AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VIRUS-CELL INTERACTIONS

TLR5-Mediated Reactivation of Quiescent Ranavirus FV3 in *Xenopus* Peritoneal Macrophages

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ABSTRACT Ranaviruses such as frog virus 3 (FV3) are large double-stranded DNA (dsDNA) viruses causing emerging infectious diseases leading to extensive morbidity and mortality of amphibians and other ectothermic vertebrates worldwide. Among the hosts of FV3, some are highly susceptible, whereas others are resistant and asymptomatic carriers that can take part in disseminating the infectious virus. To date, the mechanisms involved in the processes of FV3 viral persistence associated with subclinical infection transitioning to lethal outbreaks remain unknown. Investigation in *Xenopus laevis* has revealed that in asymptomatic FV3 carrier animals, inflammation induced by heat-killed (HK) *Escherichia coli* stimulation can provoke the relapse of active infection. Since Toll-like receptors (TLRs) are critical for recognizing microbial molecular patterns, we investigated their possible involvement in inflammation-induced FV3 reactivation. Among the 10 different TLRs screened for changes in expression levels following FV3 infection and HK *E. coli* stimulation, only TLR5 and TLR22, both of which recognize bacterial products, showed differential expression, and only the TLR5 ligand flagellin was able to induce FV3 reactivation similarly to HK *E. coli*. Furthermore, only the TLR5 ligand flagellin induced FV3 reactivation in peritoneal macrophages both *in vitro* and *in vivo*. These data indicate that the TLR5 signaling pathway can trigger FV3 reactivation and suggest a role of secondary bacterial infections or microbiome alterations (stress or pollution) in initiating sudden deadly disease outbreaks in amphibian populations with detectable persistent asymptomatic ranavirus.

IMPORTANCE This study in the amphibian *Xenopus laevis* provides new evidence of the critical role of macrophages in the persistence of ranaviruses in a quiescent state as well as in the reactivation of these pathogens into a virulent infection. Among the multiple microbial sensors expressed by macrophages, our data underscore the preponderant involvement of TLR5 stimulation in triggering the reactivation of quiescent FV3 in resident peritoneal macrophages, unveiling a mechanistic connection between the reactivation of persisting ranavirus infection and bacterial coinfection. This suggests a role for secondary bacterial infections or microbiome alterations (stress or pollution) in initiating sudden deadly disease outbreaks in amphibian populations with detectable persistent asymptomatic ranavirus.

KEYWORDS amphibian decline, infectious diseases, innate receptors, TLR, viral persistence, ranavirus

Ranaviruses, including its type species frog virus 3 (FV3), are large double-stranded DNA (dsDNA) viruses that belong to the family *Iridoviridae*. Within the last 5 decades, ranaviruses have become major pathogens of a wide variety of amphibian and reptile species, causing extensive outbreaks and morbidity worldwide (reviewed in

Citation Samanta M, Yim J, De Jesús Andino F, Paiola M, Robert J. 2021. TLR5-mediated reactivation of quiescent ranavirus FV3 in *Xenopus* peritoneal macrophages. J Virol 95: e00215-21. https://doi.org/10.1128/JVI.00215-21.

Editor Jae U. Jung, Lerner Research Institute, Cleveland Clinic

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Received 10 February 2021
Accepted 28 March 2021
Accepted manuscript posted online 7 April 2021
Published 24 May 2021

references 1–3). Since first being detected in amphibians in the 1960s and fish in the 1980s, ranaviruses have been reported to infect at least 175 species across 52 families of ectothermic vertebrates (1, 4).

In addition to the apparent increase in geographic and host ranges, ranaviruses have been associated with the population decline of amphibians around the world, affecting both ecology and international commerce (3). For example, populations of the common frog (*Rana temporaria*) in the United Kingdom have declined on average 80% where ranavirus die-offs have occurred (5). In addition, animals that are involved in international trade for human consumption or pet trade were found to harbor amphibian ranavirus (6). While some impacted species are economically important (e.g., rainbow trout and bullfrogs), many species are currently endangered (e.g., Chinese giant salamander and dusky gopher frog) (2, 7, 8). Because of these threats, ranaviruses have been designated "notifiable pathogens" by the World Organization for Animal Health (OIE) (https://www.oie.int) and must be reported to this organization by member countries.

Although some host species are highly susceptible to ranavirus, others are more resistant and can serve as asymptomatic carriers that disseminate infectious virus (9–11). While the ranavirus prevalence in some populations can reach over 80%, it does not always translate into a high death rate (12). The transition from asymptomatic or subclinical infection to deadly outbreaks is often unpredictable, and the host, viral, and environmental factors critical for this switch are poorly understood. Among host factors, macrophages (Mϕ) are innate immune effectors central to antiviral host defense (13, 14). However, macrophages are also infected by some ranavirus strains, and data from several studies suggest that macrophages can serve as a reservoir for viral persistence (15–18).

Previous studies in the amphibian *Xenopus laevis* have shown that genomic DNA of the ranavirus FV3 can be detected in frogs, months after infection, and could also be found in healthy animals from various suppliers in the United States that were not experimentally infected (10). Further studies revealed that although the immune system of adult frogs is efficient in controlling FV3 infection, quiescent, transcriptionally inactive virus is found in peritoneal macrophages (pMϕ) of otherwise healthy asymptomatic frogs (18). Further studies have revealed that inflammation and leukocyte infiltration induced by intraperitoneal (i.p.) injection of heat-killed (HK) *Escherichia coli* reactivate FV3 in asymptomatic adult frogs, which leads to active systemic infection that is often lethal (19). However, to date, the mechanism involved in HK *E. coli*-induced reactivation has remained unclear.

Microbial and viral products can be detected by various innate receptors, including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), which are critical to initiate inflammatory immune responses (20–22). Thus, we decided to investigate the possible involvement of TLRs and NLRs in the inflammation-induced reactivation of FV3. The ability of TLRs to enhance or even initiate the reactivation of latent or quiescent viral infection has been shown in mice, where the stimulation of latently murine gammaherpesvirus 68 (MHV68)-infected B cell lines with ligands for TLR3, -4, -5, and -9 enhanced viral reactivation (23). In another study, TLR3 ligand-stimulated reactivation of Epstein-Barr virus by inducing type I interferon (IFN) and proinflammatory cytokines was reported (24). While some of these TLRs (e.g., TLR3 and -9) can detect viral products, other TLRs (e.g., TLR4 and -5) are directed more toward bacterial products, which raises the possibility of some connections between viral and bacterial coinfections (reviewed in reference 22). Here, we report new evidence suggesting a mechanistic connection between the reactivation of persisting ranavirus infection and bacterial coinfection.

RESULTS

Screening for TLR or NLR gene involvement in FV3 reactivation in asymptomatic adults. To investigate whether particular TLRs or NLRs are involved in viral reactivation, we first assessed the differential expression of TLR and/or NLR genes during FV3 reactivation. Adult frogs were infected with a sublethal dose (10^6 PFU) of FV3 and

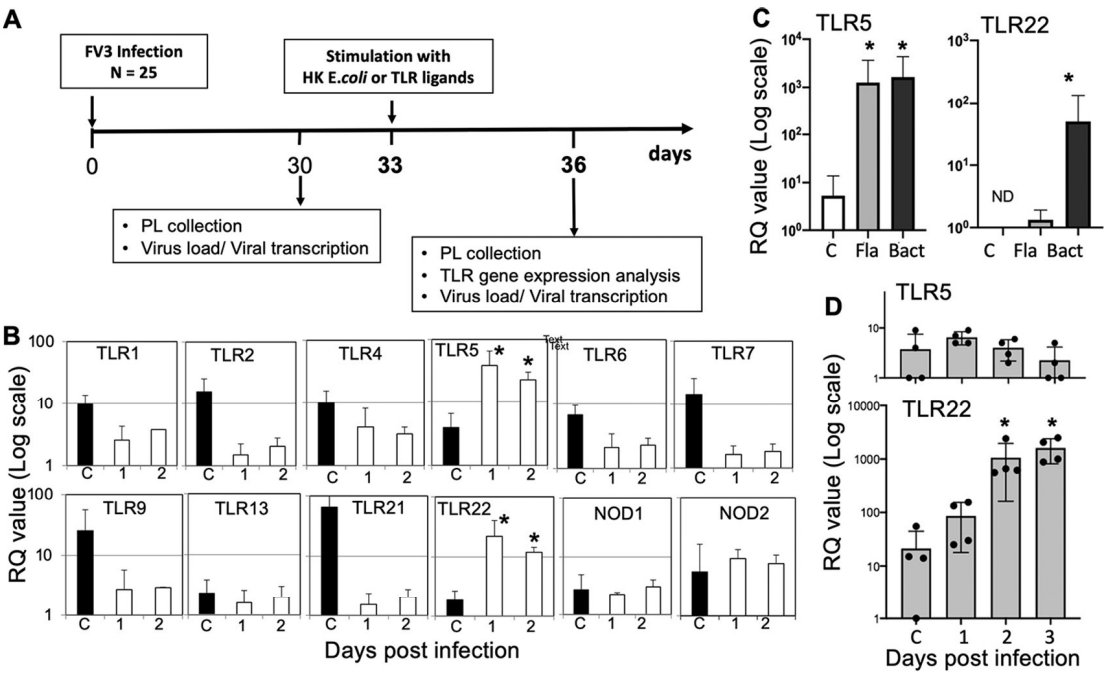

FIG 1 TLR and NOD differential gene expression in PLs of infected asymptomatic frogs following stimulation with HK *E. coli*. (A) Experimental outline. (B) Relative gene expression levels of TLR1, -2, -4, -5, -6, -7, -9, -13, -21, and -22 and NOD1 and -2 of PLs from FV3 carrier frogs treated with HK *E. coli* for 1 or 2 days or injected with APBS as a control (C). (C) TRL5 and TLR22 relative gene expression levels 3 days after i.p. injection of the TLR5 ligand flagellin (Fla), HK *E. coli* (Bact [bacteria]), or APBS as a control. (D) TLR5 and TLR22 relative gene expression levels 1, 2, and 3 days after i.p. injection of *E. coli* DNase-treated RNA (TLR22 ligand) or APBS as a control. Gene relative quantification (RQ) was determined as the fold increase relative to the GAPDH endogenous control. Bars represent standard deviations ($n = 3$ animals). Statistical significance between control and treated groups is denoted by *, where the *P* value was <0.05 using one-way ANOVA and Tukey's *post hoc* test. ND, not determined.

maintained for 30 days to ensure viral clearance. At 33 days postinfection (dpi), animals were injected with HK *E. coli* (or APBS [amphibian phosphate buffer solution] as a negative control), and peritoneal leukocytes (PLs) were isolated 1 and 2 days later (Fig. 1A). The relative gene expression levels were examined for 10 different TLRs and 2 NLRs, and among these, only the TLR5 and TLR22 genes showed noticeable induction at both days poststimulation, suggesting their involvement in HK *E. coli* pathogen-associated molecular pattern (PAMP) recognition (Fig. 1B).

Since little is known about TLR biology in *X. laevis*, we further determined the specificity of TLR5 and TLR22 stimulation in the adult frog peritoneum using their cognate ligands. In mammals, TLR5 is well known to recognize bacterial flagellin from invading motile bacteria (25). Therefore, we assessed whether stimulation with purified flagellin can similarly induce TLR5 gene expression in *X. laevis*. Indeed, i.p. injection of flagellin at a dose comparable to that in mouse (4 μg/30 g animals) resulted in a sharp increase within 3 days of TLR5 transcript levels in PLs that was comparable to that with stimulation with HK *E. coli* but not the TLR22 gene (Fig. 1C). TLR22, which is present in fish and amphibians but not in mammals, has been shown in fish to recognize bacterial RNA (26–28). To test whether *X. laevis* TLR22 would similarly be sensing bacterial RNA, we i.p. injected DNase-treated *E. coli* RNA (15 μg) and determined the relative gene expression response. A significant increase in TLR22 but not TLR5 transcript levels was detected at 2 and 3 days postinjection (Fig. 1D).

We then further characterized the cellular response to TLR5 and TLR22 stimulation. The injection of both flagellin and *E. coli* RNA induced rapid and sizable leukocyte infiltration in the peritoneum that was mainly composed of macrophages and, to a lesser extent, neutrophils (Fig. 2A to F). Macrophages exhibited an activated and vacuolated morphology.

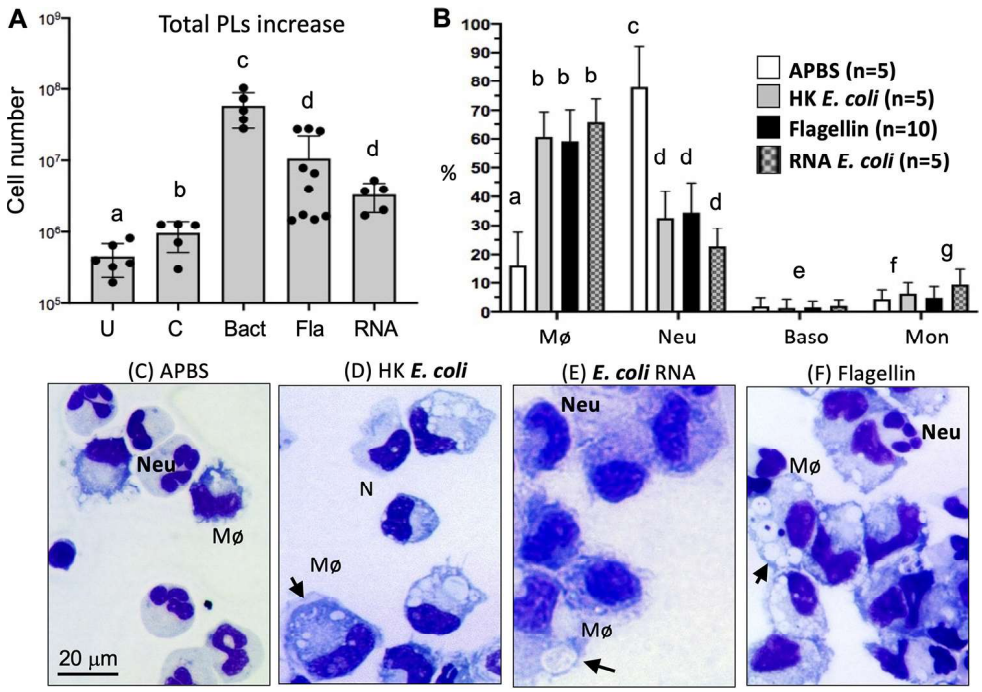

FIG 2 Cell number (A), composition (B), and morphology (C to F) of peritoneal leukocytes (PLs) recovered after stimulation by i.p. injection of different stimulants. (A) Total number of cells recovered from PLs of untreated (U) frogs 3 days after i.p. injection with either APBS (control [C]), HK *E. coli* (Bact), flagellin (Fla), or *E. coli* RNA (RNA). (B) Cell composition of the peritoneal exudates from the different treatment groups. (C to F) Cytospin analysis of cells from the peritoneal exudates from the different treatment groups stained by Giemsa stain: APBS (C), HK *E. coli* (D), *E. coli* RNA (E), and flagellin (F). The collected cells (200,000) were centrifuged on a microscope slide, fixed in methanol, and stained with Giemsa stain. Mϕ, macrophages; Neu or N, neutrophils; Baso, basophils; Mon, monocytes. Bars represent standard deviations ($n = 6$ to 9 animals). Statistical significance is denoted by different lowercase letters, where means that differ significantly ($P < 0.05$ using one-way ANOVA and Tukey's *post hoc* test) have different letters from one another, while means that do not significantly differ have the same letter.

TLR5 and -22 involvement in FV3 reactivation *in vivo*. To determine whether TLR5 and TLR22 signal transduction pathways can promote the reactivation of quiescent FV3 in the peritoneal cavity of *X. laevis*, we activated these receptors with their specific ligands. FV3 asymptomatic *X. laevis* carriers were either mock treated with APBS or treated with TLR5 ligand (flagellin at 4 μg/frog), TLR22 ligand (*E. coli* total RNA at 15 μg/frog), or HK *E. coli* as a positive control, and the reactivation of FV3 was investigated by determining the FV3 genome copy number and FV3 transcriptional activity at a later time point (days poststimulation) to allow more time for FV3 to replicate in PLs.

At 5 days poststimulation in PLs, TLR5 stimulation resulted in an increase in the viral load as marked as that with HK *E. coli* (over 1 log), whereas TLR22 bacterial RNA ligand was ineffective (Fig. 3A). FV3 reactivation by flagellin but not *E. coli* RNA was further evidenced by the significant increases in early (viral DNA polymerase II [vDNA Pol II]) and late (major capsid protein [MCP]) FV3 transcript levels (Fig. 3B and C). Consistent with these findings, TLR5 gene expression levels 5 days after stimulation with flagellin remained higher than with of the control, *E. coli* RNA, or HK *E. coli* (Fig. 3D and E). In contrast, TLR22 gene expression was only minimally sustained at this time point in animals stimulated with *E. coli* RNA, and even HK *E. coli* stimulations were ineffective (Fig. 3F).

To further examine the reactivation process induced by TLR5 and TLR22 stimulation, we determined the relative gene expression levels of several cytokine genes in PLs. Flagellin and *E. coli* RNA induced the expression of the tumor necrosis factor (TNF-α) gene comparably to HK *E. coli* but did not induce much expression of interleukin-1β

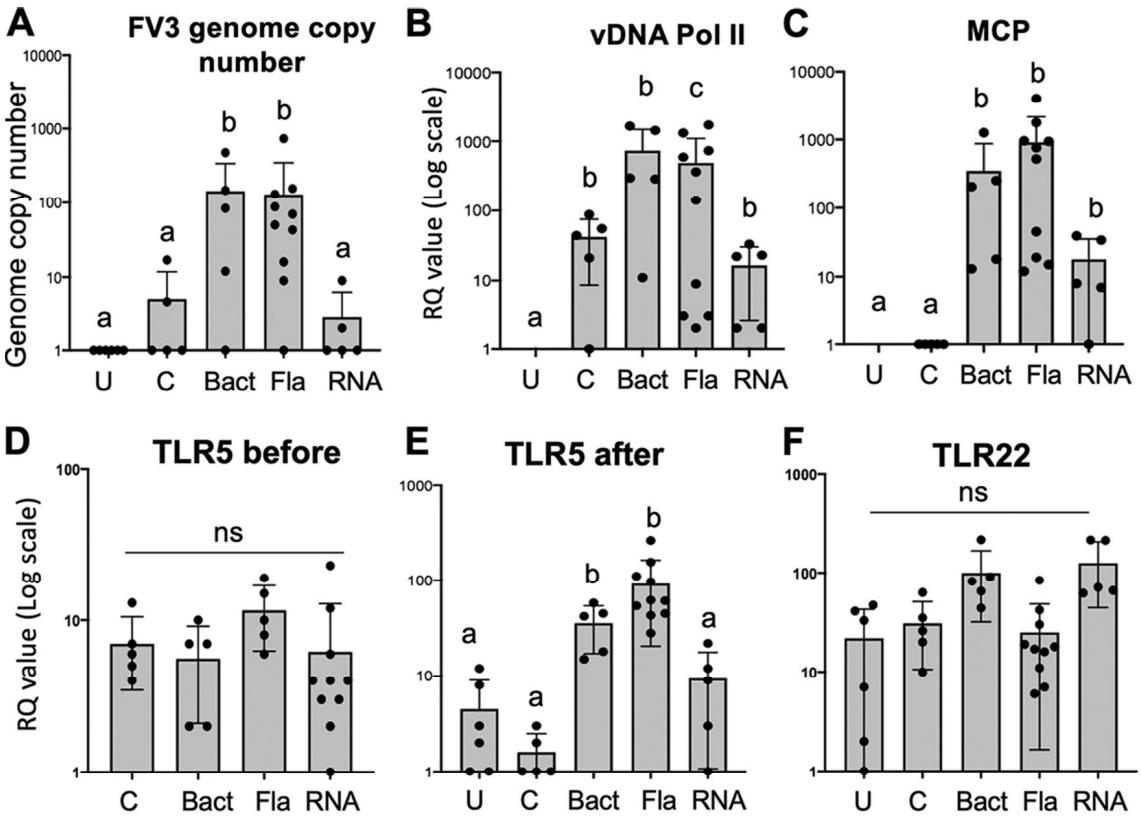

FIG 3 FV3 reactivation induced by TLR5 ligand (flagellin) but not TLR22 ligand (*E. coli* RNA). Asymptomatic frogs, 30 days after FV3 infection, were i.p. injected with either APBS (control [C]), HK *E. coli* (Bact), flagellin (Fla), or *E. coli* RNA (RNA), and 3 days later, genomic DNA and RNA from the collected PLs were used to determine the viral load (A), transcription of vDNA Pol II (B) and MCP (C), as well as eukaryotic gene expression of TLR5 before (D) and after (E) stimulation and TLR22 after stimulation (F). The genome copy number was determined by absolute qPCR. Gene relative quantification (RQ) was determined as the fold increase relative to the GAPDH endogenous control and normalized to the lowest expression level. Bars represent standard deviations ($n = 6$ to 9 animals). Statistical significance is denoted by different lowercase letters, where means that differ significantly ($P < 0.05$ using one-way ANOVA and Tukey's *post hoc* test) have different letters from one another, while means that do not significantly differ have the same letter. ns, no significance.

(IL-1β), whereas no significant increases in IFN-I transcript levels were detected at this early stage of FV3 reactivation (Fig. 4, top). A notable distinctive response to TLR22 stimulation was the high-level induction of inducible nitric oxide synthase (iNOS) that was not observed with TLR5 or HK *E. coli* (Fig. 4, bottom).

These results suggest that TLR5 but not TLR22 plays a critical and distinctive role in the reactivation of quiescent FV3.

TLR5-mediated FV3 reactivation *in vitro*. Our previous studies have suggested that peritoneal macrophages (pMϕ) residing in the peritoneal cavity harbor quiescent FV3 (18). Stimulation by i.p. injection of HK *E. coli* or TLR5 induced both the recruitment and activation of these macrophages. To obtain more direct evidence that FV3 persisting in peritoneal leukocytes present in the peritoneal cavity for over a month could be reactivated, we developed an *in vitro* experimental system (Fig. 5). Cells from the peritoneal cavity of previously FV3-infected asymptomatic frogs were collected by lavage without prior stimulation, rested in amphibian cell culture medium for 3 days, and then activated with flagellin for 1 to 3 more days (Fig. 5A). Compared to the control PL culture (cultured only in medium for 6 days), there was a significant increase in the viral load in the flagellin-stimulated cell lysates at 3 days poststimulation (Fig. 5B). The efficacy of flagellin in activating TLR5 was also confirmed in PLs (Fig. 5C). To obtain more quantitative data, the amounts of infectious particles in culture supernatants at 3 days poststimulation were also determined by a 50% tissue culture infective dose ($TCID_{50}$) assay and shown to be markedly increased (Fig. 5D).

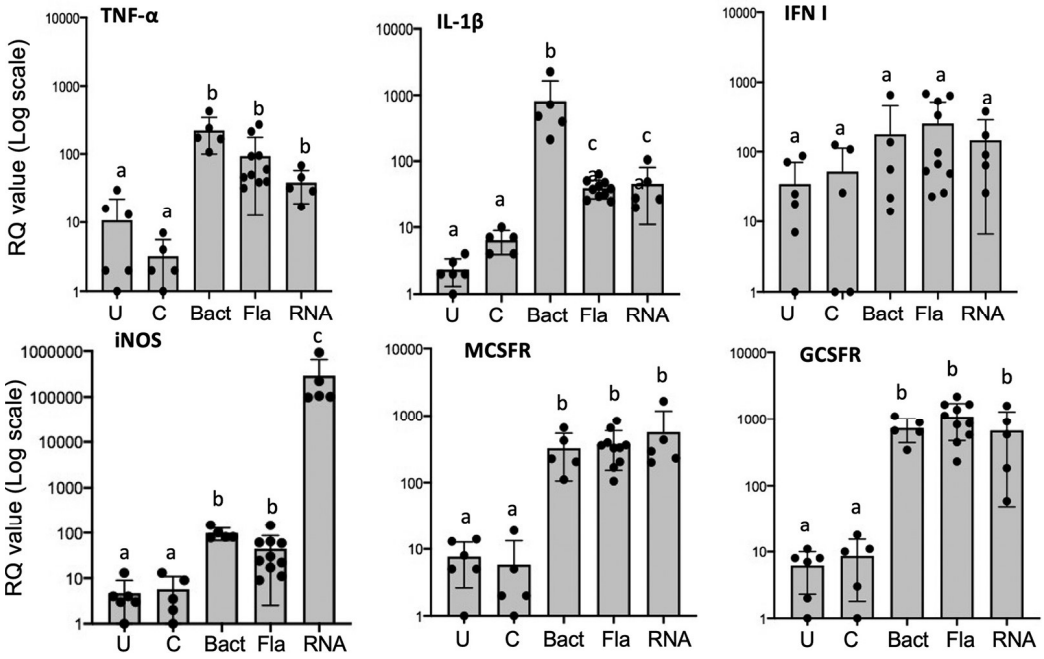

FIG 4 Relative gene expression levels of genes encoding antiviral cytokines and leukocyte receptors in FV3 carrier frogs stimulated with APBS (control [C]), HK *E. coli* (Bact), flagellin (Fla), or *E. coli* RNA (RNA) or untreated frogs (U). Asymptomatic frogs, 30 days after FV3 infection, were i.p. injected with the different stimulants, and 3 days later, RNAs from the collected PLs were used to determine gene expression for TNF-α, IL-1β, IFN-I, iNOS, macrophage colony stimulation factor 1 receptor (MCSF-1R), and GCSF-1R. Gene relative quantification (RQ) was determined as the fold increase relative to the GAPDH endogenous control and normalized to the lowest expression level. Bars represent standard deviations ($n = 6$ to 9 animals). Statistical significance is denoted by different lowercase letters, where means that differ significantly ($P < 0.05$ using one-way ANOVA and Tukey's *post hoc* test) have different letters from one another, while means that do not significantly differ have the same letter.

While there is no antibody specific for macrophage surface markers available in *Xenopus* to date, we have shown that macrophages express a bona fide CSF-1 receptor (CSF-1R) and characterized its ligand CSF-1 using tagged *X. laevis* recombinant CSF-1 (29). We used this recombinant biotinylated XlCSF-1 in combination with an *X. laevis*-specific anti-major histocompatibility complex class II (MHC-II) monoclonal antibody (mAb) to identify and sort pMϕ by flow cytometry (Fig. 6A). Sorted CSF-1-positive (CSF-1$^+$)/MHC-II$^+$ cells showed typical macrophage morphology (Fig. 6B), whereas double-negative cells were mainly composed of neutrophils (Fig. 6C). This distinction was confirmed by the prominent expression of the CSF-1R gene by CSF-1$^+$/MHC-II$^+$ cells, in contrast to the high transcript levels of CSF-3R or granulocyte colony stimulation factor (GCSF) and CXCR1, both of which are markers of neutrophils, by double-negative cells (Fig. 6D to F). While flagellin stimulation significantly induced TLR5 gene expression in both leukocyte subsets (Fig. 6G), FV3 reactivation was obtained only with the Mϕ subset, as indicated by the active transcription of both early (vDNA Pol II) and late (MCP) viral genes as well as the sizable production of infectious particles detected by a $TCID_{50}$ assay (Fig. 6H to J). These data strongly suggest that quiescent FV3 persisting in Mϕ residing in the peritoneal cavity can be reactivated by the TLR5 signaling pathway.

DISCUSSION

This study provides new evidence of the critical role of macrophages in the persistence of ranavirus in a quiescent state as well as in the reactivation of these pathogens into a virulent infection. Among the multiple microbial sensors expressed by macrophages, our data underscore the preponderant involvement of TLR5 stimulation in triggering the reactivation of quiescent FV3. Furthermore, our demonstration that FV3

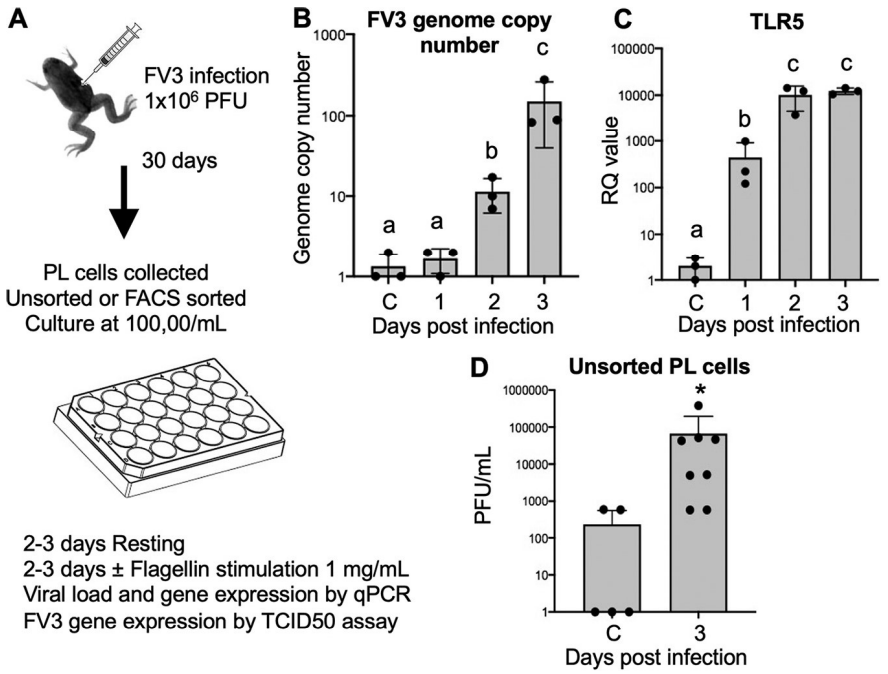

FIG 5 *In vitro* FV3 reactivation by stimulation with flagellin. (A) Experimental outline. FACS, fluorescence-activated cell sorter. (B) FV3 genome copy number. (C) Relative TLR5 gene expression. (D) Number of infectious particles determined by a $TCID_{50}$ assay of PLs harvested from asymptomatic FV3 carrier frogs rested in culture for 3 days and then stimulated with flagellin (1 μg/ml) for 1, 2, or 3 days or without flagellin for 3 days (control [C]). Bars represent standard deviations ($n = 3$ [B and C] and 6 [D]). *, $P = 0.05$ between control and treated groups. Gene relative quantification (RQ) was determined as the fold increase relative to the GAPDH endogenous control and normalized to the lowest expression level. Bars represent standard deviations ($n = 5$ to 8 animals). Statistical significance is denoted by different lowercase letters or *, where means that differ significantly ($P < 0.05$ using one-way ANOVA and Tukey's *post hoc* test) have different letters from one another, while means that do not significantly differ have the same letter.

present within a peritoneal macrophage subset coexpressing surface CSF-1R and MHC class II can be reactivated *in vitro* by stimulation with the TLR5 ligand flagellin suggests that resident peritoneal macrophages are implicated in ranavirus persistence.

Our screening methodology based on the significant increase in transcript levels following bacterial stimulation of asymptomatic FV3 carriers does not rule out a critical role of other TLRs and NOD in the host response to FV3 since the activities of these microbial sensors are regulated at multiple levels, including phosphorylation (20, 21). However, the marked increase in TLR5 and TLR22 induced by HK *E. coli* clearly distinguished these two receptors from the others. Similar to mammals, our data show that bacterial flagellin readily and specifically activates *X. laevis* TLR5. Although we did not characterize TLR5 signaling in detail, flagellin resulted in the expected response of proinflammatory genes such as TNF-α, iNOS, and IL-1β. Similarly, TLR22, which is not present in mammals and to date has been investigated only in several fish species, was activated by bacterial RNA in *Xenopus* and induced the expression of proinflammatory genes. Both TLR5 and TLR22 ligands also stimulated an active cellular response in the peritoneal cavity, including the recruitment of neutrophils and monocytic phagocytes exhibiting activation morphology. It is interesting to note that TLR22 in fish recognized both bacterial and viral RNAs (26, 27, 30). Whether TLR22 is involved in the *X. laevis* host response to FV3 remains to be determined. However, it is clear from our data that TLR22 ligands were unable to induce FV3 reactivation in asymptomatic FV3 carriers.

Indeed, TLR5 was the main pathogen recognition receptor gene induced in asymptomatic PLs by bacterial stimulation, and only the TLR5 ligand flagellin was as efficient as whole HK *E. coli* in reactivating quiescent FV3 into a transcriptionally active virus.

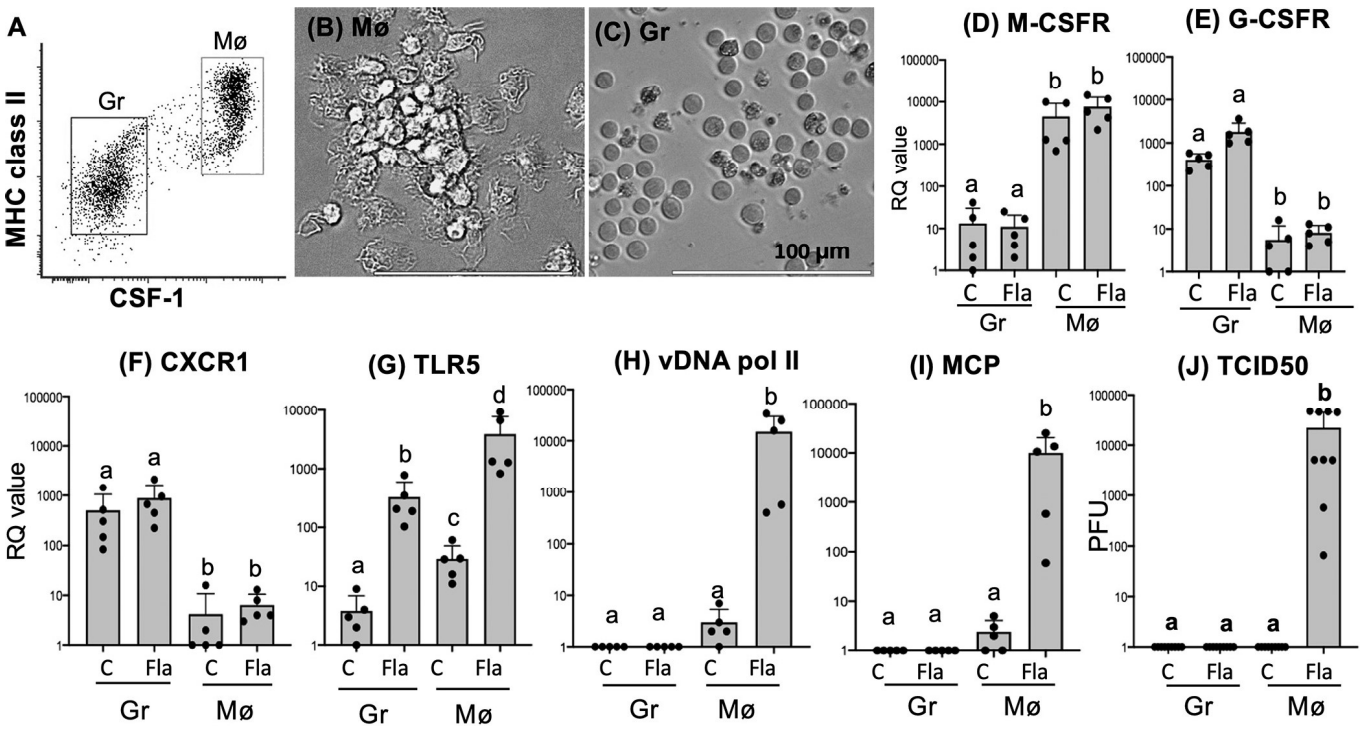

FIG 6 *In vitro* FV3 reactivation of sorted peritoneal macrophages and granulocytes by stimulation with flagellin. (A) Representative flow cytometry plot of PLs costained with recombinant CSF-1 followed by anti-V5 and FITC anti-mouse antibodies and biotinylated anti-*X. laevis* MHC class II mAb (AM20), followed by APC-conjugated streptavidin. Cells of greater size and complexity (myeloid cells) were gated for cell sorting. (B and C) Representative morphologies of sorted CSF-1$^+$/MHC-II$^+$ macrophages (Mϕ) (B) and double-negative granulocytes (Gr) (C) after stimulation with flagellin under a phase-contrast microscope. (D to I) The relative gene expression levels of MCSF-1R (D), GCSF-1R (E), CXCR1 (F), TLR5 (G), vDNA Pol II (H), and MCP (I) were determined by qPCR on sorted macrophages and granulocytes. (J) Production of infectious particles determined by a $TCID_{50}$ assay. Bars represent standard deviations (n = 9 animals). Statistical significance is denoted by different lowercase letters, where means that differ significantly ($P < 0.05$ using one-way ANOVA and Tukey's *post hoc* test) have different letters from one another, while means that do not significantly differ have the same letter.

Moreover, FV3 reactivation *in vitro* was obtained with sorted peritoneal macrophages but not granulocytes. Thus, while FV3 reactivation in asymptomatic frogs by HK *E. coli* in our initial study could be due to inflammation and/or leukocyte recruitment from other tissues and organs, the present data rather suggest direct TLR5-mediated triggering of FV3 persisting in resident peritoneal macrophages. These macrophages are characterized by the presence at the cell surface of CSF-R1, a high level of MHC class II surface expression, and a higher gene expression level of TLR5.

Recent studies on TLR expression and signaling cascades have suggested that the activation of TLRs induces not only protective effects against viral infection but also effects that contribute to viral pathogenesis (24, 31, 32). It is possible that TLR signaling is only one of the different pathways that lead to ranavirus FV3 reactivation. TLR signaling leads to the synthesis of a plethora of proinflammatory cytokines, which have autocrine and paracrine effects that may contribute to viral dissemination and expansion. Furthermore, the activation of the TLR signaling pathway can induce the expression of lectin, chemokines, and chemokine receptor genes that regulate cell migration to the inflammation site (33). The precise mechanism of how ranavirus utilizes the TLR5 signaling pathway to reactivate from its quiescent state remains to be studied.

Our present findings as well as our recent work on inflammation-mediated FV3 reactivation converge to reveal the viral immune evasion strategy that ranavirus FV3 utilizes (19). In mammals, RNA viruses such as HIV and measles virus infect macrophage subsets to evade immunity and persist in a latent or quiescent state within their hosts (34, 35). In a similar manner, FV3 evades host immune detection by disseminating and remaining in a quiescent and nonreplicating state. In the present study, we demonstrate that stimulation with a specific bacterial component, flagellin, can elicit the reactivation of the virus. Our previous FV3 infection study showed that *X. laevis*, which is

generally resistant to FV3 infections, can be an asymptomatic carrier, suggesting that it could serve as a vector of viral dissemination in the wild (10). In a subsequent study, we demonstrated that peritoneal macrophages can harbor quiescent FV3 infections for at least 3 weeks (18). These findings have relevance for global ranavirus outbreaks. Given their worldwide distribution and their ability to cause disease in numerous anuran and caudate species, ranaviruses present a significant risk to amphibian populations. Notably, ranaviral pathogens have been associated with mass die-offs in both the wild and aquaculture, causing a profound impact on commerce and international trade (36). Since ranaviruses have evolved to bypass established immune barriers by establishing a quiescent state, defining the physiological stimuli of viral reactivation from the quiescent state is a fundamental question in ranaviral epidemiology. In addition to heterologous infections triggering ranaviral reactivation, chronic inflammation may also be induced by varieties of physiological stressors such as resource competition, habitat perturbation, and pollution. It is possible that both natural and environmental stressors induce chronic inflammation and thus contribute to the rapid dissemination and expansion of these pathogens.

As such, the model system established in *X. laevis* with FV3 will be instrumental for elucidating the mechanisms of persistence and dissemination of ranaviral pathogens.

MATERIALS AND METHODS

Animals. All animals were obtained from the *X. laevis* research resource for immunology at the University of Rochester (https://www.urmc.rochester.edu/microbiology-immunology/xenopus-laevis.aspx). All animals were handled in accordance with stringent laboratory and University Committee on Animal Research regulations (approval number 100577/2003-151).

FV3 stocks and animal infections. FV3 was grown using a single passage through baby hamster kidney cells (BHK-21; ATCC CCL-10) and subsequently purified by ultracentrifugation on a 30% sucrose cushion as previously described (19). Adult frogs were infected by intraperitoneal (i.p.) injection of 1×10^6 PFU in a volume of 100 μl.

Bacterial stimulation. *E. coli* cells (XL1-Blue; Stratagene, La Jolla, CA) were cultured overnight at 37°C, boiled for 1 h, pelleted by centrifugation, and resuspended in 1/10 of the initial volume (approximately 1,500 bacteria/ml) of APBS (amphibian phosphate buffer solution). Asymptomatic infected frogs were injected i.p. with 300 μl of the heat-killed bacterial mixture (35,000 bacteria, corresponding to 3 μg of protein). Peritoneal leukocytes and kidneys were then harvested at day 3 poststimulation.

***In vivo* stimulation with TLR5 and TLR22 ligands.** Based on the dose per gram of tissue that is typically used in mouse models (23) and pilot experiments, we used 4.0 μg per ~30 g frog of flagellin (ultrapure flagellin from *Salmonella enterica* serovar Typhimurium; InvivoGen) injected i.p. Total RNA was isolated from *E. coli* using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Next, the RNA was purified following DNase (Ambion, Life Technologies) treatment. Stimulation was performed by injecting 15 μg of *E. coli* RNA.

For stimulation/reactivation, at 32 days postinfection, asymptomatic infected frogs were injected i.p. with either 4 μg flagellin or 15 μg of *E. coli* RNA in 200 μl of APBS. Peritoneal leukocytes and kidneys were then harvested at 3 days poststimulation.

***In vitro* reactivation.** Total or sorted peritoneal leukocytes (PLs) from six asymptomatic infected frogs (two independent experiments) were seeded and rested for 3 days in a 48-well culture at 100,000 cells per well in 300 μl of *Xenopus* Iscove-derived, serum-free medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 27°C (37). Cells were then stimulated for 1 to 3 days by adding 1 μg flagellin or APBS as a mock control. For cell sorting, PLs were stained on ice in APBS containing 1% bovine serum albumin (BSA) and 0.05% sodium azide with a biotinylated *Xenopus* anti-MHC class II mAb (AM20) (38) for 30 min, followed by a 1/100 dilution of allophycocyanin (APC)-conjugated streptavidin (BD Pharmingen). Cells were then incubated with 1 μg of *X. laevis* recombinant CSF-1 (29) for 1 h, followed by incubation with a 1/400 dilution of anti-V5 antibody (Invitrogen) and staining with a 1/400 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody. Cell sorting was performed by gating the cells of greater size and complexity, i.e., myeloid cells, using 4-laser BD FACSAria II and BD FACSDiva software.

Cytospin and cell staining. PLs (2×10^5 cells in a 200-μl volume) were cytocentrifuged using a Shandon Southern cytospin centrifuge (600 rpm for 5 min). A Giemsa solution (catalog number 48900; Fluka) was added directly on the cytospin slide, incubated for 1 min, and washed with APBS-diluted distilled water. Cells were then washed with double-distilled water (ddH_2O) and ethanol (EtOH). After washing, cytospin slides were dried and mounted with Permount (catalog number SO-P-15; Fisher Scientific).

Viral load quantification by qPCR and $TCID_{50}$ assays. The FV3 genome copy number was determined by absolute quantitative PCR (qPCR) by analysis of DNA isolated from PLs by TRIzol and further DNA cleaning. The transcript levels were compared to a serially diluted standard curve of an FV3 DNA Pol II PCR fragment cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). This construct was quantified and serially diluted to yield 10^{10} to 10^1 plasmid copies of FV3 DNA polymerase II. These

TABLE 1 qPCR primer sequences[a]

Primer	Primer direction, sequence
Housekeeping genes	
GAPDH	F, 5′-GACATCAAGGCCGCCATTAAGACT-3′ R, 5′-AGATGGAGGAGTGAGTGTCACCAT-3′
EF-1α	F, 5′-GCCTTCCCAGGGTTCAACAT-3′ R, 5′-TCGGTGGGTCACTCTTTGAAT-3′
Proinflammatory cytokine genes	
TNF-α	F, 5′-TGTCAGGCAGGAAAGAAGCA-3′ R, 5′-CAGCAGAGCAAAGAGGATGGT-3′
IL-1β	F, 5′-CATTCCCATGGAGGGCTACA-3′ R, 5′-TGACTGCCACTGAGCAGCAT-3′
Type I IFN	F, 5′-GCTGCTCCTGCTCAGTCTCA-3′ R, 5′-GAAAGCCTTCAGGATCTGTGTGT-3′
MCSF-R	F, 5′-GGCCTCAGTGCGCTTATATGTCAA-3′ R, 5′-AAGCAGGGTAGAGTGGCATCTTTG-3′
GCSF-R	F, 5′-ACGTGCCAGCTAAACCTCACAGAT-3′ R, 5′-TGACACAGCCTGGGCGAGAAATAA-3′
CXCR	F, 5′-TTGACTTCAGTGACATTCCTACA-3′ R, 5′-ATGACCAGAACCACAAGGCT-3′
iNOS	F, 5′-AACCGTAAGCCAAAGAAGGA-3′ R, 5′-TGGTTCTGGCAGCCACAGT-3′
FV3	
FV3 vDNA polymerase II (60R)	F, 5′-ACGAGCCCGACGAAGACTACA-3′ R, 5′-TGGTGGTCCTCAGCATCCT-3′
FV3 MCP (90R)	F, 5′-GTCCTTTAACACGGCATACCT-3′ R, 5′-ATCGCTGGTGTTGCCTATC-3′
TLRs	
TLR1	F, 5′-ATGGACTTCTCCGTGACAATAA-3′ R, 5′-GTTGATAGGAAGGCAACCAAAC-3′
TLR2	F, 5′-GCCATGGAGAAGAGCTACAA-3′ R, 5′-CAAAGAGACGGAAGTGAGAGAA-3′
TLR3	F, 5′-AGTTGGCTTAACACAACGAATG-3′ R, 5′-CTTGCAGGGAGAGCTATCAAA-3′
TLR4	F, 5′-CTGCATTCACGAGAGAGACTTC-3′ R, 5′-CGAAGTTGTGGGAGAGAACAA-3′
TLR5	F, 5′-AGGATGGGTGGTGTGTAGAA-3′ R, 5′-TCATTAGCTGGTACTGGGAGAG-3′
TLR6	F, 5′-CAGTCAGGAAGACTCAGAATGG-3′ R, 5′-CAATGATTGCTTTGCCAGGAATA-3′
TLR7	F, 5′-TATATTGCTCACCAGCGTCTC-3′ R, 5′-AACAGAGATAGCACACAATCTT-3′
TLR8	F, 5′-GCTGGCACAAAGTCTGTAAATC-3′ R, 5′-CCTGTTCTCCAAGTGATGACATA-3′

(Continued on next page)

TABLE 1 (Continued)

Primer	Primer direction, sequence
TLR9	F, 5′-AAGAAAGATGTGGTGGTCTTAGT-3′ R, 5′-TACGTGGCCAGTTCAAGAAG-3′
TLR12	F, 5′-GTGACTGGTGCTCTTTAGAAATG-3′ R, 5′-GCCGACAAATGATAACCAGAAA-3′
TLR13	F, 5′-CCAAGGAAACCCAAGTCTGT-3′ R, 5′-TTGGAGGTTCCGTCATTGTATAG-3′
TLR21	F, 5′-AGTGAGTGGTGCTCCTTAGA-3′ R, 5′-GAAAGCTCTCTGTCTGGGATTT-3′
TLR22	F, 5′-GCGTTATCAGCAGGCACTAT-3′ R, 5′-CCAGAAACAACAGAATCAGAACAC-3′
NOD1	F, 5′-TGCGAGCTGTGTTGTGATGTGAA-3′ R, 5′-TGCCTGCATCACCAAAGACAAAG-3′
NOD2	F, 5′-CTTCATCTTTGCCAGGCCCTTTA-3′ R, 5′-TCCCCAGCCTCAATGCAAGAA-3′
NLRP3	F, 5′-TTGCAACGGGGGGAATACATG-3′ R, 5′-AGTTTCCCATTGGCCCAGTCATA-3′

[a]F, Forward primer; R, reverse primer; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IFN, interferon; MCSF-R, Mϕ colony stimulation factor receptor 1; GCSF-R, granulocyte colony stimulation factor receptor 1; FV3, frog virus 3; TLRs, Toll-like receptors.

dilutions were employed as a standard curve in subsequent absolute qPCR experiments to derive the viral genome transcript copy numbers relative to this standard curve.

Infectious particles from harvested PLs were quantified by a plaque assay performed on BHK-21 monolayers in 6-well plates under an overlay of 1% methylcellulose (19). Infected cells were cultured for 7 days at 30°C in 5% CO_2. Overlay medium was aspirated, and cells were stained for 10 min with 1% crystal violet in 20% ethanol. The number of infectious viral particles *in vitro* following reactivation was determined using the *X. laevis* kidney A6 cell line by the 50% endpoint dilution ($TCID_{50}$) method (39, 40).

Quantitative gene expression analyses. Total RNA was extracted from the PLs and kidneys by using TRIzol reagent according to the manufacturer's protocol (Invitrogen). RNA (1 μg) was used to synthesize cDNA by Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) with a mixture of oligo(dT) primers (Invitrogen). For reverse transcription (RT)-PCR, 125 ng of cDNA was used to determine the expression levels of genes of interest by determining $\Delta\Delta C_T$ values with an ABI 7300 real-time PCR system and PerfeCTa SYBR green FastMix ROX. The expression levels were normalized to that of an endogenous housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then further normalized against the lowest observed expression level. Primers specific for 13 different TLRs and 3 NLRs were designed and validated by gradient PCR and with the qPCR melting curves. All the primers used in this study are listed in Table 1.

Statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test was used for statistical analysis of expression and viral load data. Analyses were performed using a VassarStats online resource (http://vassarstats.net/utest.html). Statistical analysis of survival data was performed using a log rank test (GraphPad Prism 8; GraphPad, San Diego, CA, USA). A probability (P) value of <0.05 was used in all analyses to indicate significance. Error bars on all graphs represent the standard errors of the means (SEM).

ACKNOWLEDGMENTS

We thank Tina Martin for the expert animal husbandry.

This research was supported by grant R24AI059830 from the NIH National Institute of Allergy and Infectious Diseases (NIAID), by grant IOS-1754274 from the National Science Foundation, and by an Indo-U.S. research professorship from the American Society for Microbiology.

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