺ T-cell infiltration in infected kidneys after secondary infection occur 3 days earlier (statistically significant difference at day 3 p.i.). These results indicate that pools of antiviral memory CD8⁺ T cells have been generated during the primary infection and therefore provide strong in vivo evidence of CD8⁺ T-cell memory in the amphibian *Xenopus*. Interestingly, this CD8⁺ T-cell memory response is not accompanied by a marked increase in the number of proliferating splenic CD8⁺ T cells (i.e., there were similar numbers of BrdU⁺ CD8⁺ T cells in primary and secondary infections) and results in a lower (fivefold less on average) number of infiltrating CD8⁺ T cells in infected kidneys.

We propose three explanations for these results that are not mutually exclusive. First, the modest CD8⁺ T-cell response upon secondary FV3 infection may be inherent to the *Xenopus* adaptive immune system, which represents an evolutionary transition from the advent of MHC-restricted cellular immunity to that seen in mammals. It is possible that *Xenopus* is representative of an animal that has evolved adaptive cellular immune responses that do not yet include a high degree of T-cell expansion. We have shown that although adoptively transferred carboxyfluorescein succinimidyl ester-labeled CD8⁺ T cells primed against minor H antigens specifically proliferate in the spleens of syngeneic recipients bearing antigens, the number of divisions and resulting expansions that they undergo is relatively low compared to what occurs in mammals (24). Although the architecture of the frog spleen is similar to that of mammals, there are fundamental differences (i.e., a lack of germinal centers and separation of the white and red pulp [13, 14]) that may account for differences in the magnitudes of CD8⁺ T-cell proliferation. The absence of a draining lymph node close to the site of infection (i.e., kidney) can be another limiting factor for CD8⁺ T-cell expansion.

Alternatively, the observed low CD8⁺ T-cell response during secondary infection may indicate the critical involvement of other types of effectors. It is likely that CD8⁺ T cells may not mediate immunological memory against FV3 on their own since CD8⁺ MHC class II antigen-positive cells are seen to enter the kidney early upon FV3 reinfection. These could be CD4⁺ T cells and/or B cells. Elimination of FV3 upon secondary infection happens after day 3, 6 days before that observed in primary infection. Although this correlates with the time when CD8⁺ T cells enter the kidney, the low numbers of these infiltrating cells may not be sufficient for effective elimination of virally infected cells. Neutralizing anti-FV3-specific IgY (IgG equivalent) antibodies are detectable in serum upon secondary and tertiary infections for more than 8 weeks (25). This suggests synergy between antigen-specific memory CTLs and antibody-secreting B cells for inhibition of viral entry and replication and earlier eradication. Poxviruses, such as vaccinia virus, promote CTL activation upon primary infection and B-cell memory responses involving virus neutralization by specific antibodies early upon secondary infections (7). The close phylogenetic relatedness between iridoviruses and poxviruses suggests an ancient relationship with respective hosts and similar inductions of host immune responses.

In this regard, one has to keep in mind that although a lot of attention is focused on the importance of memory CD8⁺ T cells in the protection against secondary viral infection in mammals (12), B-cell memory and the long-term maintenance of an effective antibody response has to be accounted for (18). This is particularly true for poxviruses, such as smallpox, where the presence of neutralizing antibodies after vaccination correlates with protective immunity (7). In fact, CD8⁺ T cells have been shown to be unnecessary for the resistance of mice to secondary infection by ectomelia virus (27). The importance of antibody responses against FV3 is of fundamental as well as practical interest, since ranaviruses are large double-strand DNA viruses closely related to poxviruses (10) and ranaviral diseases are becoming increasingly prevalent in amphibian populations in captivity and in the wild (10, 11). We have shown the ability

of *Xenopus* to generate neutralizing IgY anti-FV3 antibody upon secondary, but not primary, infection (25) and that B-cell memory can last at least 14 months.

A third possibility is that resident primed T cells may exist in the kidney days or weeks after primary viral clearance. This may lessen the burden for selection in the spleen upon secondary infection since the FV3-specific cells would be maintained within the initial site of infection, like the effector memory cells of mammals (22).

In summary, our data confirm the crucial role of CD8⁺ T cells in antiviral immune responses and show that in *Xenopus*, as in mammals, CD8⁺ memory T cells are generated following a primary infection, contributing to a more rapid response kinetic and faster viral clearance upon secondary infection.

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