Developmental and Comparative Immunology 106 (2020) 103610

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Catfish lymphocytes expressing CC41-reactive leukocyte immune-type receptors (LITRs) proliferate in response to *Edwardsiella ictaluri* infection *in vitro*

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ARTICLE INFO

Keywords:
Edwardsiella ictaluri
In vitro infection model
Cell-mediated immunity
Leukocyte immune-type receptors (LITRs)
Cytotoxic cells
Macrophages

ABSTRACT

Monoclonal antibodies (mAbs) CC34 and CC41 recognize overlapping subsets of leukocyte immune-type receptors (LITRs). The mAb CC34 was raised against the clonal TS32.15 cytotoxic T cell line and the mAb CC41 was raised against the clonal NK cell line TS10.1. In this study, an *in vitro* model was developed to monitor CC34- and CC41-reactive cells in response to *Edwardsiella ictaluri* infection. Briefly, head kidney leukocytes and peripheral blood lymphocytes (PBL) were isolated from individual catfish and labeled with CellTrace Violet and CellTrace FarRed dye, respectively. Head kidney-derived macrophages were infected with *E. ictaluri* and then cocultured with autologous PBL. The combined cell cultures were then analyzed using flow cytometry. A significant increase in CC41 staining was observed in the PBL population at 2, 5 and 7 days after culture, which suggest that LITRs are involved in cell-mediated immunity to *E. ictaluri*.

1. Introduction

Edwardsiella ictaluri is the causative agent of Enteric Septicemia of Catfish (ESC), which is one of the leading causes of mortality that negatively impact the catfish aquaculture industry (Hawke et al., 1981; Shotts et al., 1986; Pridgeon and Klesius, 2012; Griffin et al., 2016). These small, motile, Gram-negative, rod-shaped bacteria primarily infect macrophages and are able to establish infection in pronephros (head kidney), mesonephros (trunk kidney), and spleen within hours after exposure (Baldwin and Newton, 1993; Karsi et al., 2006). While it is well established that catfish macrophages have the ability to kill and control *E. ictaluri* when bacterial numbers are low (Shoemaker et al., 1997; Russo et al., 2009), *E. ictaluri* also have multiple virulence factors that allow them to evade innate immune defense mechanisms and facilitate their replication within macrophages (Baldwin and Newton, 1993; Booth et al., 2006; Akgul et al., 2018). Examples of these virulence factors include its LPS oligo-polysaccharide, which conveys resistance to host cationic antimicrobial peptides, and the bacteria's acid-activated urease, which is able to raise macrophage vacuolar pH levels allowing for more efficient bacterial replication (Santander et al., 2013; Martin et al., 2016; Booth et al., 2009; Baumgartner et al., 2014). Furthermore, as reviewed in Thune et al. (2007) and Dubytska et al. (2016), *E. ictaluri* possesses a type III secretion system (T3SS) that enables the transfer of bacterial effector proteins into the cytoplasm of host cells. This T3SS, provides entry of at least seven effector proteins (e.g. EseJ, EseK and EseN) that are required for *E. ictaluri* intracellular replication and virulence (Dubytska and Thune, 2018).

Notably, despite these multiple immune evasion mechanisms, successful protection against *E. ictaluri* can be provided by vaccination with live attenuated bacteria (Weise et al., 2015 and reviewed therein). For example, macrophages obtained from *E. ictaluri* vaccinated catfish produce 10-fold higher levels of reactive oxygen and nitrogen species (ROS, RNS), and nitric oxide, and are significantly more efficient at killing *E. ictaluri,* as compared to macrophages obtained from non-vaccinated catfish (Russo et al., 2009). Combined, their findings strongly suggest that *E. ictaluri* vaccination results in both activation and subsequent polarization of macrophages toward a bactericidal M1 phenotype. In mammals, classical M1 polarization occurs in response to IFN-γ produced by NK cells and several T cell subsets, including TH1, cytotoxic T lymphocytes (CTLs) and γδ T cells (Boehm et al., 1997;

Abbreviations: mAb, Monoclonal antibody; LITR, Leukocyte immune-type receptor; PBL, Peripheral blood lymphocytes

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https://doi.org/10.1016/j.dci.2020.103610
Received 23 November 2019; Received in revised form 28 December 2019; Accepted 7 January 2020
Available online 08 January 2020
0145-305X/

Stenger and Modlin, 1998; Ivashkiv, 2018). Similarly, IFN-γ has also been shown to be an important factor for macrophage activation in teleosts (Zou et al., 2005; Greyfer and Belosovic, 2009). Since catfish cytotoxic cells produce INF-γ, we took advantage of two monoclonal antibodies (mAbs), CC34 and CC41, that recognize LITRs expressed on catfish cytotoxic cells, to examine if the numbers of cytotoxic cells increase in the presence of *E. ictaluri*-infected macrophages. LITRs are immunoregulatory receptors specific to teleosts, and phylogenetically related to Fc-receptors and receptors encoded within the Leukocyte Receptor Complex (LRC; Stafford et al., 2006, 2007). Previously, mAb CC41 was shown to bind a subset of LITRs and members of this subset were identified as markers for catfish NK cells, alloantigen specific CTLs, and anti-viral cytotoxic cells (Shen et al., 2004; Taylor et al., 2016). In the study by Taylor et al. mAb CC41 was used to follow both the *in vivo* and the *in vitro* cytotoxic cell response to channel catfish virus.

Here, we develop an *in vitro* stimulation model based on autologous mixed leukocyte reactions (MLR) to examine if lymphocytes that express CC41-reactive and/or CC34-reactive LITRs proliferate in response to *E. ictaluri*. In this cell culture platform, the *E. ictaluri*-infected head kidney-derived macrophages (HKDM) are functioning as a source of stimulation for autologous PBL. Using this model, we demonstrate that the number of $CC41^+$ cells increased in response to *E. ictaluri* infection. In addition, we also provide evidence that mAbs CC34 and CC41 bind to epitopes within the D2-D3 domains of LITR1.1a expressed by clonal TS32.15 CTLs.

2. Materials and methods

2.1. Experimental animals

Outbred and gynogenetic channel catfish, *Ictalurus punctatus*, were obtained from the Warmwater Aquaculture Research Unit in Stoneville, MS (USDA-Agriculture Research Service) and were maintained and handled according to relevant institutional and national guidelines using protocols approved by the University of Mississippi Medical Center (UMMC) Institutional Animal Care and Use Committee (IACUC). Catfish were individually housed with constant aeration as described by van Ginkel et al. (1992), and maintained on a high protein floating feed diet.

2.2. Monoclonal antibodies

The mouse mAbs used in this study were: 1) anti-LITR CC41 (IgG1, κ) reacts with a subset of catfish LITRs found on catfish CTLs and NK cells (Shen et al., 2003; Taylor et al., 2016), 2) anti-catfish IgM 9E1 (IgG1, κ) reacts with catfish Igμ chain (Miller et al., 1987), and 3) anti-rainbow trout IgM 1.14 (IgG1, κ) reacts with the rainbow trout Igμ chain (DeLuca et al., 1983), and was used as isotype (or negative) control. In addition, a second anti-LITR mAb, CC34 (IgG1, κ) used in this study was produced according to standard protocols (Morretta et al., 1985; Campbell, 1991) using a BALB/c mouse immunized with the catfish clonal alloantigen specific CTL line, TS32.15 (Stuge et al., 2000). Hybridomas were grown in advanced Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and 15% fetal bovine serum (FBS). All hybridoma culture supernatants were collected and passed through a 0.22 μm bottle-top vacuum filters (Corning), aliquoted and stored at −20 °C until titered and used.

2.3. Catfish cell culture media and buffers

Catfish RPMI (cfRPMI) wash media consists of Roswell Park Memorial Institute (RPMI)-1640 powdered media mix (Sigma-Aldrich) dissolved in sterile deionized water, supplemented with 50 U/ml Penicillin/50 μg/ml Streptomycin solution (HyClone), and 1 μg/ml sodium bicarbonate ($NaHCO_3$; Fisher Scientific), adjusted to catfish tonicity using 10% (v/v) deionized H_2O (Miller et al., 1994). Catfish AL-4 media consists of equal parts Leibovitz's L-15 (Corning) and AIM-V (Gibco) media adjusted to catfish tonicity with 10% (v/v) H_2O, and supplemented with 1 μg/ml $NaHCO_3$, 100 U/ml penicillin/100 μg/ml streptomycin solution (HyClone), 20 μg/ml gentamicin (American Pharmaceutical Products), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 4% heat-inactivated, pooled catfish serum. Channel catfish macrophage media (CCMM), consists of cfRPMI, 15 mM HEPES, 0.18% sodium bicarbonate, 50 μM 2-mercaptoethanol, and 5% heat-inactivated, pooled catfish serum. Incomplete CCMM was prepared as CCMM without the addition of catfish serum, and was used for preparing and washing head kidney derived macrophages (HKDM).

2.4. Catfish clonal cell lines

The catfish clonal cell lines used in this study were 28S.3, an autonomous T cell line (which does not require repeated *in vitro* stimulation) derived from an outbred catfish (Wilson et al., 1998) and TS32.15, a non-autonomous antigen-dependent CTL line, which requires weekly stimulation with irradiated catfish 3B11 B cells (6000 rads for 30 min) for its continuous proliferation (Stuge et al., 2000). The 28S.3 cell line was grown in AL-4 media and TS32.15 CTLs were grown in conditioned complete catfish medium. Conditioned complete catfish medium, consists of AL-5 medium (AL medium with 5% heat-inactivated catfish serum), supplemented with 5% (v/v) each of 42TA and 28S.3 culture supernatants. 42TA is an autonomous macrophage cell line derived from an outbred catfish (Vallejo et al., 1991). Both 42TA and 28S.3 supernatants are included as a source of cytokine-like growth factors for culturing catfish leukocytes.

2.5. Recombinant LITR proteins and mAb binding assay

Total RNA was extracted from the catfish clonal TS32.15 CTL cell line using the RNAqueous-4PCR total RNA extraction Kit (Ambion) and treated with DNase according to the manufacturer's recommendations. Two μg of RNA was then reversed transcribed using an oligo (dT) primer and 200 units of SuperScript III Reverse Transcriptase (total reaction volume 20 μl; Invitrogen). The resulting cDNA was diluted with 30 μl PCR-grade H_2O, and RT-PCR was performed as described by Stafford et al., (2007), using the universal LITR1/2 forward primer and the LITR1-specific reverse primer, with 1 μl of cDNA template (Advantage 2 Polymerase Kit; Clontech Laboratories, see supplemental Table 1). Cycling parameters were: 5 min at 95 °C, followed by 32 cycles of 95 °C for 30 s, 59 °C for 1 min, and 72 °C 3 min, followed by a final extension at 72 °C for 5 min. The PCR products were separated on 1% agarose gels containing 0.005% Ethidium Bromide (EtBr) in Tris-Acetate EDTA Buffer, excised from the agarose gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen). A PCR product of ~2430 bp was cloned into pCR4-TOPO (Invitrogen Life Technologies) and sequenced (Eurofins Genomics USA). The full length TS32.15 LITR1.1a cDNA sequence was verified to match the TS32.15L1.1a nucleotide sequence originally described by Stafford et al. (2007; accession number: DQ858227).

Specific primers for TS32.15L1.1a immunoglobulin (Ig) domains were designed using SnapGene (GSL Biotech; see supplemental Table 1) and RT-PCR was performed using Phusion Green High-Fidelity DNA Polymerase as described above. The PCR products were gel purified, digested with the restriction enzymes specific for the sites introduced by the forward and reverse PCR primers, and ligated into pSecTag 2B (Invitrogen) using T4 Ligase (Promega) at 4 °C overnight. The resulting plasmids were transformed into TOP10 *E. coli*, plated on LB agar containing Carbenicillin (50 μl/ml) and grown for 16 h at 37 °C. Individual colonies were selected for plasmid DNA isolation and the inserts were confirmed by sequencing.

FLAG-tagged recombinant LITR proteins were expressed in HEK-

293T cells (ADCC CRL-3216), which were seeded into 6-well culture plates (Nunc) and grown to ~80% confluency. Transfections were performed using GeneJuice Transfection Reagent (Novagen) at an optimized ratio of 3 μl GeneJuice: 1 μg DNA. Briefly, 3 μl of GeneJuice was added drop-wise to 100 μl of serum free DMEM, mixed by vortexing and incubated for 5 min at room temperature. One μg of pSecTag-LITR plasmid DNA was then added to the GeneJuice media mixture and allowed to complex for 45 min at room temperature. The DNA-GeneJuice complexes were diluted into 1 ml serum free DMEM and added to the seeded wells, which were then incubated for 1 h at 37 °C. DMEM supplemented with 15% FBS was then added to the culture plates. At 72 h the cells were harvested, pelleted at 500 × *g*, and resuspended (5×10^5 cells/50 μl) in Lysis Buffer (10 mM Tris, 150 mM NaCl, 1% NP-40, pH 7.4) containing 1 Protease Inhibitor tablet (Pierce). After a 15 min incubation on ice, the cell lysates were separated from the cellular debris by centrifugation at 12,000 × *g* for 10 min, and the lysates were stored at 4 °C until used.

Monoclonal Abs CC34, CC41, and anti-rainbow trout IgM 1.14 (as a negative control) were separately coupled to M-270 Epoxy DynaBeads (Invitrogen) according to the manufacturer's protocol. Briefly, 120 μg of purified mAb in a volume of 120 μl was combined with 6 mg of freshly washed DynaBeads suspended in 120 μl 0.1 M sodium phosphate, pH 7.4. To initiate covalent antibody coupling, 120 μl of fresh 3 M ammonium sulfate was added and the mixture was incubated overnight at 37 °C with end-over-end mixing. Antibody conjugated beads were washed in PBS-0.1% BSA, and aliquoted into separate Eppendorf tubes at 2 mg/100 μl PBS-0.1% BSA. For each sample, 20 μl of cell lysate was brought to a final volume of 100 μl in PBS, and combined with 2 mg of Ab-conjugated beads. The samples were incubated for 1.5 h at 4 °C with end-over-end mixing, and washed three times with PBS using magnetic separation. The captured target proteins were then eluted with 0.1 M citrate pH 3.1.

For western blot analysis, samples of 45 μl of each collected fraction (unbound, wash, and elute) were separated on 10% SDS-PAGE under reducing conditions, and transferred to Immobilon-P Transfer PVDF membranes (EMD Millipore). The membranes were then blocked with 5% Milk (Instant Nonfat Dry Milk; Best Choice) in PBS-Tween 0.01% (PBS-T) for 1 h at room temperature before adding the anti-FLAG M2 (Sigma) primary antibody (diluted into 5% milk PBS-T; 1:5000). After 1 h of incubation, the membranes were washed in PBS-T three times before adding the secondary goat anti-mouse IgG (H + L)-HRP antibody (1:5000; Southern Biotech) in 5% milk PBS-T. FLAG-tagged proteins were visualized using a SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific) and imaged using a ChemiDoc XP Imager (Bio-Rad).

2.6. Bacterial strains and culture conditions

The *E. ictaluri* strain 93-146 used in this study was derived from a clinical isolate (Booth et al., 2006) and was generously provided by Dr. A. Karsi (College of Veterinary Medicine, Mississippi State University). *Edwardsiella ictaluri* bacteria were grown in Bacto™ Brain-Heart Infusion (BHI) broth (Becton Dickinson) in a 30 °C shaking incubator at 200 rpm for 16 h to obtain a log-phase culture. All bacterial stocks were stored at −80 °C in 15% glycerol. Growth curves were generated at the beginning of the study by plotting bacterial growth as absorbance/colony forming units (CFU) numbers versus time in culture. To verify bacterial cell counts and viability, triplicates of serial dilutions in BHI broth were plated on tryptic soy agar (TSA) plates supplemented with 5% defibrinated sheep blood (Thermo Scientific) and incubated at 30 °C. CFUs were counted 48 h after plating.

2.7. In vitro stimulation protocol

Here, we modified the gentamicin survival assay described by Booth et al. (2006) to examine if the numbers of CC34- and CC41-reactive LITR expressing lymphocytes increased when cultured in the presence of *E. ictaluri*-infected macrophages.

2.7.1. Tissue collection, PBL and head kidney derived macrophage isolation

Adult catfish (> 2 kg) were euthanized by immersion in 300 mg/L tricaine methanesulfonate (MS-222; Western Chemical). Upon loss of the tail reflex, 15–18 ml of blood was drawn from the caudal vein into heparinized vacutainer tubes, and the head kidneys were subsequently removed and transferred to sterile cfRPMI. To isolate PBL, whole blood was diluted 1:3 in cfRPMI and layered over Accu-Prep™ Lymphocytes Solution (Axis-Shield) as previously described by Miller et al. (1994). The PBL were then resuspended in cfRPMI at 1×10^7 cells/ml and labeled with CellTrace™ Violet (see below), or were directly resuspended in AL-4 media at 1×10^7 cells/ml for culturing overnight at 27 °C in 5% CO_2.

Catfish monocyte/macrophages were enriched from head kidney leukocytes according to the protocol described by Booth et al. (2006). Briefly, harvested tissues were washed, sliced into small pieces, and disassociated through a 100 μm cell strainer (BD Falcon) by using a syringe rubber plunger into incomplete CCMM. The isolated cells were then washed in incomplete CCMM, passed through a second cell strainer (pore size 70 μm), and counted. Head kidney leukocytes were either stained immediately with CellTrace™ Far Red (see below), or placed in culture overnight. In the latter case, cells were resuspended at 2×10^7 cells/ml in CCMM, and seeded into poly-D-lysine coated 24-well plates (0.5 ml/well) or into T-75 culture flasks (18 ml/flask). Both 24-well plates and T-75 flasks were coated with 0.1 mg/ml poly-D-lysine solution (Gibco). The cells were then allowed to adhere overnight (~16 h) at 27 °C in 5% CO_2. Afterwards, the plates (or flasks) were carefully washed by gentle pipetting four times with cfRPMI to remove any nonadherent cells and to enrich for HKDM. In a pilot experiment, the adherent cells were confirmed to be predominantly HKDM by staining for nonspecific esterase as previously described (Ellsaesser et al., 1984).

2.7.2. Cell labeling

Peripheral blood leukocytes, and head kidney leukocytes were counted and labeled according to the manufacturer's directions, with CellTrace™ Violet and CellTrace™ FarRed (Invitrogen), respectively. Briefly, leukocytes were resuspended in serum free catfish media at 1×10^7 cells/ml, and a volume of the 5 mM CellTrace™ dye stock solution (in DMSO) was added to each tube to obtain a final dye concentration of 1.25 μM. The leukocytes were allowed to stain for 30 min at room temperature in the dark with gentle rocking to ensure uniform labeling. Any excess free dye was then quenched by adding five volumes of complete CCMM. The labeled cells were resuspended in fresh prewarmed AL-4 or complete CCMM and cultured overnight (16 h) at 27 °C in 5% CO_2.

2.7.3. Plasma IgM opsonization of E. ictaluri

Plasma was separated from freshly isolated catfish blood by centrifugation at 1100 × *g* for 5 min, collected, and used as a source of IgM. An *E. ictaluri* culture was grown to log-phase in BHI broth, and 1 ml aliquots (~1×10^8 bacteria) were added into 1.5 ml Eppendorf tubes. The bacteria were pelleted by a quick spin at 3000 × *g* and resuspended in 1 ml of freshly isolated plasma. After a 30 min incubation at room temperature (~25 °C), the opsonized bacteria were diluted in cfRPMI to a concentration of 1×10^6 cfu/ml. To demonstrate that bacteria had been bound by plasma IgM, 1×10^6 bacteria were pelleted, resuspended in 100 μl of anti-catfish IgM hybridoma supernatant, and incubated on ice for 30 min. The bacteria were washed in 3 ml cfRPMI and stained with 100 μl of goat anti-mouse IgG (H + L)-PE antibody diluted 1:80 in cfRPMI for 30 min on ice. After a second wash in cfRPMI, the bacteria were then resuspended in 0.5 ml of cfRPMI. Anti-trout IgM mAb was used as an isotype control. The stained bacteria were analyzed using a NovoCyte Flow Cytometer (ACEA Biosciences)

with a double threshold set at 5000 for both forward scatter (FSC) and side scatter (SSC).

2.7.4. HKDM infection and PBL stimulation

Experiments were performed either in 24-well plates or in T-75 flasks.

24- well plates: 16 h cultures containing ~5 × 10^5 CellTrace™ Far Red labeled HKDM in 0.5 ml of CCMM per well, were either infected with 10 μl of opsonized *E. ictaluri* (2.2 × 10^4 bacteria) at a multiplicity of infection (MOI) of ~0.05, or mock-infected by adding 10 μl of cfRPMI. To promote bacteria:HKDM interaction, the plates were centrifuged at 200×*g* for 2 min, and incubated for 1.5 h at 27 °C in 5% CO_2. The free bacteria were removed from cultures by gentle aspiration and 0.5 ml CCMM media containing a lethal dose of gentamicin (100 mg/ml) was added to each well. The plates were placed back into the incubator, and after 1 h, the treated HKDM were washed twice in incomplete CCMM. The CellTrace™ Violet labeled autologous PBL that had been cultured overnight in cfRPMI were resuspended at 1 × 10^6 cells/ml in AL-4 media (containing 20 mg/ml gentamicin). One ml of the labeled PBL was then added to each HKDM culture well. At 2, 6, 12, 24, 48, 72 and 96 h, after adding the PBL, wells of combined PBL and HKDM were harvested by pipetting across the well surface to remove the adherent HKDM. Cell samples were then stained in triplicates with anti-LITR CC34, anti-CC41 or anti-trout IgM 1.14 (isotype control).

T-75 flasks: 16 h cultures containing ~4 × 10^6 CellTrace™ FarRed labeled HKDM in 4.6 ml of AL-4 media were either infected with 400 μl of plasma opsonized *E. ictaluri* (4 × 10^5 cfu) for a MOI of 0.1, or mock-infected by adding 400 μl of cfRPMI. The flasks were incubated at 27 °C in 5% CO_2 for 1.5 h, the free bacteria were then removed, and cultures were treated with gentamicin as above. The HKDM were washed twice in incomplete CCMM, and then 6 ml of complete CCMM containing a static dose of gentamicin (0.35 μg/ml) was added to each flask. The CellTrace™ Violet labeled autologous PBL from the overnight culture were washed in cfRPMI and resuspended in AL-4 at 1 × 10^7 cells/ml. Four mls of the labeled PBL were then added to each culture flask for a ratio of 10:1 (PBL:HKDM). The culture flasks were monitored daily for 7 days by microscopy and/or flow cytometry. For flow cytometry, 1.5 ml of suspended PBL were collected by careful pipetting to minimize disruption to the HKDM layer. Media was not added to replace the withdrawn volume.

2.7.5. Three-color flow cytometry

For flow cytometry analysis, 3 × 10^5 leukocytes were washed in cfRPMI at 500×*g* for 5 min, resuspended in 100 μl of hybridoma supernatant from either CC34, CC41, 9E1 or 1.14 and incubated on ice for 30 min. The leukocytes were then washed in cfRPMI and incubated with 100 μl of goat anti-mouse IgG_1-PE (1:80 v/v in cfRPMI; Southern Biotech) for 30 min on ice. The stained cells were then washed in cfRPMI, resuspended in 0.5 ml of cfRPMI, and analyzed using a NovoCyte 3000 Flow Cytometer (ACEA Biosciences). The FSC threshold was set at 10,000 and the residual noise (< 10^3 FSC-H, < 10^3 SSC-H) was routinely gated out. Phycoerythrin (PE) was excited by the 488 nm blue laser and detected using the PE channel (572/28). CellTrace™ Violet was excited by the 405 nm violet laser and detected using the Pacific blue channel (445/35). CellTrace™ Far Red was excited by the 635 nm red laser and the fluorescence emission was collected using the APC channel (675/30).

2.7.6. Fluorescence microscopy

For fluorescent and brightfield images of *E. ictaluri*-infected leukocyte cultures, an EVOS FL fluorescent microscope (Life Technologies) was used. The Texas Red emission channel was used to visualize CellTrace™ FarRed labeled cells. Files were exported as.TIFF images and optimized for printing using Keynote software (Apple Inc.). Video was captured using an iPhone 8-Plus camera.

2.8. Statistical analysis

Data are presented as mean ± SD, statistical analyses were performed using GraphPad Prism 8. Independent paired t-tests were used to analyze the differences between pairs of infected and mock-infected samples from each individual fish using two-tailed comparisons. Outliers were identified using ROUT (Q = 1%).

3. Results

3.1. Monoclonal antibody CC34 binds a subset of LITRs that overlaps with CC41-reactive LITRs

To verify that mAb CC34 binds to catfish LITRs, three overlapping regions of TS32.15 LITR1.1a were expressed as 3xFLAG-tagged recombinant proteins in HEK293T cells and used in a mAb binding assay. TS32.15 LITR1.1a, which consists of 7 Ig-domains was chosen as a template since it was identified as the longest LITR expressed in clonal TS32.15 CTLs (Stafford et al., 2007). In addition, previous studies from our laboratory had demonstrated that mAbs CC41 and CC34 specifically immunoselect proteins of high molecular weight (180-115 kDa) from surface biotinylated TS32.15 CTLs (Taylor et al., 2016, and unpublished). Here in the binding assays, mAbs CC41, CC34, and 1.14 (negative control) were covalently conjugated to magnetic beads and incubated with total cell lysate containing FLAG-tagged TS32.15 LITR1.1a recombinant proteins including domains D2-D3, D3-D4-D5, or D5-D6-D7. Fractions of the unbound cell lysates, wash and eluted proteins were analyzed by western blot using anti-FLAG. As shown in Fig. 1A, the recombinant LITR1.1a D2-D3 protein was bound by both mAb CC41 and mAb CC34 coupled magnetic beads and not by the isotype control mAb 1.14 coupled beads. Recombinant LITR1.1a proteins D3-D4-D5, and D5-D6-D7, however were not bound by any of the antibody conjugated beads. When used in flow cytometry, both mAbs stained the clonal TS32.15 CTLs as well as the majority of the cells (87%) in a mixed leukocyte culture derived from PBL from a naive outbred fish stimulated with irradiated allogeneic 3B11 B cells (Fig. 1B and Supplemental Figure 1). In contrast, mAb CC34 did not stain the clonal long-term T cell line, 28S.3 at the same level as mAb CC41. Taken together these results strongly suggest that mAb CC34 recognizes a subset of LITRs that partially overlaps with the subset that is recognized by mAb CC41.

3.2. Coculturing of catfish PBL with E. ictaluri-infected macrophages results in increased numbers of $CC34^+$ and $CC41^+$ cells

To examine if the numbers of mAb $CC34^+$ and mAb $CC41^+$ lymphocytes increase in response to *E. ictaluri* infection, a pilot experiment with two fish was first performed. Peripheral blood leukocytes and head kidney leukocytes were harvested from each fish, and 5 × 10^6 PBL and 5 × 10^6 head kidney leukocytes were set aside for flow cytometry to determine their baseline expression levels of CC34- and CC41-reactive LITRs. While mAbs CC34 and CC41 each stained a small percentage of PBL (~2–4%), head kidney cells within the lymphocyte gate were negative for both mAbs (Fig. 2A). Similarly, only 1.36% of the large granular head kidney cells stained with mAb CC41. Also, as expected, a large population of PBL (23%) stained positive for catfish IgM, and only a small population of IgM^+ lymphocytes (4%) were found in the head kidney (data not shown). The remaining PBL and head kidney cells were then placed in culture overnight. Head kidney leukocytes were cultured on poly-D-lysine coated 24-well culture plates to enrich for HKDM as described by Booth et al. (2006). After 16 h, the plates were washed three times to remove any nonadherent cells, and the HKDM were then infected with *E. ictaluri* that had been opsonized with autologous plasma (MOI of 0.1). When an aliquot of infected HKDM was examined at 2 h post infection, a small increase in the staining of large granular cells was observed with mAb CC34 (from 0.43% to 1.08%) and

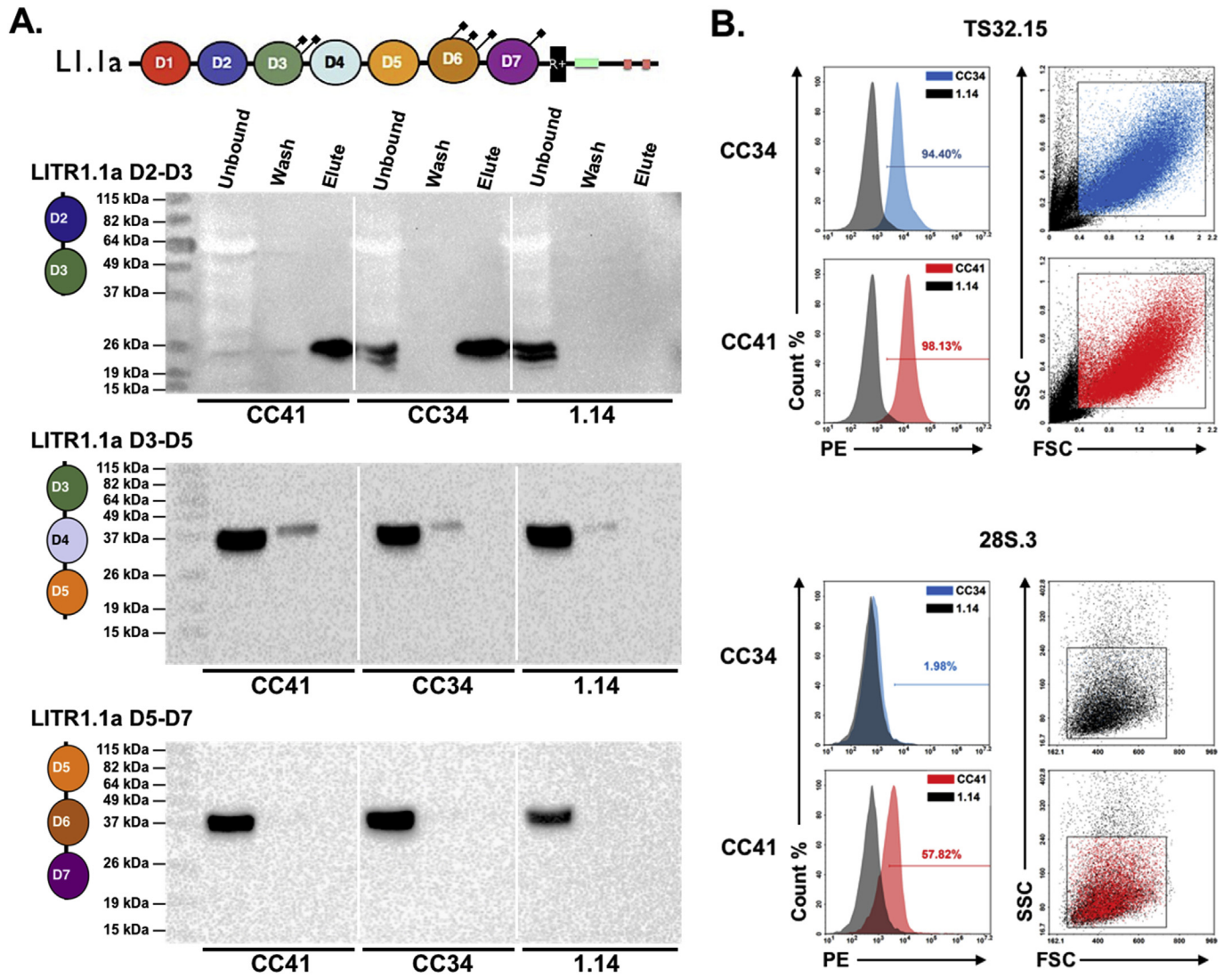

Fig. 1. Monoclonal Abs CC41 and CC34 recognize subsets of LITRs that are partially overlapping. (**A**) The schematic of TS32.15 LITR1.1a (ABI16036) is shown above the western blots. N-linked glycosylation sites are marked as black lines. The predicted ITAM and the two ITIMs in the cytoplasmic tail are shown in green and red, respectively. Schematics of the recombinant 3X-FLAG-tagged LITR D2-D3, LITR D3-D4-D5, and LITR D5-D-6-D7 proteins expressed in HEK-293T cells are shown on the left next to each respective blot. Immunoprecipitations were performed using total cell lysates and antibody-conjugated dynabeads: anti-catfish LITR CC34, anti-catfish LITR CC41, or anti-trout IgM 1.14 as a negative control. Fractions of the unbound cell lysates, wash and eluted proteins were separated by 10% SDS-PAGE under reducing conditions. FLAG-tagged proteins were visualized by western blot using anti-FLAG M2 mAb followed by goat-*anti*-mouse IgG-HRP as the secondary antibody. (**B**) Catfish clonal TS32.15 CTLs and 28S.3 T cells were stained with mAbs CC34 and CC41 followed by goat-*anti*-mouse IgG1-PE (1:80) as the secondary antibody. The percent positive cells within each gate as compared to the isotype control, anti-rainbow trout IgM mAb 1.14 (gray) are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mAb CC41 (from 1.36% to 3.37%; Fig. 2B). Autologous PBL were then added to the remaining HKDM wells at a ratio of 2:1 and cells were examined by flow cytometry at 2, 6, 12, 24, 48 and 96 h. At 48 h after infection, the large granular cell population was greatly reduced and the numbers of CC34 and CC41 staining cells were increased to 5.44% and 7.06%, respectively (Fig. 2C).

As shown in the time course panels, at 2 and 6 h post infection, infected HKDM appeared more diverse in their size and internal complexity as compared to mock-infected HKDM, which is likely due to their uptake of bacteria (Fig. 3). However, at 12 and 24 h post infection the numbers of large granular cells were reduced from 45% at 6 h, to 25% and 16%, respectively. In contrast, while the percentages of HKDM in mock-infected cultures remained relatively constant (between 36 and 56%), there was an observed shift in their morphology to smaller and less internally complex cells by the end of the time course. The loss of infected macrophages may be partially due to their increased fragility associated with increased numbers of intracellular bacteria, i.e. the numbers of macrophages as analyzed by FACS would be underestimated. A reduction in HKDM numbers at 12 h post infection is also similar to the observations originally reported by Booth et al. (2006). Moreover, Russo et al. (2009) observed lysed HKDM at 36 h post infection in cultures established from unvaccinated fish, and their numbers continued to increase until the experiment ended at 96 h. However, in our cultures at 96 h intact macrophages containing live bacteria could be observed (see Supplemental video).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.dci.2020.103610.

It may be that the observed increase in mAb CC34 and mAb CC41 staining in Fig. 2C is due to upregulation of specific LITR subsets on cytotoxic lymphocytes, or it is possible that $CC34^+$ and $CC41^+$ lymphocytes can survive better in culture. Alternatively, $CC34^+$ and $CC41^+$ lymphocytes may only proliferate when *E. ictaluri*-infected

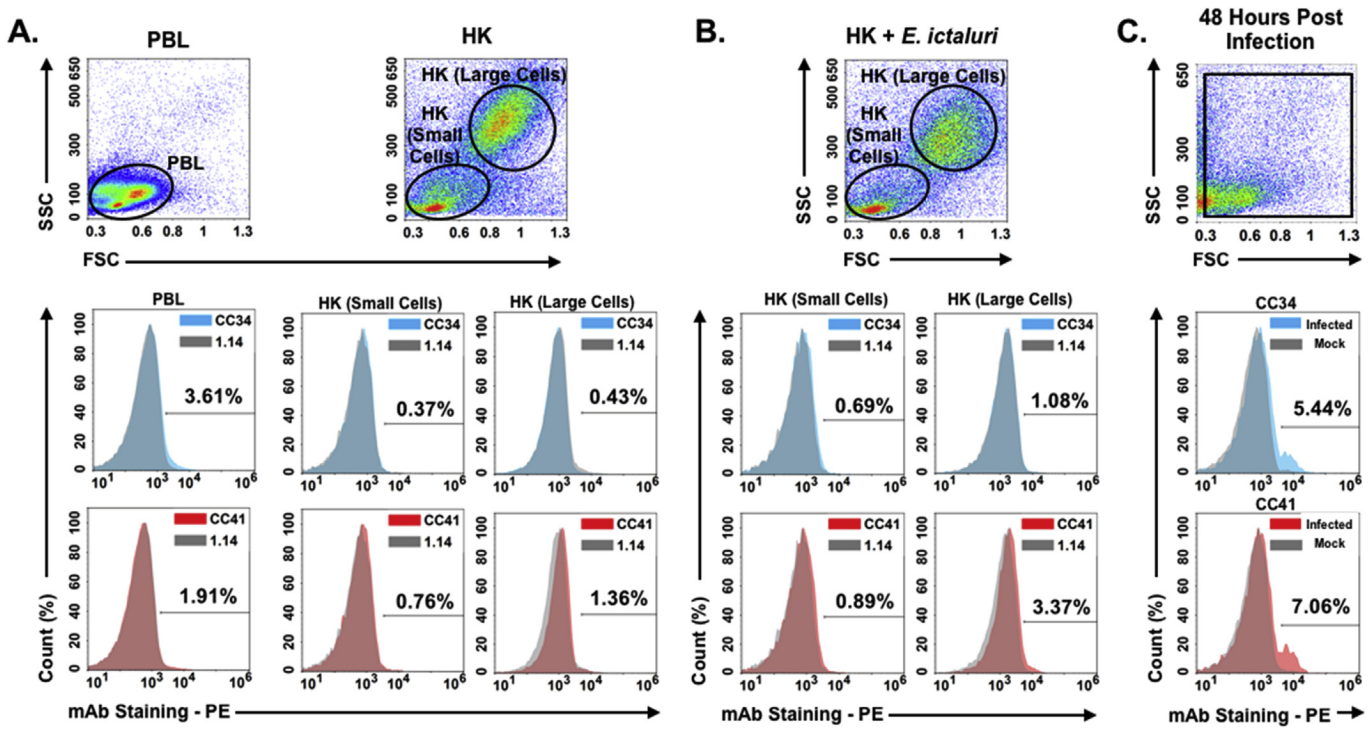

Fig. 2. Coculturing of catfish PBL with *E. ictaluri*-infected macrophages results in increased numbers of CC34$^+$ and CC41$^+$ cells. (**A**) Freshly isolated PBL and head kidney (HK) leukocytes were collected from a naive fish and analyzed by flow cytometry. Density plots of unstained PBLs (left) and HK leukocytes (right) are shown with gates indicated for lymphocytes (small agranular cells) and large internally complex leukocytes. Normalized histograms of the gated populations after staining with anti-LITR CC34 (blue) and anti-LITR CC41 (red) followed by goat-*anti*-mouse IgG-PE are shown below each plot. The percent positive cells within each gate as compared to the isotype control, mAb 1.14 is indicated in gray. (**B**) Adherent HK leukocytes were infected with *E. ictaluri* (MOI 0.05), removed from the plate at 2.5 h after infection, and analyzed as in panel A. (**C**) *E. ictaluri*-infected adherent head kidney leukocytes were cocultured with PBL for 48 h and analyzed as in panel A. The staining of the mock-infected control culture is overlaid in gray in each histogram. Data are representative of two independent experiments using two fish. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

macrophages are present. In order to explore these possibilities, an *in vitro* model where *E. ictaluri*-infected HKDM and autologous leukocytes could be monitored independently was developed.

3.3. Leukocyte proliferation in response to E. ictaluri-infected macrophages can be monitored using cell Trace labeling

To determine how the CC34$^+$ and CC41$^+$ leukocyte populations change when cocultured with *E. ictaluri*-infected HKDM, CellTrace labeling was used. Briefly, as described in Fig. 4A, CellTrace Violet labeled PBL and CellTrace FarRed labeled head kidney leukocytes were cultured separately overnight, and the head kidney leukocytes were either infected with plasma opsonized *E. ictaluri* or mock-infected. The labeled HKDM cultures were then treated with gentamicin to kill any remaining extracellular bacteria before the labeled PBL were added to the cultures at a ratio of 10:1. In this context, it important to note that natural IgM present in plasma from uninfected catfish readily bound *E. ictaluri* (Fig. 4B).

After 6 h of coculturing (22 h after cell labeling), the combined HKDM-PBL cultures were harvested and examined by flow cytometry

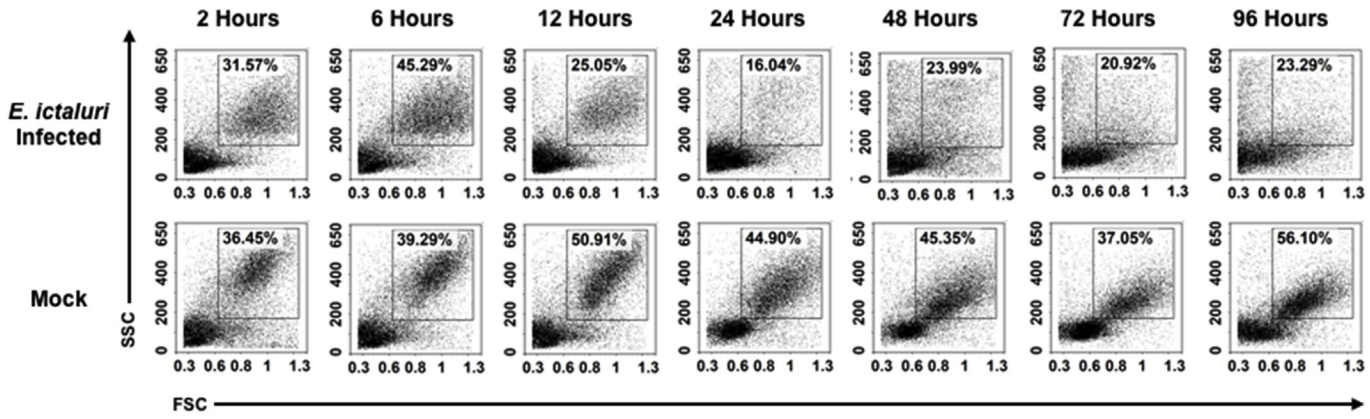

Fig. 3. The relative number of large granular leukocytes is reduced as a result of infection with *E. ictaluri*. Adherent HK leukocytes were infected with *E. ictaluri* (MOI 0.05) or mock-infected, and cocultured with autologous PBL in 24-well plates. Cells were harvested from individual wells at the indicated time points and analyzed by flow cytometry; 18,000 cells are displayed in each panel. Large granular cells including HKDM are gated. The indicated percentage of cells within each gate is calculated as the average of triplicates. Data are representative of two independent experiments.

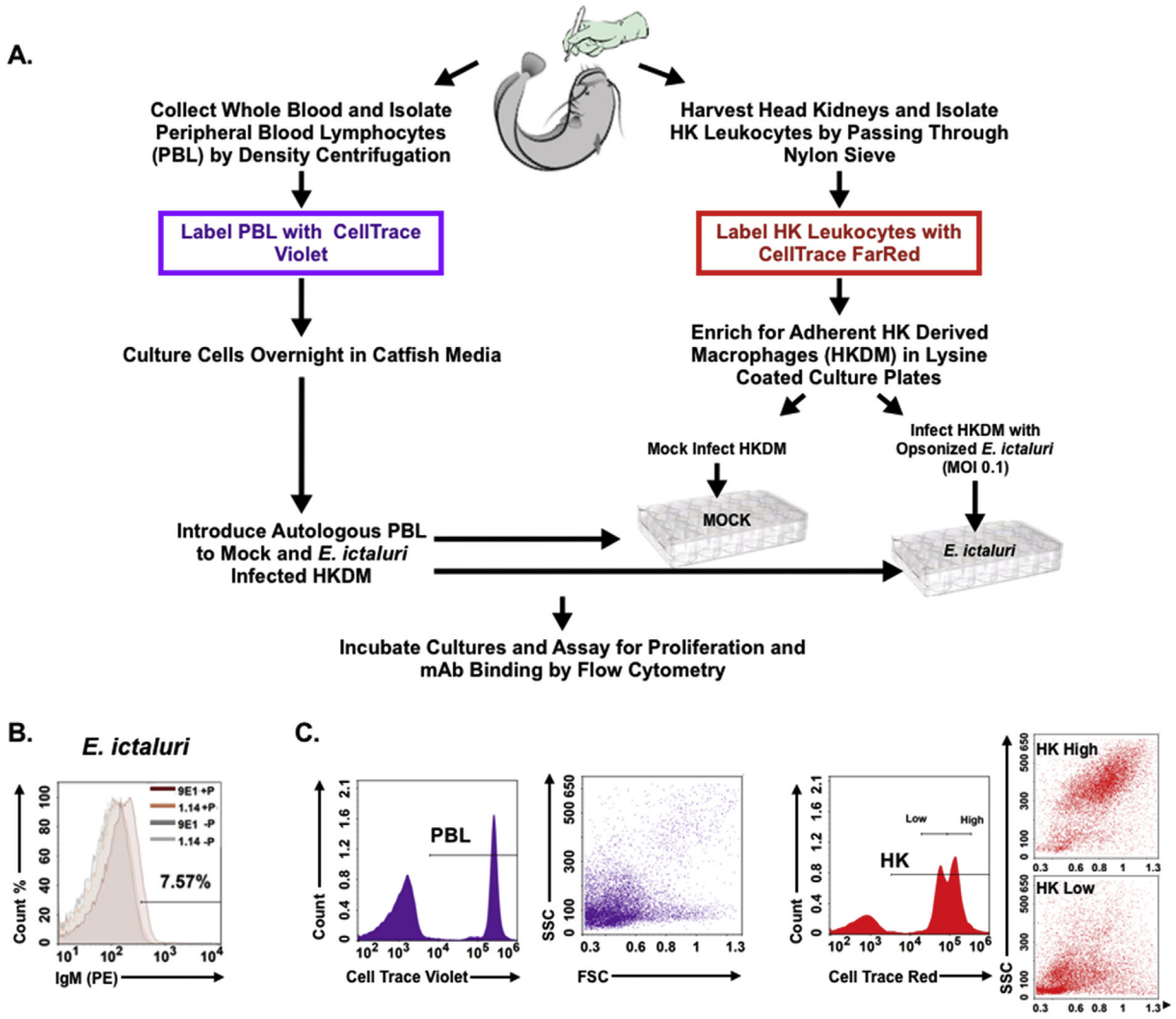

Fig. 4. *In vitro* model to examine lymphocyte stimulation in response to *E. ictaluri* infection. (**A**) Peripheral blood and head kidney tissues were harvested from individual catfish. For each fish, PBL were labeled with CellTrace Violet and cultured overnight (16 h) in AL-4 media. In parallel, head kidney tissue slices were disassociated through nylon cell strainers, and the collected leukocytes were labeled with CellTrace FarRed, and cultured overnight in CCMM on poly-D-lysine coated plates. The head kidney leukocytes were then washed in incomplete CCMM, and the adherent HKDM were infected for 1.5 h with *E. ictaluri* previously opsonized with autologous plasma (MOI 0.1). Gentamicin was added to each well to kill any remaining extracellular bacteria. After 1 h, the plates were washed in incomplete CCMM. CellTrace Violet labeled autologous PBL were introduced to the cultures at a ratio of 10:1 and maintained in media containing a static dose of gentamicin. Cells were monitored daily by fluorescent microscopy and/or flow cytometry. **(B)** *E. ictaluri* (1×10^6) was incubated with plasma (+P) as a source of IgM or without plasma (-P). The bacteria were then washed and incubated with anti-catfish IgM mAb 9E1 followed by goat anti-mouse IgG-PE. Staining with the isotype control anti-rainbow trout IgM, mAb 1.14 is shown in either orange or gray. (**C**) CellTrace Violet labeled PBL were cocultured with CellTrace FarRed labeled mock-infected HK-derived leukocytes for 6 h and analyzed by flow cytometry. Fluorescence histograms of cells examined for CellTrace Violet staining or CellTrace FarRed staining are shown. Positive gates for CellTrace Violet and CellTrace FarRed were set based on unlabeled cells. The peaks of two different staining intensities with CellTrace FarRed were gated separately (high and low). The scatter profiles of the cells within each gate are shown next to each histogram. Notably at this time point, the ratio of CellTrace Violet labeled cells to CellTrace FarRed labeled cells were the same for infected and non-infected cultures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for fluorescence using the Pacific blue (445/35) and APC channels (675/30). At this time point, CellTrace Violet labeled PBL stained uniformly and 92% of the PBL were found in one sharp peak, well-separated from the negative population of CellTrace FarRed labeled head kidney leukocytes (Fig. 4C). In contrast, CellTrace FarRed labeled head kidney leukocytes were found in two major peaks of different staining intensity (low and high; at a ratio of 0.6:1). This was unexpected since significant cell division within this 22 h timeframe was not anticipated. When the head kidney leukocytes within the two peaks were gated separately and examined by forward and side scatter, the cells within the high intensity gate consisted mainly of large internally complex HKDM, while the cells with lower fluorescence were smaller and less complex. Also, examination of replicate cultures from this time point by microscopy showed numerous smaller, less complex cells including lymphocytes, monocytes, red blood cells, and platelets. It is likely that the intense staining of the larger HKDM with CellTrace FarRed is due to their more efficient uptake of the dye.

At 48 h, an overall reduction in PBL number was observed when cocultured with mock-infected HKDM, i.e. 26,900 CellTrace Violet PBL at 6 h vs 10,660 CellTrace Violet PBL at 48 h (see Figs. 4C and 5A). Similarly, the number of labeled PBL was also reduced when cultured with *E. ictaluri*-infected macrophages (26,160 PBL at 6 h vs 9580 PBL at 48 h). This reduction was not unexpected, since a decline in lymphocyte numbers during the first two days in culture is routinely observed when culturing freshly isolated PBL (Barker et al., 2000; Miller et al., 1994). In comparison, when CellTrace FarRed labeled head kidney leukocytes

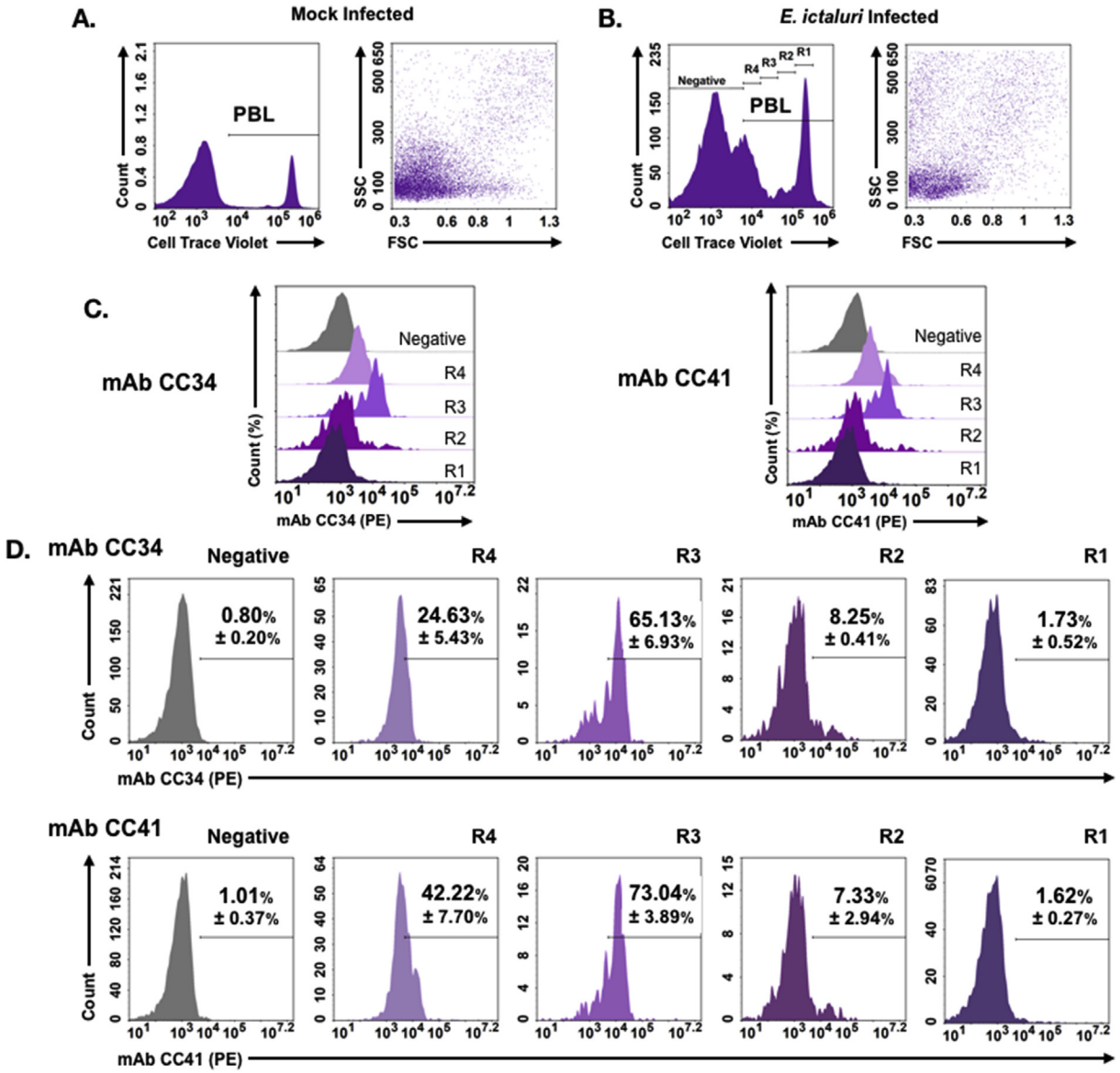

Fig. 5. Catfish PBL cocultured with autologous *E. ictaluri*-infected macrophages proliferate and are mAb CC34 and mAb CC41 positive. CellTrace Violet labeled PBL were combined with CellTrace FarRed labeled HKDM that were infected with *E. ictaluri* or mock-infected, cultured for 48 h in 24 well plates, and then examined by flow cytometry. (**A**) The histogram of a mock-infected cell culture was gated for CellTrace Violet staining based on an unstained control sample; the cells within the PBL gate are presented in the scatter profile. **(B)** The histogram for the *E. ictaluri*-infected culture was gated into four regions (R1-R4), based on decreasing CellTrace Violet staining intensity. The scatter profile displays all events within the positive PBL gate of the histogram. (**C**) Overlays of normalized histograms of mAb CC34 staining (left) and mAb CC41 staining (right) of cells within each region gated on CellTrace Violet staining in panel A. (**D**) Individual histograms of mAb CC34 staining (top) and mAb CC41 staining (bottom) of cells within each region gated on CellTrace Violet staining in panel A. Percentage of positive cells within each gate is set based on the staining with the isotype control anti-rainbow trout IgM, mAb 1.14. Data is representative of two independent experiments performed in triplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were analyzed, their numbers remained constant, or even increased in the mock-infected cultures (44,330 at 6 h versus 46,076 at 48 h). In contrast, the numbers of CellTrace FarRed cells in the *E. ictaluri*-infected cultures were significantly reduced due to the loss of the macrophage population that stained with high intensity (40,433 head kidney cells at 6 h versus 15,527 head kidney cells at 48 h; Supplemental Fig. 2). More importantly, a reduction in CellTrace Violet staining intensity occurred in 19.1% ± 3.6% of the PBL in the presence of *E. ictaluri*-infected HKDM as compared to 4% ± 1.1% in the presence of mock-infected HKDM (Fig. 5A and B). This decrease in staining demonstrated that at least some CellTrace Violet lymphocytes had proliferated. Furthermore, as the cells proliferated, there was an increase in LITR expression as assessed by both CC34 and CC41 staining (Fig. 5C). For example, lymphocytes within the R1 gate, had not proliferated and stained very weakly with CC34 and CC41 (< 2.5%; Fig. 5D). Cells within the R2 and R3 gates, however had replicated and a larger percentage of cells within these gates stained positive with mAb CC34 and mAb CC41. Together, these staining patterns imply that as the lymphocytes begin to proliferate (R2), they upregulate their expression of CC34-reactive LITRs and CC41-reactive LITRs. The high percentages of LITR positive cells (64.4% and 70.4%) found within R3, also suggest that lymphocytes can be mAb CC34 and mAb CC41 double positive. While cells within the R4 gate had proliferated the most and were only dimly positive for CellTrace Violet, a significant portion of these cells were still positive for mAb CC34 (25.4%) and mAb CC41 (39.6%).

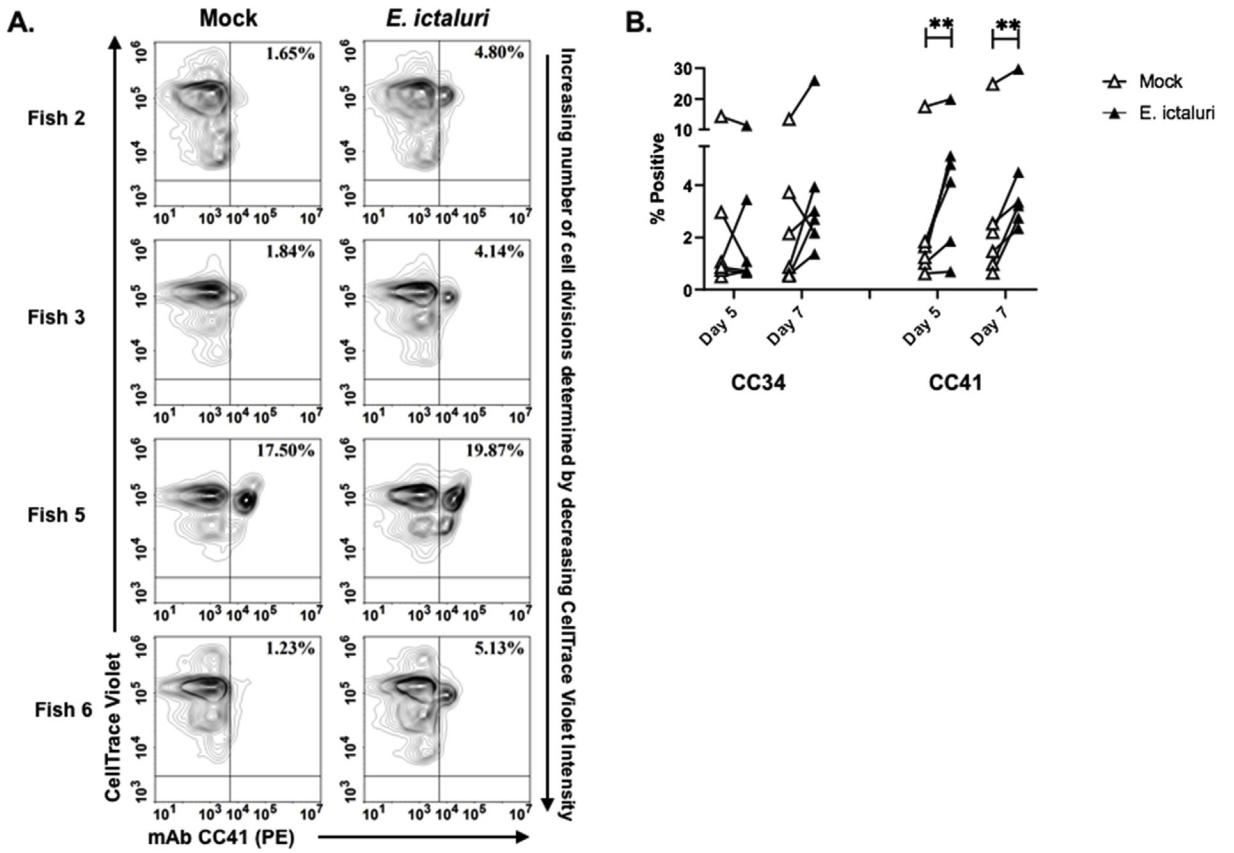

Fig. 6. Numbers of CC41$^+$ PBL increase when cocultured with *E. ictaluri*-infected macrophages. CellTrace Violet labeled PBL were combined with CellTrace FarRed labeled HKDM that were infected with *E. ictaluri* or mock-infected, cultured for 7 days in T-75 flasks. Samples from the cultures were examined every other day by flow cytometry. (**A**) Contour plots of cells collected on day 5 and stained with CC41 are shown. Cells are gated on CellTrace Violet fluorescence (y-axis) and analyzed for CC41 staining (x-axis). The percentages of double positive cells are indicated in the top right quadrants. Data from four representative fish are shown (N = 6). (**B**) Percentages of CC34$^+$ and CC41$^+$ cells within the PBL (CellTrace Violet) gate are presented. The percent positive PBL from mock-infected and *E. ictaluri*-infected cultures were analyzed using a two-tailed paired *t*-test. The pairs of mock-infected (△) and *E. ictaluri*-infected (▲) cultures from each fish are shown for day 5 and day 7; n = 6, ** *p*-value < 0.015.

3.4. CC41$^+$ lymphocytes proliferate when cocultured with E. ictaluri-infected macrophages

Since the results from the pilot study demonstrated that LITR expressing lymphocytes proliferated in response to *E. ictaluri*-infected HKDM, the *in vitro* infection model was scaled up to include six fish. In this experiment all of the cultures were grown in T-75 flasks for seven days. As above, CellTrace Violet labeled PBL were incubated with mock-infected or *E. ictaluri*-infected HKDM, and aliquots (1×10^6 cells) were collected for flow cytometry at days 3, 5 and 7. The cell samples were stained with anti-LITR CC34 or CC41, and also with anti-catfish IgM 9E1. Anti-trout IgM 1.14 was used as the isotype control. The contour plots in Fig. 6A and Supplemental Fig. 3 show distinct populations of CellTrace Violet cells, which demonstrate that several rounds of lymphocyte proliferation occurred in both infected and mock-infected cultures. Importantly, there was a significant increase in the percentage of CC41$^+$ cells on days 5 and 7 for each of the six infected cultures as compared to their paired mock-infected cultures ($p < 0.015$; Fig. 6 and Supplemental Table 2). In contrast for mAb CC34, the staining data was more variable. On day 5, only one of the infected cultures exhibited a noticeable increase in CC34 staining and in two of the cultures the percentages of CC34$^+$ cells decreased (see Fig. 6B). However on day 7, an increase in CC34$^+$ PBL was observed in infected cultures from five out of six fish, while the culture from the 6th fish, exhibited a lower percentage of CC34$^+$ PBL. Similarly the staining with anti-catfish IgM was also variable. On day 5, four of the six cultures showed an increase in the percentage of IgM$^+$ PBL, while in the other two cultures a decrease in the staining with anti-catfish IgM occurred as compared to the mock-infected cultures (Supplemental Fig. 3). On day 7, multiple populations of IgM$^+$ cells were present including cells staining with low, intermediate and high intensity. While there was an increase in the percentage of IgM$^+$ cells in infected cultures from five of the six fish, this increase was not significant when the percent IgM$^+$ positive PBL from mock-infected and *E. ictaluri*-infected cultures were analyzed using a two-tailed paired *t*-test ($p = 0.09$). Here it should be noted that IgM$^+$ cells include not only B cells, but also FcμR bearing cells such as NK cells.

That *E. ictaluri*-infected HKDM and lymphocytes readily interact was demonstrated using fluorescent microscopy. The fluorescence microscopy overlay in Fig. 7A shows a cluster of activated lymphocytes (CellTrace FarRed negative) on day 5, surrounding and interacting with *E. ictaluri*-infected CellTrace FarRed labeled HKDM. Notably, some of these lymphocytes exhibit morphologies similar to the elongated morphologies of activated catfish CTLs as described by Spencer et al. (2019), while other lymphocytes were more rounded in shape. Also, at lower magnification, light microscopy images of *E. ictaluri*-infected cultures showed differentiated cells with diverse morphologies, and cell to cell contact was readily observed (Fig. 7B). In contrast, the lymphocytes in the paired mock-infected cultures were small and rounded, even so proliferating lymphocytes could also be observed.

4. Discussion

In this study we determined that mAb CC34 and mAb CC41 binds to

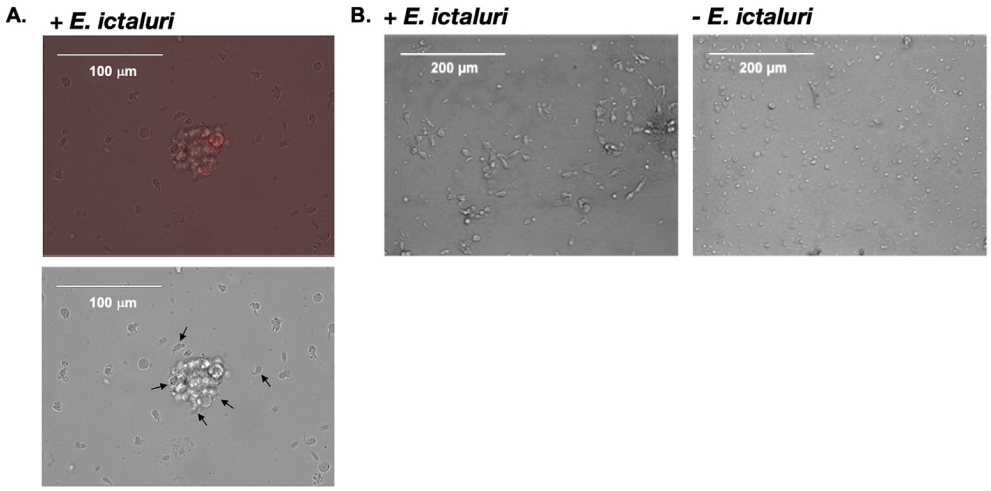

Fig. 7. *E. ictaluri*-infected macrophages stimulate PBL. (**A**) Fluorescent microscopy overlay and brightfield image of CellTrace FarRed labeled HKDM infected with *E. ictaluri* (MOI 0.1) cultured with PBL for 5 days. Arrows indicate different examples of cytotoxic lymphocytes. Scale bar: 100 μm. (**B**) Bright-field microscopy of the paired *E. ictaluri*-infected HKDM and PBL (left), and mock-infected HKDM and PBL (right) co-cultured for 5 days. Scale bars: 200 μm.

TS32.15 LITR1.1a, and like CC41, CC34 is predicted to bind within the D2-D3 domains of certain LITRs. Furthermore, we developed an *in vitro* model based on autologous MLR to examine the lymphocyte response to *E. ictaluri* infection, and demonstrated that CC41-reactive cytotoxic lymphocytes increase in number and proliferate in the presence of infected HKDM. Also, by using two color CellTrace labeling, it was possible to independently track PBL and head kidney derived leukocytes from individual fish. Moreover, paired mock-infected cultures from each fish provided the control. Therefore, even though the number of $CC41^+$ cells that were present in the freshly isolated PBL varied between fish, that CC41-reactive cytotoxic cells increased after coculture with *E. ictaluri*-infected HKDM was consistent in all fish (Figs. 5 and 6). For example, fish 5 exhibited much higher numbers of both $CC34^+$ and $CC41^+$ cells in its PBL *ex vivo* and was identified as an outlier by the Prism software using ROUT (Q = 1%). Even so, the increases in the number of $CC41^+$ cells were statistically significant, regardless if fish 5 was included or excluded from the analyses ($p < 0.0150$). These observed increases in $CC41^+$ cells appear to be due to 1) an increase in CC41 staining intensity, i.e. more LITRs are expressed on the cell surface of each cell, and 2) the proliferation of $CC41^+$ cells as determined by decreased CellTrace Violet staining intensity (see Figs. 5 and 6). In contrast, even though an increase in the number of $CC34^+$ cells was also observed with some fish, the results were more variable and the numbers were not statistically significant. While it is possible that a study with larger numbers of fish could prove the increase in the $CC34^+$ cell population to be statistically significant, it should be emphasized that mAbs CC34 and CC41 recognize overlapping (but not identical) subsets of LITRs. For example, CC34 and CC41 both bind to epitopes within the D2-D3 domains of TS32.15 LITR1.1a, however their staining profiles of long-term cell lines are not identical, i.e. 28S.3 T cells only stain with CC41. Using our coculture model it was clear that different populations of IgM^+ cells also proliferated in response to *E. ictaluri*-infected macrophages. However, there was not a significant increase in the percentages of IgM^+ cells in *E. ictaluri*-infected cultures as compared to mock-infected cultures. Even so, the nature of these IgM^+ cells is difficult to interpret without performing flow cytometry experiments that include staining with mAbs to both IgL κ light chains (Lobb et al., 1982, 1984). Dual staining with both anti-IgL F and anti-IgL G discriminates between B cells that express only one IgL isotype and cells that acquired exogenous IgM via an IgM binding receptor. For example, catfish NK cells, like mammalian NK cells express IgM binding receptors (Shen et al., 2003, 2004), and more recently Taylor et al. (2016, 2019) demonstrated that cytotoxic T cells also can acquire exogenous IgM.

In this current study, while we did not characterize the $CC41^+$ cells in terms of cytotoxicity and mRNA expression, we previously established that $CC41^+$ cells are cytotoxic and express perforin (Taylor et al., 2016). Furthermore, in earlier studies using our clonal cell lines it was also demonstrated that $CC41^+$ cells include both innate and adaptive cytotoxic cells, e.g. clonal NK cells, and Group I CTLs (Shen et al., 2002, 2004; Taylor et al., 2016). Therefore, and because the fish used in this current *in vitro* stimulation were naïve, it seems likely that the responding $CC41^+$ cells were NK cells that were stimulated by cytokines, i.e. TNF-α and IL-12, secreted by the infected macrophages. In this context, it is important to note that catfish NK cells can stain with anti-IgM mAb 9E1. For example, we previously determined that catfish NK cells armed with exogenous IgM can kill target cells via antibody-dependent cell-mediated cytotoxicity (ADCC; Shen et al., 2002, 2003). Also in 2004, Shen et al. examined individual clonal NK cell lines and observed differential staining for IgM, i.e. some clonal cell lines exhibited low levels of staining while others stained with high intensity. More recently, it was also demonstrated that the number of IgM positive cells increase in virally infected cells and that clonal virus specific CTLs also are IgM^+ (Taylor et al., 2019). Importantly, catfish clonal NK cell lines, TS32 Group I CTLs and TS32 Group II CTLs all express message for INF-γ and INF-γrel (Milev-Milovanovic et al., 2006). Fish INF-γ share several features of mammalian INF-γ including the ability to stimulate the production of reactive oxygen species (ROS), as demonstrated in cultures of goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), and carp (*Cyprino carpio*) primary kidney macrophages (reviewed by Grayfer et al., 2018). Similarly, large yellow croaker (*Larimichthys crocea*), and goldfish INF-γ were shown to induce the expression of iNOS and proinflammatory cytokines, including TNF-α in kidney-derived macrophages. Also, teleost INF-γ, like mammalian INF-γ, are critically important for classical activation of M1 macrophages and host defense against intracellular pathogens, e.g. mycobacterium species. It is interesting that in ginbuna carp (*Carassius auratus langsdorfii*), adoptive transfer of *Edwardsiella tarda* sensitized $CD4^+$ or $CD8^+$ T cells mediated protection when fish were challenged one day after T cell transfer (Yamasaki et al., 2014). In addition, the fish that received either $CD4^+$ or $CD8^+$ T cells expressed higher levels of perforin mRNA, and exhibited lower bacterial counts in their kidneys and spleens. Moreover, the fish that received *E. tarda* sensitized $CD8^+$ T cells had the lowest bacterial counts, exhibited fewer and less severe clinical symptoms, and the IFN-γ gene expression in their kidneys were elevated.

Therefore, we believe that it is reasonable to speculate that catfish $CC41^+$ cells can participate in the activation and polarization of macrophages towards a microbicidal phenotype via their secretion of INF-γ, and perhaps also by their release of perforins and granzymes. In the future, it should be possible to modify our current model to determine how $CC41^+$ cells are activated, and also define how $CC41^+$ cells contribute to macrophage-mediated immunity against *E. ictaluri*.

Declaration of competing interest

None.

Acknowledgements

We thank Dr. Atilla Karsi at Mississippi State University for providing the *Edwardsiella ictaluri* strain 93-146, and Dr. Norma Ojeda (MS CEPR Biostatistics/Bioanalytical Sub-Core) for assistance with the statistical analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103610.

Funding

This work was supported by the U.S. Department of Agriculture, National Institute of Food and Agriculture Predoctoral Fellowship 2017-67011-26282 to (L. E. B.), and the National Science Foundation Grant IOS-1655,419. In addition, research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P20GM121334. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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