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Cloning and characterization of antiviral cytotoxic T lymphocytes in channel catfish, *Ictalurus punctatus*

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ABSTRACT

To determine the role of piscine anti-viral cytotoxic cells, we analyzed the response of channel catfish to *Ictalurid herpesvirus 1*, commonly designated channel catfish virus (CCV). Peripheral blood leukocytes (PBL) from catfish immunized with MHC-matched, CCV-infected G14D cells (G14D-CCV) showed marked lysis of G14D-CCV but little to no lysis of uninfected allogenic (3B11) or syngeneic (G14D) cells. Expansion of effectors by *in vitro* culture in the presence of irradiated G14D-CCV cells generated cultures with enhanced cytotoxicity and often broader target range. Cytotoxic effectors expressed rearranged TCR genes, perforin, granzyme, and IFN-γ. Four clonal cytotoxic lines were developed and unique TCR gene rearrangements including γδ were detected. Furthermore, catfish CTL clones were either $CD4^+/CD8^-$ or $CD4^-/CD8^-$. Two CTL lines showed markedly enhanced killing of G14D-CCV targets, while the other two lines displayed a broader target range. Collectively, catfish virus-specific CTL display unique features that illustrate the diversity of the ectothermic vertebrate immune response.

1. Introduction

Although cytotoxic T lymphocytes (CTL) play a critical role in mammalian antiviral immunity, their role in teleost fish and other lower vertebrates is poorly understood. In mammals, both NK cells and CTL are responsible for the destruction of virus-infected cells (Zinkernagel, 1993). In teleosts, much has been learned from ginbuna crucian carp (*Carassius auratus langsdorfii*) and rainbow trout (*Oncorhynchus mykiss*) model systems that utilize clonal fish and MHC-matched cell lines (Nakanishi et al., 2011, 2015). Virus-specific cell-mediated cytotoxicity was first shown in clonal triploid ginbuna carp immunized with syngeneic cells infected with infectious pancreatic necrosis virus (IPNV) and eel virus from America (EVA; Somamoto et al., 2000). PBL cytotoxicity peaked seven days after a secondary immunization with virus-infected cells (Somamoto et al., 2000, 2002), and lymphocytes displayed elevated levels of TCRβ and CD8α message and killed virus-infected cells in a perforin-dependent manner (Toda et al., 2009, 2011). Moreover, cytotoxic effectors proliferated *in vitro* following stimulation with virus-infected syngeneic targets (Somamoto et al., 2009). Similar studies using the rainbow trout clone C25 and the MHC-matched rainbow trout gonad cell line (RTG-2) demonstrated that PBL from viral hemorrhagic septicemia virus (VHSV)-infected rainbow trout killed both VHSV-infected RTG-2 cells and also an infected xenogeneic carp cell line (Fischer et al., 2003). However, compared to cytotoxicity directed towards syngeneic targets, cytotoxicity towards xenogeneic cells was reduced and did not appear until 11 days after a second VHSV injection (Utke et al., 2007). In addition, C25 rainbow trout immunized with a DNA vector encoding the VHSV glycoprotein G killed both VHSV-infected syngeneic and xenogeneic targets, while fish immunized with the N nucleocapsid protein only lysed VHSV-infected syngeneic targets (Utke et al., 2008). In addition to trout and carp, $CD8^+$ anti-viral cytotoxic cells have also been identified in grouper using virus-infected autologous cells as targets (Chang et al., 2011). Lastly, studies in amphibians have identified not only anti-viral $CD8^+$ T cells, but cells that may correspond to innate-like T (iT) cells that recognize virus-infected cells in a non-MHC-I restricted fashion (Edholm et al., 2019; Morales and Robert, 2007). However, in both fish and amphibians, the lack of long-term *in vitro* culture systems for cytotoxic cells precluded the generation of CTL lines for study of antiviral cytotoxic cells.

Channel catfish, an important aquacultural crop and a well-established model of teleost immunity, has provided insights into both piscine humoral and cell-mediated immunity (Clem et al., 1996; Miller et al., 1998; Bengten and Wilson, 2015). In catfish, NK and alloantigen-

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specific CTL lines have been established and characterized (Shen et al., 2002, 2004; Stuge et al., 2000; Zhou et al., 2001, 2003; Taylor et al., 2016; Spencer et al., 2019). NK cell lines were derived from PBL of naïve fish and generated after long-term alloantigen stimulation *in vitro*. Catfish NK clones contain granular lymphocytes that do not express TCR message and kill allogeneic targets in a non-MHC restricted fashion (Shen et al., 2004). Clonal alloantigen-specific CTL lines were generated from the PBL of a fish immunized with 3B11, an allogeneic B cell line, and two types of CTL lines were established. Group I CTL specifically lyse only 3B11 B cells that were used for immunization and these CTL express message for rearranged TCR α and β. In contrast, group II CTL lyse multiple allogeneic targets and express message for rearranged TCR α, β, and γ chains. Interestingly, neither group I or group II CTL express message for the typical CTL cell co-receptor CD8, however group II CTL express message for both of the CD4 co-receptors found in catfish (Stuge et al., 2000; Zhou et al., 2001; Edholm et al., 2007). Although antiviral cytotoxic T cells have not been extensively examined in catfish, NK-like cells present within the PBL of unimmunized fish have been described and shown to lyse CCV-infected autologous cells (Hogan et al., 1996).

Ictalurid herpesvirus 1 (IcHV1, family *Alloherpesviridae*), commonly designated channel catfish virus (CCV), is capable of triggering severe hemorrhagic disease in fry and fingerling catfish with mortality approaching 95% (Davison, 2009; Wolf, 1988). Although vaccination with a DNA vector expressing CCV ORF6, a putative membrane protein, and ORF59, the major viral envelope glycoprotein, elicited a protective antibody response and protected fish from subsequent CCV challenge (Nusbaum et al., 2002), the role of CTL could not be evaluated because a MHC-matched system was not available. To determine the role of anti-viral CTL in catfish, we made use of a family of homozygous gynogenetic catfish and the MHC-matched T cell line G14D that supports CCV replication. The G14D cell line was derived from the same lineage of gynogenetic catfish as those used in this study (Hogan et al., 1999). With this MHC-matched system, we have the ability to evaluate the virus-specific CTL response in catfish.

Here, we report the generation and characterization of clonal virus-specific CTL lines in catfish. After developing an immunization protocol using CCV-infected G14D cells (G14D-CCV), the cytotoxic effector population was expanded *in vitro* using mixed lymphocyte culture (MLC) and clonal cell lines were established by limiting dilution. The resulting cell lines exhibited differential cytotoxicity: some were cytotoxic toward CCV-infected syngeneic targets alone, whereas others lysed G14D-CCV, vesicular stomatitis virus (VSV)-infected G14D cells (G14D-VSV), and uninfected allogeneic cells. None of the cell lines expressed CD8, however three of the clones expressed message for both CD4-1 and CD4-2. These data provide evidence of specific antiviral cell-mediated cytotoxicity, and suggest that catfish CTL effectors differ from their mammalian and amphibian counterparts by their expression of CD4 rather than CD8.

2. Materials and methods

2.1. Viruses and cell lines

The Auburn CCV strain was propagated in channel catfish ovary (CCO) cells as previously described (Bowser and Plumb, 1980; Chinchar et al., 1993). Briefly, confluent CCO monolayers were infected with CCV at a MOI of 0.01 PFU/cell. After 48 h, or after extensive cytopathic effects were observed, the cell suspension was subjected to three freeze-thaw cycles and cellular debris was removed by centrifugation at 1000 × *g* for 10 min at 4 °C. The virus titer was determined by plaque assay and the resulting stock stored at −80 °C until use. Recombinant vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP) was obtained from SP Whelan (Harvard Medical School). VSV-GFP was propagated and titered in BSR-T7 cells (Whelan et al., 1995).

Catfish clonal leukocyte cell lines were grown at 27 °C in AL-4 medium consisting of equal parts of AIM-V and L-15 (Invitrogen Life Technologies, Carlsbad, CA) adjusted to catfish tonicity with 10% (v/v) H_2O and supplemented with 1 μg/mL $NaHCO_3$, 50 U/mL penicillin, 50 μg/mL streptomycin, 20 μg/mL gentamicin, 50 μM 2-mercaptoethanol and 4% heat-inactivated catfish serum (Miller et al., 1994a). 3B11 is a cloned autonomous B cell line generated from an outbred catfish by mitogen stimulation (Miller et al., 1994b). The catfish autonomous cell lines do not require repeated *in vitro* stimulations for their continued proliferation. G14D is an autonomous clonal T cell line derived from gynogenetic catfish #14 (Hogan et al., 1999; Zhou et al., 2003). In contrast, TS32.15 is an alloantigen-specific CTL line that was cloned from an outbred fish, which had been immunized with 3B11 B cells (Stuge et al., 2000). These CTL require weekly stimulation with irradiated 3B11 B cells for their continuous proliferation and are grown in conditioned complete catfish medium, which consists of AL-5 (supplemented with 5% heat-inactivated catfish serum) medium supplemented at 5% (v/v) with 42TA and 28S.3 culture supernatants. 42TA is an autonomous macrophage cell line derived from an outbred catfish (Vallejo et al., 1991), and 28S.3 is a clonal autonomous T cell line derived from another outbred catfish (Wilson et al., 1998). Both 42TA and 28S.3 secrete growth factors.

For this study, G14D cells infected with CCV at a MOI of 10 and irradiated at 4 h post infection were used for immunizations of gynogenetic catfish, and as stimulator cells in MLCs. In addition, CCV infected G14D cells (G14D-CCV) and VSV infected G14D cells (G14D-VSV) were used as targets in 4-h ^{51}Cr-release assays, as outlined below. To demonstrate that infection had occurred, CCV gene expression was analyzed by end-point reverse transcription PCR. The infection of G14D cells by recombinant VSV-GFP was confirmed by flow cytometry (Supplemental Fig. 1).

2.2. Experimental animals, immunizations, and lymphoid cell isolation

The third generation homozygous gynogenetic catfish lines, G-05 and G-16, used in this study are MHC-matched with and derived from the same gynogenetic line of fish as described by Hogan et al. (1999). All gynogenetic lines were provided by Geoff Waldbieser at the Warmwater Aquaculture Research Unit, USDA-ARS (Stoneville, MS). Fish (1–2 kg) were maintained in individual tanks as previously described (van Ginkel et al., 1992). Experiments involving live catfish were performed in accordance with relevant institutional and national guidelines and regulations, and approved by the UMMC Institutional Animal Care and Use Committee. To perform immunizations, gynogenetic catfish were anesthetized with tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO) and immunized intracoelomically with 3×10^6 G14D cells infected 4 h earlier with CCV at a MOI of 10 (G14D-CCV) or with 3×10^6 mock infected G14D. Fish were subsequently re-immunized 14 days later, and PBL were isolated three days after the second immunization by centrifugation onto a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical Corp. Westbury, NY) as previously described (Miller et al., 1994a).

2.3. Monoclonal antibodies and flow cytometry

The mAbs used in this study included: mouse anti-catfish IgM 9E1 (IgG1, κ), reacts with Igμ heavy (H) chain (Miller et al., 1987); mouse anti-catfish immunoglobulin light chain (IgL) 3F12 (IgG1, κ), reacts with the IgL F isotype (Lobb et al., 1984); mouse anti-catfish IgL 11A2 (IgG2b, κ), reacts with IgL G isotype (Lobb et al., 1984); rat anti-trout Lck (Laing et al., 2007), cross-reacts with catfish Lck (Taylor et al., 2015). Mouse anti-trout IgM 1.14 (IgG1, κ), which reacts with trout Igμ chains, was used as an isotype control (DeLuca et al., 1983).

PBL, cells from MLCs, or individual cell lines were resuspended at 10^7 cells/mL in RPMI medium adjusted to catfish tonicity using 10% water (catfish RPMI; cfRPMI) and analyzed for surface staining using flow cytometry. For single color flow cytometry, 1×10^6 cells were incubated with 50 μL of anti-catfish IgM mAb (9E1) hybridoma

supernatant for 30 min on ice, washed with cfRPMI, and incubated with 50 μL of goat anti-mouse IgG1-PE (1:80 v/v in cfRPMI) for 30 min on ice. After washing, samples were resuspended in 500 μL of cfRPMI and analyzed on a FACScan (Beckman Coulter). For two color flow cytometry, 1×10^6 cells were incubated with 50 μL of anti-catfish IgL F and 50 μL of anti-catfish IgL G for 30 min on ice, washed with cfRPMI and incubated with 50 μL of goat anti-mouse IgG1-FITC (1:40 v/v in cfRPMI) and goat anti-mouse IgG2b (1:80 v/v in cfRPMI). After washing, samples were resuspended in 500 μL of cfRPMI and analyzed as above.

2.4. Generation of cytotoxic cell lines

To establish cytotoxic cell lines, MLCs were generated by co-culturing PBL from G14D-CCV immunized fish with irradiated G14D-CCV targets. Briefly, 5×10^7 PBL were isolated from fish three days after G14D-CCV booster injection and cultured with 1×10^7 irradiated G14D-CCV in conditioned complete catfish medium containing 1 μg/mL acycloguanosine. Conditioned complete catfish medium contains 5% catfish serum and 5% (v/v) each of 42TA and 28S.3 culture supernatants, as described above. Acycloguanosine, an inhibitor of herpesvirus DNA polymerase, was used to prevent full CCV replication and the infection of effector cells in the culture. MLCs were maintained by weekly re-stimulations with 5×10^6 irradiated G14D-CCV. After 40 days and four *in vitro* stimulations, MLC-generated effectors were cloned by limiting dilution. Briefly, PBLs derived from two different fish, G-05-35 and G-05-6, were plated in round bottom 96-well plates at 0.33 cells/well in the presence of 10^5 irradiated G14D-CCV cells in 100 μL conditioned catfish medium containing 1 μg/mL acycloguanosine. After 10 days in culture, proliferating responder cells were transferred to flat bottom 96 well plates containing 2×10^5 irradiated G14D-CCV cells in 100 μL conditioned catfish medium. After one week, proliferating responders were transferred to 24-well plates containing 2×10^6 irradiated G14D-CCV cells in 1.5 mL conditioned catfish medium. After an additional week, cells were transferred to 12.5 cm^2 flasks in 4 mL of conditioned medium with 3×10^6 irradiated G14D-CCV. The resulting cell lines were maintained in 25 cm^2 flasks by weekly re-stimulation with 1×10^7 irradiated G14D-CCV, and four days after stimulation, cells were split into two 25 cm^2 flasks. In total, six cell lines were established from MLC G-05-6 MLC and three cell lines from MLC G-05-35. Of these, six cell lines were cytotoxic and were used for further analyses.

2.5. ^{51}Cr-release assays

^{51}Cr-release assays were performed as previously described (Yoshida et al., 1995). Briefly, 3×10^6 G14D or 3B11 target cells were labeled with 0.25 mCi $Na_2{}^{51}CrO_4$ (PerkinElmer) for 1.5 h at 27 °C/5% CO_2, washed twice, and incubated for an additional 30 min in AL-4 medium. For virus-infected targets, G14D cells were simultaneously labeled and infected with CCV or VSV-GFP at a MOI of 10. The virus inoculum was removed after 1.5 h by centrifugation and the cells were incubated for an additional 30 min in AL-4 medium. One hundred μL of effector cells in AL-4 medium were combined, at the indicated effector:target (E:T) ratio, with ^{51}Cr-labeled targets in 100 μL AL-4 medium in round bottom 96-well tissue culture plates. Experiments at each E:T ratio was performed in triplicates. The plates were centrifuged at 200 × g for 2 min to facilitate contact between effectors and targets, and then incubated for 4 h at 27 °C/5% CO_2. After 4 h, the cells were resuspended by pipetting and centrifuged at 500 × g for 5 min. One hundred μL of cell-free supernatant was removed from each well and released radioactivity was determined in a COBRA II auto gamma-counter (Packard, Meriden, CT). Percent specific release was calculated using the following formula:

$$\%\ specific\ release = \frac{cpm\ (experimental) - cpm\ (spontaneous\ release)}{cpm\ (maximum\ release) - cpm\ (spontaneous\ release)} \times 100$$

Spontaneous release wells received 100 μL AL-4 medium instead of effector cells, and maximum release wells received 100 μL 2% IGEPAL CA-630 in AL-4 medium. Standard deviation of triplicates never exceeded 2%.

2.6. RNA extraction, RT-PCR, 5′- rapid amplification of cDNA ends (RACE), and sequence analysis

Total RNA from MLC and clonal cell lines was isolated and treated with DNase I using the RNAqueous-4PCR kit (Ambion) according to the manufacturer's directions. Two μg of RNA were subsequently reverse transcribed into cDNA using an oligo[dT] primer and 200 U of Superscript III reverse transcriptase (Invitrogen). RT-PCR was performed using primers specific for each gene (Supplemental Table 1). Elongation factor-1α (EF-1α) was used as a template control. Typical PCR parameters were 3 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, and a final extension of 72 °C for 5 min. Unique TCR gene rearrangements were confirmed in each cell line using 5′ RACE with the SMARTer RACE cDNA amplification kit (Clontech) and primers specific for the constant regions of catfish TCR α, β, γ, and δ. All 5′ RACE products were cloned into pCR4-TOPO (Invitrogen) and sequenced using the Sanger method. Productive V(D)J gene rearrangements were analyzed for V- D- J-segment usage based on nucleotide identity to published catfish TCR cDNA and gene sequences using DNASTAR software. Sequences were submitted to GenBank under the accession numbers: MN313366-MN313378. The accession number for the G14D TCR Vδ sequence is HQ913599.

2.7. Western blotting

Total protein was extracted from 1×10^7 cells using 100 μL lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% IGEPAL CA-630, cOmplete Protease Inhibitor Cocktail Tablets (Roche Applied Science). The lysates were cleared of cellular debris by centrifugation at 12,000 × *g* for 15 min. Protein lysates corresponding to 3×10^6 cells were separated on 10% SDS-PAGE gels, transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare Life Sciences) and incubated in Tris-buffered saline containing 5.0% bovine serum albumin (BSA) and 0.1% Tween-20 (TBST-BSA) overnight at 4 °C. The membranes were incubated for 1 h with rat anti-trout Lck (1:500 in TBST-BSA), washed four times in TBST, followed by incubation with horseradish peroxidase conjugated goat anti-rat IgG (1:5,000 in TBST-BSA) for 1 h. After four 10-min washes with TBST, immunoreactive bands were visualized using Supersignal West Pico chemiluminescent substrate (Thermo Scientific).

3. Results

3.1. Cytotoxic cells are present within the PBL population of G14D-CCV immunized fish

To identify virus-specific CTL from catfish, we developed the following immunization protocol. Because initial attempts to generate virus-specific CTLs by injection of CCV virions into adult fish were unsuccessful, we immunized gynogenetic catfish with 3×10^6 G14D cells infected with CCV (G14D-CCV) or the same number of uninfected G14D cells and boosted 14 days later. In contrast to ginbuna carp, where cytotoxic activity was maximal seven days after booster immunization (Somamoto et al., 2000), catfish demonstrated optimal cytotoxic activity three days after booster immunization, and displayed minimal cytotoxicity when sampled at seven days (data not shown).

PBL from two fish immunized with G14D-CCV (G-05-6 and G-16-20)

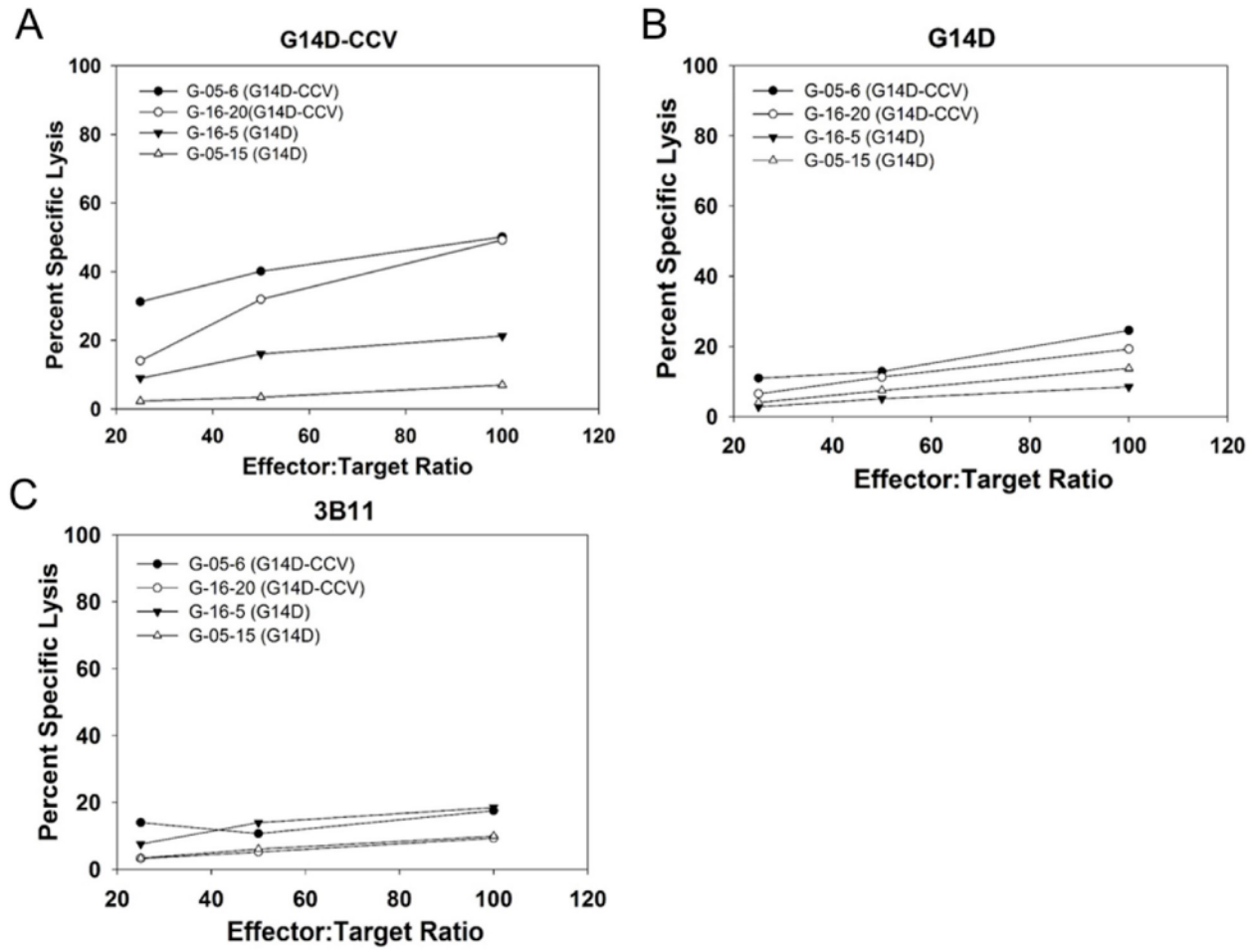

Fig. 1. PBL from G14D-CCV immunized fish preferentially lyse CCV-infected targets. PBL were isolated from G14D-CCV and G14D immunized fish three days after a booster immunization and used in 4-h ^{51}Cr-release assays with *A,* G14D-CCV; *B,* G14D; and *C,* allogeneic 3B11 target cells. Two fish (G-05-6 and G-16-20) immunized with G14D-CCV and two fish (G-16-5 and G-05-15) immunized with G14D are shown. Assays at each effector:target ratio were performed in triplicate. SD of the triplicates never exceeded 2%. Results are representative of five fish from each treatment group.

and two fish immunized with uninfected G14D cells (G-16-5 and G-05-15) were used as effectors in cytotoxic assays targeting G14D-CCV cells, uninfected G14D cells, and an uninfected allogeneic B cell line, 3B11 (Fig. 1). PBLs from fish immunized with G14D-CCV cells displayed marked lysis of G14D-CCV targets (~50% lysis at 100:1 effector:target (E:T) ratio) and minimal lysis of uninfected syngeneic (G14D) and allogeneic (3B11) targets (< 20% lysis at 100:1 E:T ratio). In contrast, PBL from fish immunized with uninfected G14D cells showed minimal lysis of all targets. These results suggest that fish immunized with G14D-CCV mounted a specific cell-mediated response that targeted virus-infected, MHC-matched targets, but not uninfected, MHC-matched targets, or allogeneic cells. The low level of lysis of G14D-CCV targets by PBL from mock immunized fish, as well as the minimal lysis of allogeneic targets indicates that NK-like activity in PBL from these fish is low. Collectively, these results suggest that PBL obtained from fish immunized with CCV-infected G14D cells contain a population of putative CTL effectors that specifically recognize CCV-infected MHC-matched targets.

Since catfish clonal NK cells have been shown to possess putative FcμR on their surface (Shen et al., 2003), we examined if PBL from G14D-CCV immunized fish exhibited an increase in FcμR$^+$ cells compared to G14D immunized fish. Freshly isolated PBL were stained with anti-catfish IgM mAb (9E1), or double stained with both anti-IgL F and anti-IgL G mAbs. Together these isotypes represent 90% of the light chains associated with serum IgM (Lobb et al., 1984). Lymphocytes that have passively acquired IgM via an FcμR stain positive with both anti-IgL antibodies. PBL of naïve fish generally contain between 4-8% FcμR$^+$ cells (Shen et al., 2003). Flow cytometric analysis showed that G14D-CCV immunized fish displayed an increase in total IgM positive cells compared to mock-immunized fish (Supplemental Fig. 2). Among five G14D-CCV immunized fish 11% of the PBL population was FcμR$^+$, while among five mock immunized catfish only 3% were FcμR$^+$ cells (Fig. 2). Collectively, these data indicate that immunization with G14D-CCV resulted in measurable anti-viral cytotoxic activity in the PBL population, and that these cytotoxic cells, like catfish NK cells, are FcμR$^+$.

3.2. *In vitro* expansion of cytotoxic effector cells

To expand the cytotoxic cell population, PBL from G14D-CCV immunized fish were co-cultured with irradiated G14D-CCV in complete catfish medium supplemented with 5% (v/v) each of 42TA and 28S.3 culture supernatants. 42TA and 28S.3 are macrophage and T cell lines, respectively, that secrete growth factors required for *in vitro* proliferation of non-autonomous cell lines. The cultures were restimulated every seven days with irradiated G14D-CCV, and after 15 days, expression of TCR and cytotoxic cell markers were compared between three original PBL populations and the resulting MLCs. As shown in Fig. 3, expression of constant (C) regions from all TCR genes, including those that were undetectable in the original PBL populations, was observed in all three MLCs. This result indicates that both αβ and γδ T cells proliferated in the MLCs. In addition, transcripts encoding membrane-bound IgM were

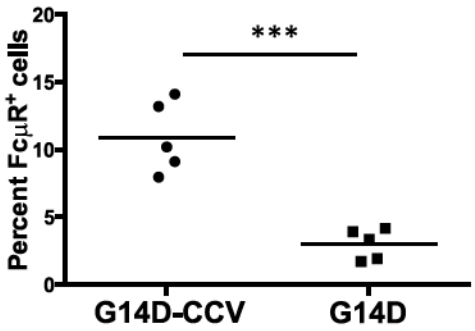

Fig. 2. The percentage of FcμR$^+$ cells is increased in the PBL of G14D-CCV immunized fish. Freshly isolated PBL were collected from five G14D-CCV and five G14D immunized fish three days after a booster immunization and stained with anti-catfish IgL F (3F12), and anti-catfish IgL G (11A2) mAbs. The percentage of FcμR$^+$ cells was determined as the percentage of cells present in the upper right quadrant (See Supplemental Fig. 2). The average of the five fish in each group is shown. ***$p < 0.0005$.

absent in all three MLCs after 15 days of culture. In all three MLCs, both isoforms of CD4-like molecules were expressed, while CD8α was absent and CD8β showed only low levels of expression. Finally, the cytotoxic cell markers perforin, granzyme, FasL, IFN-γ1, and IFN-γ2 were all expressed in MLCs. These results suggest that catfish cytotoxic effector cells are likely composed of αβ and well as γδ T cells, and that CD4$^+$ CTL may comprise the bulk of the CTL effector cell population.

We next examined the ability of this expanded population of putative CTL effectors to lyse G14D-CCV, G14D, and 3B11 targets (Fig. 4). Lysis of G14D-CCV target cells was increased in all three MLC, with an average of 40% lysis at a 1:1 E:T ratio. This value represents an approximately 100-fold increase in cytotoxicity compared to freshly isolated PBL. Surprisingly, in two of the three MLC, there was also an increase in cytotoxicity toward allogeneic 3B11 targets. Since lysis of allogeneic targets was not detected in the PBL of any immunized fish, the observed cytotoxicity could be due to expansion of either NK-like effectors or CTL with both viral- and/or allo-specificity. As before, MHC-matched, uninfected G14D cells were generally not lysed. We also examined surface expression of IgM, and found that ~80%, of the cells displayed IgM surface staining (data not shown), although message for IgM was not expressed. These findings indicate that *in vitro* culturing of PBL from G14D-CCV immunized fish result in expansion and proliferation of CTL.

3.3. Cloning and phenotypic analysis of CTL lines

After four re-stimulations in culture, cell lines were cloned by limiting dilution from two independent MLCs. Nine cell lines were cloned; six were cytotoxic and were further characterized. To determine whether the cytotoxic cell lines were derived from the same or different precursors, the TCR rearrangements for each clone were obtained by 5′RACE. The three cell lines isolated from the G-05-35 MLC (ET35.1, ET35.2, ET35.5) expressed identical TCRα and TCRβ rearrangements, exhibited similar cytotoxicity as measured by ^{51}Cr-chromium release assays, and most likely were derived from the same precursor. In contrast, all three CTL clones isolated from the G-05-56 MLC expressed identical TCRγ and TCRδ rearrangements. Two of the clones, ET56.2 and ET56.13, expressed different functional TCRα and TCRβ rearrangements. However, due to the lack of anti-catfish TCR antibodies it is unknown whether αβ or γδ TCR is expressed on the surface of these clonal cells. ET56.17, which functionally rearranged TCR γ and δ, is the first γδ T cell line established in a teleost fish (Table 1).

We next used RT-PCR to examine the expression of T cell co-receptors and cytotoxic effector molecules in each of the four CTL with unique TCR genomic configurations, ET35.1, ET56.2, ET56.13, and ET56.17 (Fig. 5A). All of the CTL lines isolated in this study express the cell adhesion and co-receptor molecule CD2. However, in contrast to most mammalian and amphibian cytotoxic T cells, ET35.1 did not express CD4 or CD8 co-receptor molecules. All three ET56 clones, ET56.2, ET56.13, and ET56.17, expressed message for both CD4-1 and CD4-2, and did not express CD8α or CD8β message. Clonal CTL lines were also

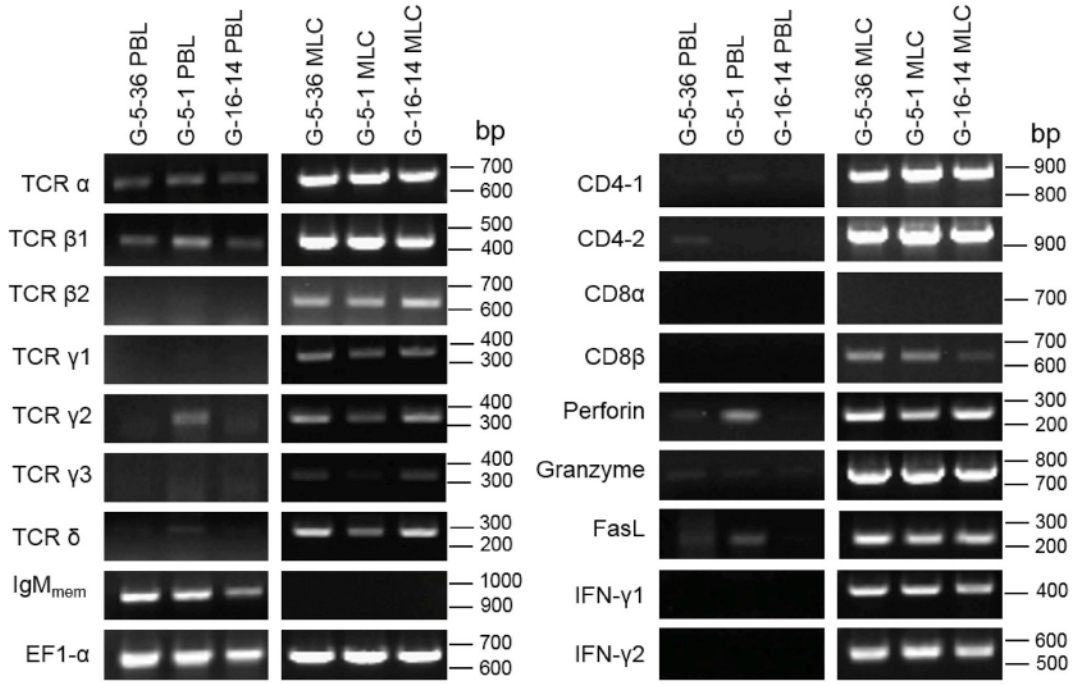

Fig. 3. Expression of TCR and cytotoxic cell marker genes in PBL and MLC from G14D-CCV immunized fish. Total RNA was extracted from PBL of G14D-CCV immunized fish three days after booster injection and from MLC derived from G14D-CCV immunized fish after 15 days *in vitro* culture. RT-PCR was performed using primers specific for each TCR constant (C) region, the Igμ heavy chain as well as the T cell co-receptors CD4-1, CD4-2, CD8, and the cytotoxic cell markers perforin, granzyme, FasL, and IFN-γ. EF1-α was amplified as a positive control. Base pair markers are indicated at right. All PCR products were verified by sequencing.

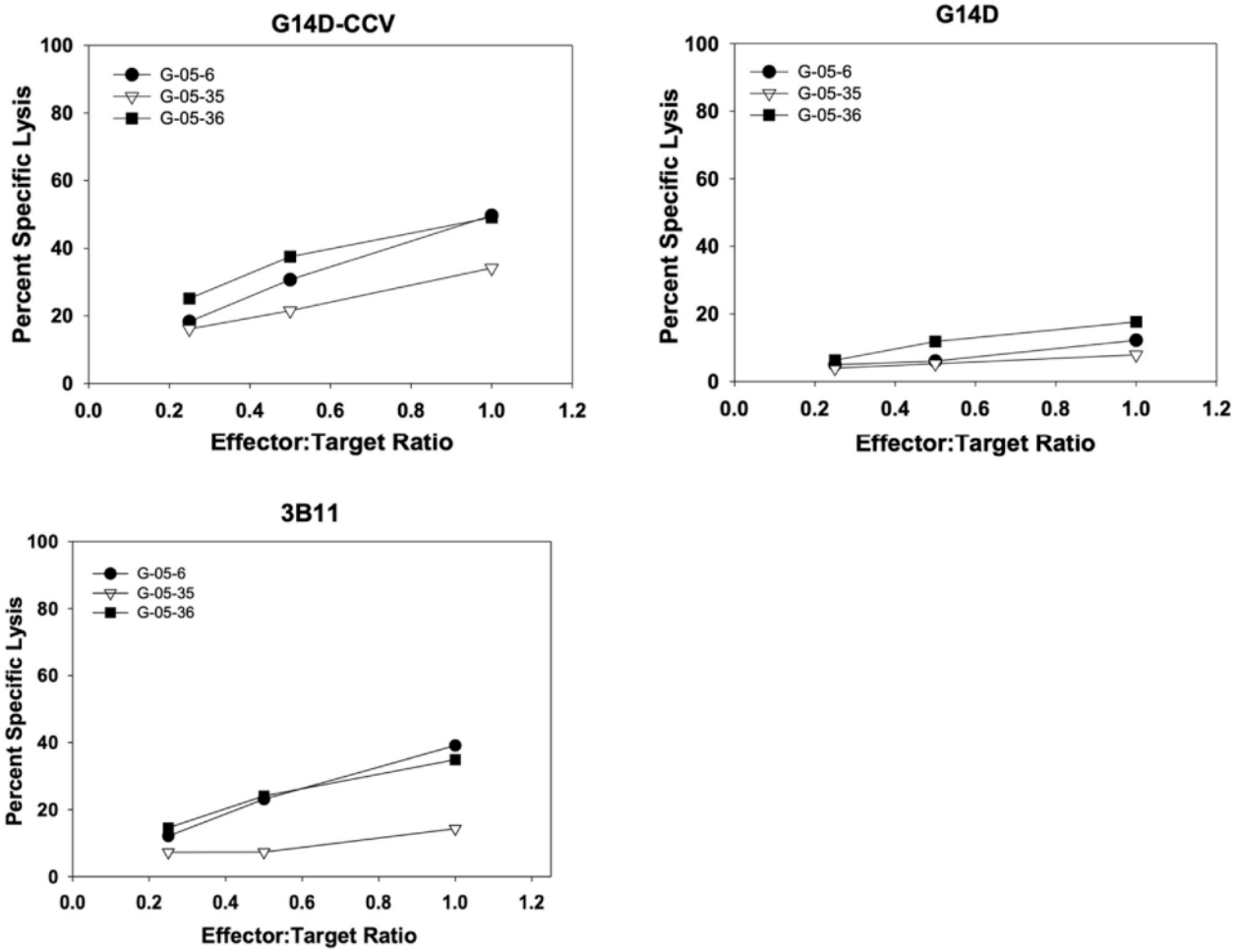

Fig. 4. PBL from G14D-CCV immunized fish exhibit increased cytotoxicity following *in vitro* stimulation. PBL were isolated from G14D-CCV immunized fish three days after a booster immunization and cultured with irradiated G14D-CCV cells in the presence of acycloguanosine. After 15 days in culture, MLC effectors were used in 4 h ^{51}Cr-release assays with G14D-CCV, G14D, and allogeneic 3B11 cells target cells. Results from three different MLC are shown. Assays at each effector:target ratio was performed in triplicate and standard deviations (SD) never exceeded 2%.

monitored for expression of perforin, granzyme, IFN-γ1 and -γ2. Consistent with their identity as cytotoxic lymphocytes, we found that all four lines expressed message for these genes with the exception of ET56.13, which transcribed little to no message for perforin (Fig. 5A). In addition, each of the anti-viral clones was tested for the expression of the T cell specific kinase, Lck by western blot. All four cell lines, as well as the control, TS32.15, express a protein consistent with the size of Lck (Fig. 5B). Lastly, each anti-viral cell line was assayed for surface IgM staining two days after stimulation using flow cytometry. All cell lines exhibit low intensity surface IgM staining suggesting that CTL activation is accompanied by IgM binding (Fig. 6).

To determine target specificity, each clonal cell line was tested for cytotoxicity against CCV infected G14D and VSV infected G14D targets (Fig. 7). In addition, we also tested for reactivity against allogeneic 3B11 B cells and two types of MHC matched syngeneic target cells, mock-infected G14D and ET56.10 T cells. G14D is a long-term autonomous MHC-matched cell line established in 1999 and ET56.10 is a non-cytotoxic, non-autonomous MHC-matched T cell line generated in this study. The TCR αβ CTL line, ET35.1 displayed a high level of cytotoxicity against G14D-CCV targets (71% lysis at a 10:1 E:T ratio), but did not lyse allogeneic 3B11 targets. G14D-VSV targets and G14D-mock infected targets were also killed, although at much lower levels (20% and 10% at a 5:1 E:T ratios, respectively). This low level killing may be due to recognition of stress molecules expressed on their surface, e.g. Fas. In comparison, the high level killing of G14D-CCV cells suggests that ET35.1-mediated killing is virus–specific. To examine if ET35.1

Table 1
TCR gene rearrangements in clonal cell lines.

Cell line	α	β1	β2	γ1	γ2	γ3	δ
ET35.1	V4J8	none	V4D1J30	none	none	none	none
ET56.2	V2J9	V6D1J4	none	unproductive	V3J6	unproductive	V1D3D4J1
ET56.13	V2J10	V7D1J14	none	unproductive	V3J6	V1J7	V1D3D4J1
ET56.17	none	none	none	unproductive	V3J6	unproductive	V1D3D4J1
G14D	ND	V4D1J12	none	none	ND	ND	V2D1D2D4J1

The TCR α, β, γ, and δ gene rearrangements from the indicated clonal catfish CTL lines were identified by 5′ RACE. Sequences are submitted to GenBank under the accession numbers: MN313366-MN313378. The accession number for the G14D TCR Vδ sequence is HQ913599.
Unproductive rearrangements are indicated; none indicates not expressed; ND indicates not determined.

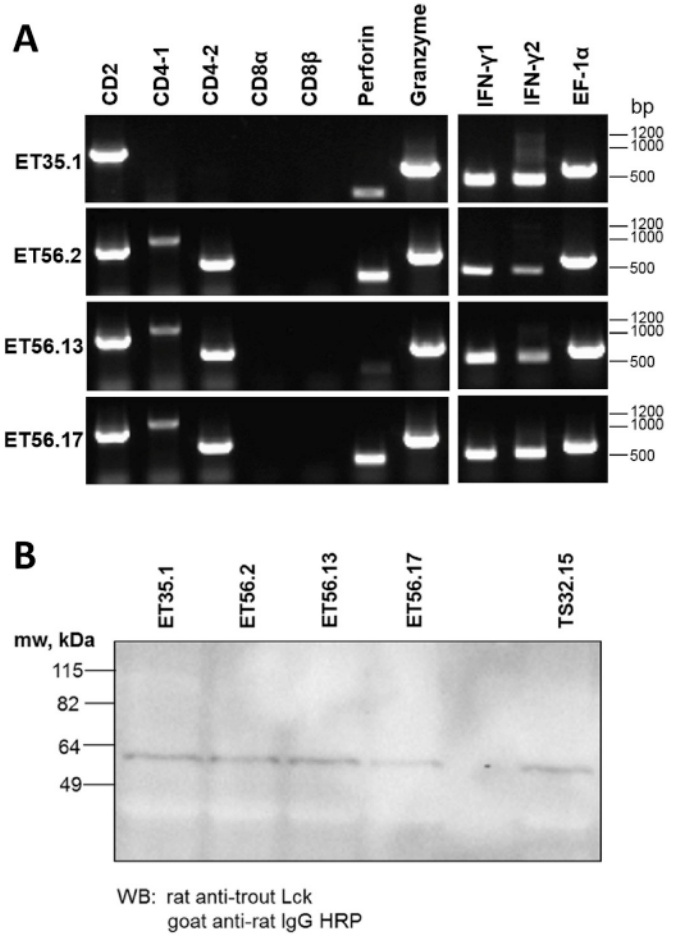

Fig. 5. Clonal cell lines express T cell and cytotoxic cell markers, but do not express CD8. *A*, Total RNA was extracted from each cell line and RT-PCR was performed using primers specific for the T cell co-receptors CD2, CD4-1, CD4-2, and CD8 and the cytotoxic cell markers perforin, granzyme, and IFN-γ. *B*, Total cell lysates (2×10^6 cells/lane) from each cell line were analyzed by 10% SDS-PAGE under reducing conditions and the separated proteins transferred to nylon membranes. Proteins were visualized using western blot with rat anti-trout Lck as the primary antibody and HRP-conjugated goat anti-rat IgG as the secondary antibody. Size markers in kDa are at left.

killed G14D-CCV cells via the calcium-dependent perforin-granzyme pathway, a cytotoxic assay was performed in the presence of 2mM EGTA. In the presence of EGTA, killing was reduced from 71% specific lysis to 31% at a 10:1 E:T ratio. Virus infected target lysis was restored to 63% when 5mM $CaCl_2$ was added back to the culture medium (data not shown). Together, these findings demonstrate that ET35.1 kills not only by the perforin and granzyme pathway, but by another mechanism as well, e.g. Fas ligand. Clone ET56.2, which expresses message for rearranged TCR αβγδ exhibited a pattern of cytotoxicity similar to that of ET35.1. ET56.2 shows a high level of cytotoxicity toward G14D-CCV targets, and markedly lower levels toward G14D-VSV, G14D, and allogeneic targets (Fig. 7). A third clone, ET56.13 was less cytotoxic than the other cell lines, and lysed both G14D-CCV and 3B11 equally well, while displaying reduced killing of G14D-VSV and G14D targets. The γδ T cell line, ET56.17 had the highest level of cytotoxicity of all the cell lines tested. At the lowest E:T ratio (1.25:1), ET56.17 cytotoxicity was similar to that seen with ET56.2, i.e., killing of G14D-CCV > G14D-VSV = 3B11 > G14D. However, at higher E:T ratios (i.e., E:T = 10) G14D-CCV, G14D-VSV, and 3B11 were lysed equally well (i.e., > 80% lysis) and at twice the rate (~40%) of uninfected G14D cells. None of the four cytotoxic cell lines exhibited any significant cytotoxic activity toward the syngeneic non-autonomous ET56.10 cell line. Collectively, these results indicate that catfish clonal CTLs generated following immunization with CCV-infected G14D targets displayed different specificities. Two of the CTL lines preferentially lysed G14D-CCV and demonstrated reduced lysis of G14D-VSV, G14D, and 3B11, while the other two cell lines displayed enhanced killing of a broader range of targets.

4. Discussion

In this study, we provide evidence that channel catfish, as other with teleosts, possess anti-viral cytotoxic T lymphocytes. More significantly, this is the first report of the cloning of virus-specific CTL lines in a teleost fish. Briefly, we developed an immunization strategy in which virus-infected, MHC-matched cells were used to induce a CTL response in catfish. PBL from fish immunized with CCV-infected G14D cells lysed virus infected targets at more than twice the rate of PBL from fish immunized with uninfected G14D cells, and 40% lysis was routinely achieved at 100:1 E:T ratios. Furthermore, consistent with the generation of virus-specific CTL, PBL effectors from G14D-CCV immunized fish spared uninfected G14D and allogeneic 3B11 cells. Subsequently, we demonstrated that CTL effectors could be expanded by *in vitro* culture in the presence of gamma-irradiated G14D-CCV and that these expanded effector populations expressed message for TCRα, β, γ, and δ, CD4-1, and CD4-2, as well as perforin, granzyme, FasL, IFNγ-1 and IFNγ-2 (Fig. 2). Very little CD8β message was expressed and CD8α message was not detected. Moreover, the expanded effector populations lysed G14D-CCV cells 100x more efficiently than *ex vivo* PBL i.e., 40% lysis was observed at an E:T ratio of 1:1. However, in contrast to results with *ex vivo* PBL, two of the three expanded populations also lysed 3B11 cells which suggested that alloreactive cells were proliferating. Finally, continued *in vitro* stimulation of the effector cell populations allowed us to establish a number of clonal CTL lines, four of which were characterized. In contrast to the bulk expanded cultures, none of the clonal lines expressed message for CD8, instead three of the cell lines, ET56.2, ET56.13, and ET56.17 expressed message for both CD4-1 and CD4-2. In this regard they resemble the catfish Group II TS32.17 clonal CTL, which kills 3B11 cells and several allogeneic targets (Edholm et al., 2007). The fourth clonal CTL line ET35.1 did not express message for either CD4 or CD8. This is reminiscent of the previously identified Group I TS32.15 CTL line, which is strictly alloantigen dependent and only kills 3B11 (Stuge et al., 2000; Zhou et al., 2001). As with the alloantigen specific CTL TS32.15 and TS32.17, cytotoxic assays demonstrated heterogeneity with respect to target specificity among the antiviral clonal lines. While ET35.1 and ET56.2 readily lysed CCV-infected G14D cells, they did not kill 3B11 B cells. In contrast, ET56.13 and ET56.17 killed allogeneic 3B11 targets as well as they killed CCV-infected G14D cells. In addition, all of the CTL clones lysed G14D-VSV cells at a level intermediate to that observed with CCV-infected G14D cells and mock-infected G14D cells. Collectively, these results demonstrate that following G14D-CCV immunization, catfish respond by producing virus-specific cytotoxic T cells. In addition, immunization with G14D-CCV resulted in an increase in the percentage of $FcμR^+$ cells present in the PBL population, and each of the resulting cytotoxic cell lines continued to display a putative FcμR. Although the functional significance of the FcμR on these cells is unknown, it may play a role in antibody-dependent cell-mediated cytotoxicity as in catfish NK cells (Shen et al., 2003).

Sequencing of the TCR gene rearrangements of the four clonal cell lines confirmed that they are unique clones and provided insight into their molecular phenotype. Two cell lines, ET56.2 and ET56.13 have productive rearrangements of TCRα, β, γ, and δ genes, and in the case of ET56.13, a productive rearrangement for both Cγ2 and Cγ3. Here it is important to note, that the gene rearrangement identified in the antiviral clonal lines are distinct from the rearrangements expressed by G14D target cells, and therefore are not due to contamination by G14D RNA (Table 1). It is also important to realize that the genomic

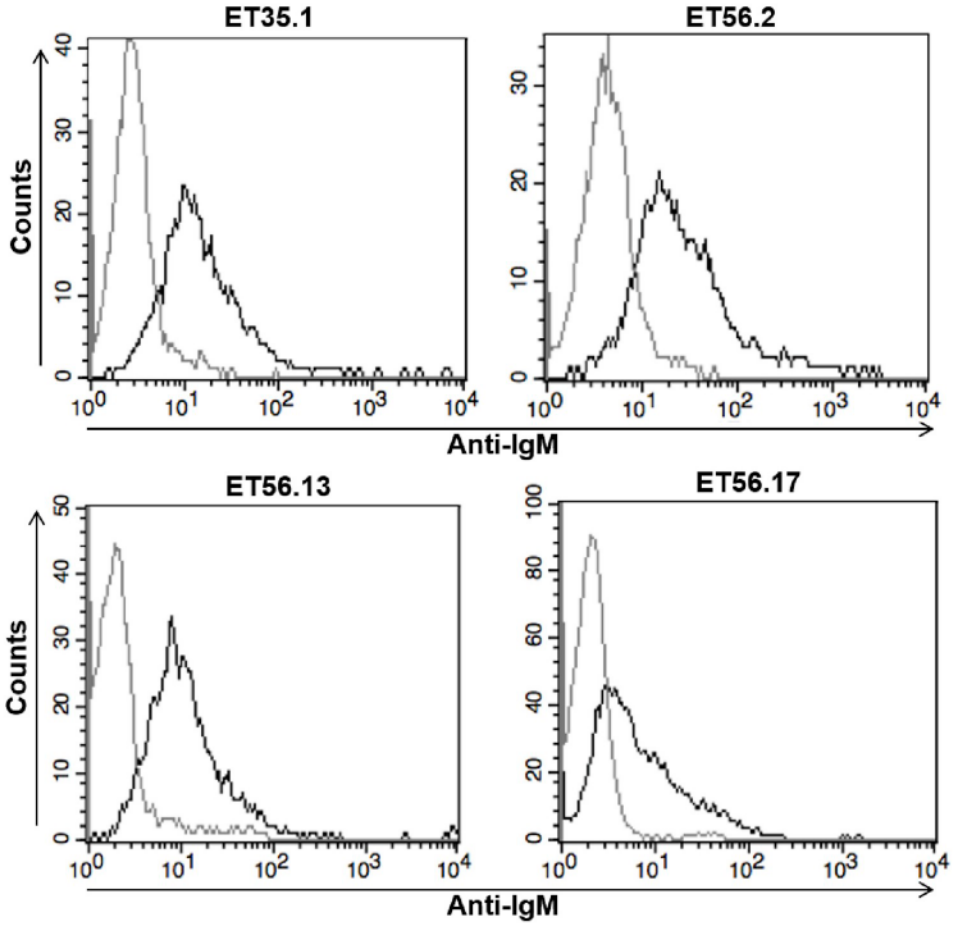

Fig. 6. Clonal CTL generated from G14D-CCV immunized fish exhibit surface IgM staining. Each cell line was analyzed for surface IgM staining two days after stimulation with irradiated G14D-CCV using flow cytometry. Histogram of IgM staining (outlined in black) is shown. Staining with the isotype control antibody 1.14 is outlined in gray.

organization of the T cell receptor genes in catfish and in other teleost species allows for the simultaneous rearrangement on multiple loci. For example, many teleost species, including the catfish, have the *TRA* and *TRD* gene loci linked to each other in an inverted orientation. Thus, rearrangement of *TRA* does not preclude rearrangement of *TRD* (Fischer et al., 2002; Schorpp et al., 2006). In contrast, in humans and mice, all *TRD* genes are found within the *TRA* locus and are in the same transcriptional orientation as the *TRA* genes. As a consequence, *TRA* rearrangements result in deletion of the *TRD* locus. Also, since the catfish *TRG* locus contains tandem duplications of Vγ-Jγ-Cγ, more than one rearrangement can occur on each chromosome (Moulana et al., 2014). In the ET56.13 clonal cell line, productive rearrangements occur for both Cγ2 and Cγ3; however, whether both Cγ2 and Cγ3 are expressed on the cell surface cannot be determined until specific Cγ2 and Cγ3 mAbs become available. Interestingly, in humans, between 1-6% of γδ T cells express two functional TCRγ chains on their cell surface (Davodeau et al., 1993). Also, studies in mice indicated that 10% of γδ T cells rearrange two functional TCRγ chains, although only one γ chain was expressed at the cell surface (Boucontet et al., 2005).

Even though ET56.2 and ET56.13 have identical rearrangements of TCR Cγ2 and TCR δ, they differ in their target specificities. ET56.2, like ET35.1 which expresses an αβTCR, specifically kills G14D-CCV, does not lyse allogeneic B cells, and exhibits a low level of cytotoxicity toward G14D-VSV and G14D targets. This pattern of cytotoxicity suggests that ET35.1 and ET56.2 are αβ CTL that kill in an MHC-restricted fashion. However, further studies are needed to confirm this hypothesis. In contrast, ET56.13, like the γδ T cell line ET56.17, is less specific and kills both allogeneic 3B11 B cells and G14D-CCV targets. Therefore, it is likely that ET56.13 and ET56.17 recognize their targets in an MHC-unrestricted manner. Overall, ET56.17 exhibited the highest degree of cytotoxicity and also lysed G14D-VSV targets efficiently. Thus, ET56.17 is similar to human γδ T cells, which lyse allogeneic and virus-infected syngeneic targets. For example, TCR γδ T cells from HSV seropositive individuals were shown to lyse both autologous HSV- and vaccinia virus-infected targets, as well as allogeneic Daudi cells, but not mock-infected autologous PBMC (Bukowski et al., 1994). We note that the γδ ET56.17 showed robust killing of allogeneic 3B11 targets and the long-term autonomous MHC-matched G14D target cells, whereas cytotoxicity towards the non-autonomous ET56.10 MHC-matched T cell line generated in this study was low. It may be that catfish γδ T cells recognize molecules present on both virus-infected cells and stressed cells, analogous to the recognition of MICA and MICB by mammalian γδ T cells (Bauer et al., 1999).

Notably, the catfish CTL population was dominated by CD4 effectors, although $CD4^-/CD8^-$ effectors were also detected. This finding is in contrast to what is observed in other fish (carp, trout, and grouper) and in the amphibian *Xenopus laevis* where CTL effectors are $CD8^+$ (Chang et al., 2011; Morales and Robert, 2007; Nakanishi et al., 2011; Somamoto et al., 2009, 2014; Tajimi et al., 2019; Toda et al., 2011). In the ginbuna crucian carp however, it was observed that the CD8-depleted fraction of peripheral blood lymphocytes from crucian carp hematopoietic necrosis virus (CHNV)-infected fish exhibited higher level of cytotoxicity against syngeneic CHNV-infected targets when compared to $CD8^+$ CTLs (Somamoto et al., 2013). Though it is not known if the CD8-depleted fraction contained any $CD4^+$ CTLs or if NK cells were responsible for the observed cytotoxicity. In this study, all three cell lines cloned from the G-05-6 MLC expressed message for CD4-1 and CD4-2. While it is somewhat surprising that the γδ T cell line ET56.17 expressed CD4 message, $CD4^+$ γδ T cells have been identified in pigs (Sinkora et al., 2005, 2007). In contrast, $CD4^+$ γδ T cells are rare in humans, i.e. the majority of human γδ T cells are $CD4^-CD8^-$ (Victor and Koning, 1990; Ziegler et al., 2014). This preferential expression of CD4

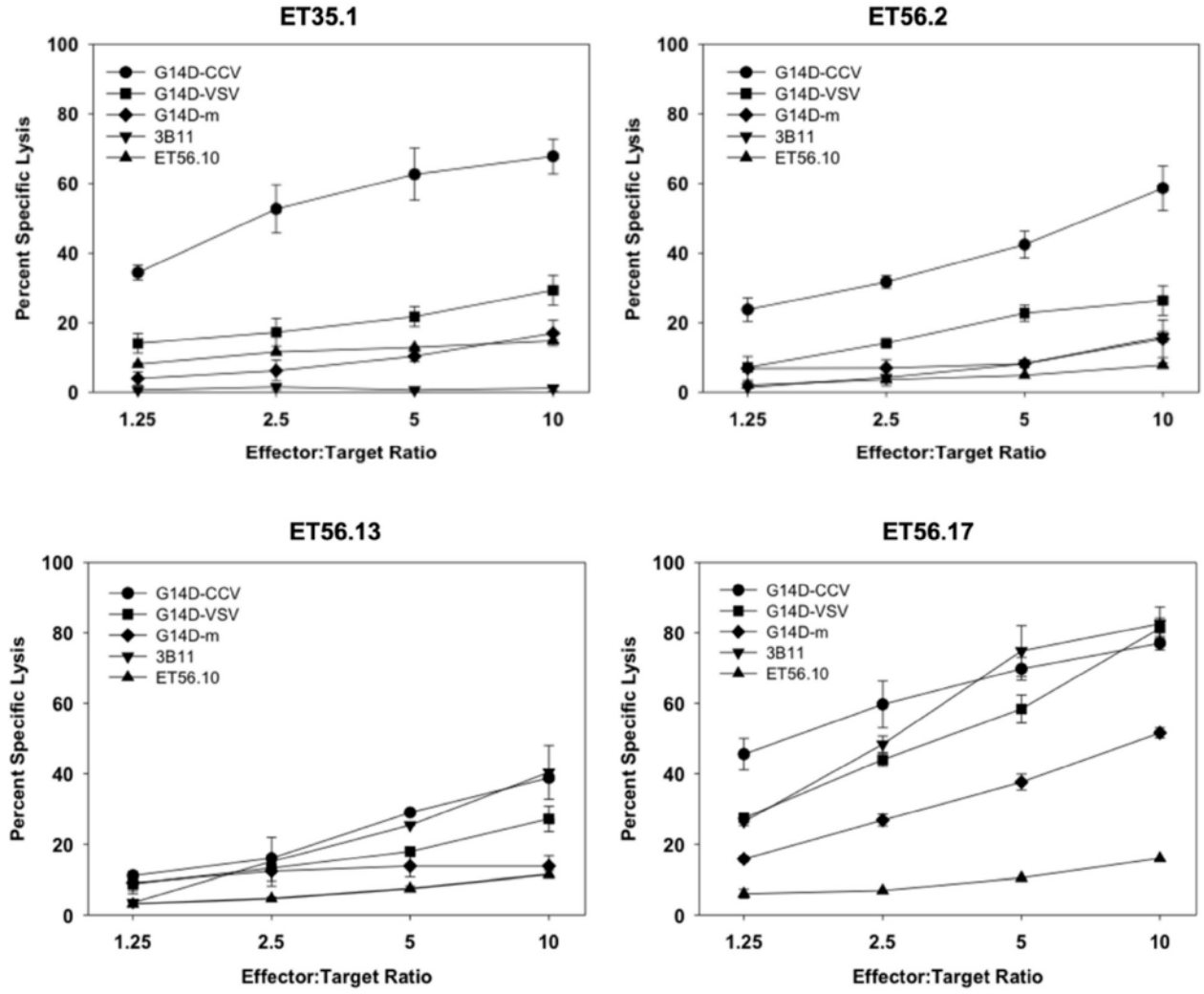

Fig. 7. Differential cytotoxicity of clonal CTL lines. Each catfish CTL line was tested for cytotoxicity in ^{51}Cr-release assays using G14D-CCV, G14D-VSV, G14D-m, 3B11, and ET56.10 cells as targets. Data show the mean ± SD of three independent experiments for each cell line.

was not unique to the G-05-6 MLC since MLCs generated from G14D-CCV immunized fish (G-05-36, G-05-1, and G-16-14) also displayed increases in CD4-1 and CD4-2. In comparison, CD8β message levels were low in all of the MLCs and CD8α message was not expressed. While $CD4^+$ CTL have often been associated with long-term *in vitro* culture of CTL (Fleischer, 1984; Lukacher et al., 1985), it is well established that $CD4^+$ CTL are present in the peripheral blood of individuals with chronic viral infections such as HIV-1 (Appay et al., 2002; Zaunders et al., 2004) and human cytomegalovirus (Appay et al., 2002). Since many viruses including EBV, CMV, and herpes viruses downregulate MHC I expression to escape CD8 CTL mediated immunity, it has been speculated that CD4 CTL may lyse target cells in a MHC class II dependent manner (Takeuchi and Saito, 2017). In this regard, it should be noted that we previously demonstrated that surface MHC expression on G14D cells is unaffected by CCV infection (Taylor et al., 2015). Therefore, even though it has been suggested that CCV, similar to other herpesviruses, establishes latency in fish that survive a primary infection (Gray et al., 1999), we do not believe that chronic viral infection alone is responsible for the prevalence of $CD4^+$ CTL and lack of $CD8^+$ CTL in this study.

Understanding the role of CD8 in the channel catfish has been challenging for a variety of reasons. Quiniou et al. (2011) showed that catfish CD8α and CD8β were expressed at their highest levels in the thymus of two month old catfish, and were only expressed at low levels in lymphoid tissues of adult fish. Consistent with that observation, neither group I nor group II clonal alloantigen-specific CTL lines expressed CD8, whereas group II CTL expressed both CD4-1 and CD4-2 (Stuge et al., 2000; Zhou et al., 2001; Edholm et al., 2007). Moreover, Quiniou et al. (2011) also showed that the cytoplasmic tails of catfish CD8α and CD8β lack the consensus Lck binding motif that is necessary for initiating T cell signal transduction, while CD4-1, CD4-2 and CD2 each express the Lck binding motif. Furthermore, *in vitro* binding assays demonstrated that the cytoplasmic tails of catfish CD4-1, CD4-1 and CD2 reproducibly bound recombinant catfish Lck, whereas catfish CD8α and CD8β were unable to bind Lck (Taylor et al., 2015). We believe that this failure of Lck to bind CD8 provides a reasonable explanation for the apparent absence of $CD8^+$ CTL and the prevalence of $CD4^+$ CTL in catfish. That catfish CD2, like mammalian CD2, was able to bind Lck also offers an explanation for how T cell signal transduction in $CD4^-CD8^-$ CTL lines, e.g. ET35.1 and TS32.15, can be initiated.

We have also used this model of homozygous gynogenetic fish and the clonal MHC-matched G14D T cell line to study catfish antiviral cytotoxic cell responses *in vivo* (Taylor et al., 2016). In the 2016 study, we took advantage of the mAb antibody CC41, which is specific for a subset of immunoregulatory receptors termed leukocyte immune-type receptors (LITRs) expressed on catfish group I and group II CTL, and on NK cells. Using magnetic cell sorting and cytotoxic assays, it was clearly demonstrated that $CC41^+$ PBL obtained from a G14D-CCV immunized fish were responsible for the lysis of G14D-CCV targets. Additionally, flow cytometry analysis using mAb CC41 showed that the percentage of

CC41$^+$ cells significantly increased in fish on day 5 after primary immunization with G14D-CCV (25% vs 5%), and on day 3 after booster immunization (36% vs 9.5%), compared to control fish injected with mock-infected G14D. Furthermore, when CC41 positive PBL collected three days after booster immunization were phenotypically characterized by RNA flow cytometry, they were shown to express CD3γδ, perforin, CD4-1 and CD4-2 (Taylor et al., 2016). Combined, the data demonstrated that the cell population responsible for killing CCV infected cells in immunized fish was dominated by CD4$^+$ CTL.

The current study has demonstrated the feasibility of *in vitro* culturing and cloning of antiviral cytotoxic cells and allowed us to examine the target specificity and phenotype of individual clones. Taken together, our results indicate that catfish anti-viral cytotoxic T cells are heterogeneous and include CD4$^+$ γδ T cells. Finally, the generation of long-term, clonal anti-viral CTL lines permits a more detailed analysis of piscine cytotoxic T cells including their target cell recognition mechanism(s) and increases our understanding of cell-mediated immunity in teleosts.

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Author contribution statement

Erin B. Taylor: Conceptualization, Methodology, Validation, Investigation, Funding acquisition, Writing - Original Draft, Writing - Review & Editing, **V. Gregory Chinchar:** Conceptualization, Methodology, Project administration, Supervision, Funding acquisition, Writing - Review & Editing, **Sylvie M.A. Quiniou:** Resources, Investigation, Data Curation, Writing - Review & Editing, **Melanie Wilson:** Supervision, Project administration, Funding acquisition, Writing - Review & Editing, **Eva Bengtén:** Conceptualization, Project administration, Supervision, Funding acquisition, Writing - Review & Editing.

Declaration of competing interest

The authors whose names are listed below wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and has been configured to accept email from this journal.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.11.014.

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