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The amphibian (Xenopus laevis) colony-stimulating factor-1 and interleukin-34-derived macrophages possess disparate pathogen recognition capacities

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ABSTRACT

Pathogens such as the Frog Virus 3 (FV3) ranavirus are contributing to the worldwide amphibian declines. While amphibian macrophages (Mϕs) are central to the immune defenses against these viruses, the pathogen recognition capacities of disparate amphibian Mϕ subsets remain unexplored. In turn, Mϕ differentiation and functionality are interdependent on the colony-stimulating factor-1 receptor (CSF-1R), which is ligated by colony-stimulating factor-1 (CSF-1) and the unrelated interleukin-34 (IL-34) cytokines. Notably, the *Xenopus laevis* frog CSF-1- and IL-34-derived Mϕs are functionally distinct, and while the CSF-1-Mϕs are more susceptible to FV3, the IL-34-Mϕs are highly resistant to this pathogen. Here, we elucidate the pathogen recognition capacities of CSF-1- and IL-34-differentiated Mϕs by evaluating their baseline transcript levels of key pathogen pattern recognition receptors (PRRs). Compared to the frog CSF-1-Mϕs, their IL-34-Mϕs exhibited greater expression of PRR genes associated with viral recognition as well as PRR genes known for recognizing bacterial pathogen-associated molecular patterns (PAMPs). By contrast, the CSF-1-Mϕs displayed greater expression of toll-like receptors (TLRs) that are absent in humans. Moreover, although the two Mϕ types possessed similar expression of most downstream PRR signaling components, they exhibited distinct outcomes upon stimulation with hallmark PAMPs, as measured by their tumor necrosis factor-alpha and interferon-7 gene expression. Remarkably, stimulation with a TLR2/6 agonist conferred FV3 resistance to the otherwise susceptible CSF-1-Mϕs while treatment with a TLR9 agonist significantly ablated the IL-34-Mϕ resistance to FV3. These changes in Mϕ-FV3 susceptibility and resistance appeared to be linked to changes in their expression of key immune genes. Greater understanding of the amphibian macrophage pathogen-recognition capacities will lend to further insights into the pathogen-associated causes of the amphibian declines.

1. Introduction

The alarming global amphibian population declines continue to pose a threat to world-wide biodiversity, with emerging pathogens significantly contributing to these catastrophic die-offs (Hayes et al., 2010; Stuart et al., 2004). Frog Virus 3 (FV3) and other members of the

Abbreviations: BHK, baby hamster kidney; CDS, cytosolic DNA sensor; CSF-1, colony-stimualting factor-1; CSF, 1R-CSF-1 receptor; Ddx41, DEAD-box helicase 41; Dhx36, DEAH-box helicase 36; Dhx58, DExH-box helicase 58; ds, double-stranded; FLA-ST, *Salmonella typhimurium* flagellin; Fln29, TRAF-type zinc finger domain-containing protein 1; FSL1, *Mycoplasma salivarium* lipopeptide; FV3, Frog Virus 3; HKLM, heat killed *Listeria monocytogenes*; HMW, high molecular weight; HSV-60, herpes simplex virus 1 genome-derived DNA; IFN, interferon; IL-34, interleukin-34; imiquimod, imidazoquinoline amine analog to guanosine; ISD, interferon stimulatory DNA/non-CpG oligomer from *Listeria monocytogenes* genome; LMW, low molecular weight; LPS-EK, *Escherichia coli* lipopolysaccharide; Mϕ, macrophage; Myd88, myeloid differentiation primary response 88; ODN2006, CpG DNA; PAMP, pathogen-associated molecular pattern; Pin1, Peptidylprolyl cis/trans isomerase 1; PRR, pattern recognition receptor; RIG-I, retinoic acid-inducible gene I; Sarm1, sterile alpha and tyrosine inhibitory receptor motif containing 1; Socs1, supressor of cytokine signaling 1; ss, single-stranded; ssRNA40, uridine-rich single-stranded RNA derived from the HIV-1 long terminal repeat; TLR, toll-like receptor; TNF, tumor necrosis factor; Tollip, toll-interacting protein; Traf6, tnf receptor associated factor 6; Triad3A, E3 ubiquitin-protein ligase; VACV-70, vaccinia virus genome-derived DNA; Yy1, Ying yang 1; Zbp1, Z-DNA binding protein 1

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ranavirus genus (family *Iridoviridae*), are notable contributors to these declines, where ranaviruses cause extensive levels of morbidity and mortality among amphibian species around the globe (Chinchar, 2002; Gray et al., 2009). Although amphibians possess sophisticated immune systems (Flajnik, 2018; Robert and Ohta, 2009; Rollins-Smith, 1998; Yaparla et al., 2017), the mechanisms conferring their susceptibility and resistance to emerging pathogens remain to be adequately understood. Thus, garnering greater insights into the capacities of amphibians to recognize and respond to distinct pathogens is critical to devising more-effective strategies by which to counteract their declines.

Macrophages (Mϕs) are considered to be amongst the most important sentinel cells of the immune system due to their refined capacities to detect infiltrating pathogens (Grayfer et al., 2014; Hirayama et al., 2017). Vertebrate Mϕ differentiation and functionality depend on the activation of the Mϕ colony-stimulating factor-1 receptor (CSF-1R) (Hanington et al., 2007; Pixley and Stanley, 2004), which in addition to colony-stimulating factor-1 (CSF-1) is also activated by the unrelated interleukin-34 (IL-34) cytokine (Chihara et al., 2010; Liu et al., 2012; Ma et al., 2012; Wei et al., 2010). While the biological necessity for two CSF-1R ligands remains to be fully explored, our studies using the amphibian *Xenopus laevis* indicate that the frog CSF-1 and IL-34 cytokines give rise to morphologically and functionally distinct Mϕs (Grayfer and Robert, 2015, 2014). Frog CSF-1-Mϕs are highly susceptible to the emerging FV3 ranavirus and worsen frog FV3 infections (Grayfer and Robert, 2015, 2014) by increasing the frog tissue viral loads, presumably by disseminating the virus. By contrast, IL-34-Mϕs are resistant to this pathogen, which is consistent with their elevated expression of type I and type III interferon (*ifn*) cytokine genes as well as an array of antiviral restriction factors (Yaparla et al., 2018). However, the pathogen recognition capacities of these respective Mϕ subsets remain to be fully explored and are therefore the main focus of this study.

The success of pathogen recognition by immune cells is extremely important to effective clearance of invading pathogens, and pathogen pattern recognition receptors (PRRs) represent a major immunological strategy for this pathogen detection (Mogensen, 2009; Takeuchi and Akira, 2010). Distinct families and groups of PRRs are strategically expressed on immune cell plasma membranes to recognize extracellular pathogens such as bacteria or fungi. Other PRRs are localized to endosomal membranes and within the cytosol, thus facilitating the recognition of intracellular invaders such as viruses, intracellular bacteria and protozoans (Mogensen, 2009; Takeuchi and Akira, 2010; Thompson et al., 2011). The specificities of disparate PRRs are dictated by their respective recognition of distinct repetitive pathogen-associated molecular patterns (PAMPs). The PAMP-PRR binding results in the coordinated immune cell activation, culminating in pathogen-appropriate downstream immune gene expression including the upregulated expression of proinflammatory and/or antiviral cytokines, depending on the PRR/PAMP interaction (Mogensen, 2009; Takeuchi and Akira, 2010; Thompson et al., 2011).

The best-characterized PRRs are the toll-like receptors (TLRs), which are expressed on either plasma membranes, thereby facilitating the immune recognition of extra-cellular pathogens; or are present within the endomembrane system and recognize intracellular microbes (Takeda and Akira, 2005). Notably, in addition to membrane-bound PRRs, vertebrate species possess an array of cytosolic RNA and DNA sensors, which recognize a wide variety of intracellular PAMPs, resulting in pro-inflammatory and/or IFN responses (Ori et al., 2017).

Here, we report that the *X. laevis* frog CSF-1- and IL-34-derived Mϕs possess distinct PRR expression and PAMP recognition capacities. Moreover, we show that the respective FV3 susceptibility and resistance of the CSF-1- and IL-34-Mϕs to FV3 may be altered by exposure to certain PAMPs.

2. Materials and methods

2.1. Animals

Outbred adult *X. laevis* (~1–2 years old, 2–3 inch males and females) were purchased from Xenopus1 (Dexter, MI), housed and handled under strict laboratory regulations of Animal Research Facilities and IACUC at the George Washington University.

2.2. Recombinant cytokine production

X. laevis recombinant CSF-1 and IL-34 were generated via previously described methods (Grayfer and Robert, 2015). Briefly, PCR amplicons corresponding to the signal-peptide cleaved CSF-1 and IL-34 open reading frames were ligated into the pMIB/V5 His A vector. These expression constructs were then transfected into Sf9 insect cells (Cellfectin II, Invitrogen) and positive transfectants were selected with 10 μg/ml blasticidin. The Sf9 supernatants were screened for recombinant CSF-1 or IL-34 by western blot against the V5 epitope on the recombinants. The cultures expressing CSF-1 or IL-34 were scaled up and grown as 500 ml liquid cultures, pelleted and the supernatants concentrated using polyethylene glycol flakes (8 kDa) at 4 °C and subjected to overnight (16 h) dialysis against 150 mM sodium phosphate at 4 °C. The concentrated supernatants were then subjected to Ni-NTA agarose column purification (Qiagen). The bound recombinants were washed with 2 × 10 vol of high stringency wash buffer (0.5% tween 20, 20 mM sodium phosphate, 500 mM sodium chloride, 100 mM imidazole) and 5 × 10 vol of low stringency wash buffer (as above, but with 40 mM imidazole). The recombinant CSF-1 and IL-34 were then eluted in fractions with 250 mM imidazole. Western blot analysis was performed against the V5 epitope on these recombinant proteins to confirm the presence of CSF-1 or IL-34. The protein concentrations were determined by Bradford protein assays (BioRad) and a protease inhibitor cocktail (Halt protease inhibitor cocktail; Thermo Scientific) was added to the purified CSF-1 and IL-34, which were then aliquoted and stored at −20 °C until use.

2.3. FV3 stocks

FV3 production has been described previously (Morales et al., 2010). Briefly, baby hamster kidney (BHK-21) cells were subjected to FV3 infection at a 0.1 multiplicity of infection (MOI), followed by incubation at 30 °C and 5% CO_2. The FV3-containing cell lysates were collected over 30% sucrose by ultracentrifugation, resuspended in saline and quantified using plaque assays over BHK-21 cells.

2.4. Differentiation and culture of CSF-1- and IL-34-Mϕs

The isolation of *X. laevis* bone marrow cells and culture conditions have been previously described (Yaparla and Grayfer, 2018). For each experiment, bone marrow cells were derived from 5 to 6 healthy adult frogs (N = 5–6, mixed gender) and enumerated by trypan blue (Invitrogen) live/dead exclusion, cultured at a density of 10^4 cells per well with 250 ng/ml of recombinant *X. laevis* CSF-1 or IL-34 and incubated at 27 °C with 5% CO_2. The cells were restimulated with each of the respective cytokines on day 3 and harvested on day 5 in Trizol (Invitrogen).

2.5. Isolation of RNA and DNA from CSF-1- and IL-34-Mϕs

For all experiments, the cells were harvested into Trizol reagent (Invitrogen) and the RNA was extracted following manufacturer's instructions. Following RNA isolation, the remaining trizol layer was used to isolate DNA from samples infected with FV3 by mixing with back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1M Tris pH.8), centrifugation to obtain the DNA containing aqueous

phase, followed by overnight (16 h) precipitation with isopropanol. The isolated DNA was then pelleted by centrifugation, washed with 70% ethanol and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA).

2.6. Quantitative analyses of gene expression and FV3 DNA loads

Five hundred nanograms of total RNA were used for all samples to synthesize cDNA, using iScript cDNA synthesis kits (Bio-Rad) and 2.5 μl of cDNA was used as a template for gene expression analyses. All gene expression analyses were performed via the delta^delta CT method, with expression examined relative to the *gapdh* endogenous control and normalized against the lowest observed expression. The PAMP-stimulated CSF-1- and IL-34-Mϕ immune genes expression was normalized against the expression of the respective genes in the corresponding unstimulated Mϕ subsets. CFX96 Real-Time System (BioRad) and iTaq Universal SYBR Green Super mix were used for all the experiments. The expression analyses were performed using the BioRad CFX Manager software. All primers were validated prior to use against serially diluted *X. laevis* spleen-derived cDNA, ensuring that the amplification of each transcript corresponded to the magnitudes of cDNA dilution and exhibited single melt curves. All primer sequences are listed in Supplementary Table 1.

FV3 loads were quantified via absolute qPCR using primers specific for viral (v)DNA Polymerase, using a previously described approach (Grayfer and Robert, 2014). Briefly, an FV3 vDNA Pol PCR fragment was cloned into the pGEM-T Easy vector (Promega), amplified in bacteria, following quantification and serial dilutions to yield 10^8 to 10^1 plasmid copies of vDNA Pol II. A standard curve of these dilutions was used in all absolute qPCR experiments, with 50 ng of individual DNA samples screened for the absolute FV3 DNA loads.

2.7. PAMP stimulation and FV3 infection of CSF-1- and IL-34-Mϕs

X. laevis bone marrow-derived CSF-1- and IL-34-Mϕs from 5 individual adult frogs ($N = 5$), were treated with the following TLR 1–9 agonists (InvivoGen; using doses based on published literature): triacylated lipopeptide (Pam3CSK4)-TLR1/2 agonist (10 μg/ml); heat-killed *Listeria monocytogenes* (HKLM)-TLR2 agonist (25:1) high molecular weight (HMW) double-stranded RNA (Poly I:C)-TLR3 and RIG-I agonist (10 μg/ml) (Fujimoto and Naka, 2010; Guven-Maiorov et al., 2015); low molecular weight double-stranded RNA (LMW) of (Poly I:C)-TLR3 and RIG-I agonist (10 μg/ml) (Zhou et al., 2013); *Escherichia coli* lipopolysaccharide (LPS-EK)-TLR4 agonist (10 μg/ml) (Yumoto et al., 2005); *Salmonella typhimurium* flagellin (FLA-ST)-TLR5 agonist (1 μg/ml) (Ferrero et al., 2012); *Mycoplasma salivarium* lipopeptide (FSL1)-TLR2/6 agonist (1 μg/ml) (Grassin-Delyle et al., 2018); imidazoquinoline amine analog to guanosine (imiquimod)-TLR7 agonist (10 μg/ml) (Kim et al., 2016); uridine-rich single-stranded RNA derived from the HIV-1 long terminal repeat (ssRNA40)-TLR8 agonist (10 μg/ml) (O'Hara et al., 2011); CpG DNA oligonucleotide (ODN2006)-TLR9 agonist (10 μg/ml) (O'Hara et al., 2011). The cells were subjected to overnight treatment (16 h) with the agonists and RNA was extracted using Trizol reagent the next day, followed by cDNA synthesis and qPCR analyses. Mϕs were also stimulated with 10 μg/ml (as per working concentration suggested by the manufacturer, InvivoGen) each of the following cytosolic DNA sensor (CDS) agonists: HSV-60 (herpes simplex virus 1 genome-derived DNA), VACV-70 (vaccinia virus genome-derived DNA) and non-CpG oligomer from *Listeria monocytogenes* genome (referred to as interferon stimulatory DNA; ISD) (Invivogen). All CDS agonists were transfected into cells using the cationic lipid transfection reagent LyoVec, according to manufacturer instructions (InvivoGen) with the Mϕs stimulated overnight (16 h) and processed the following day.

For the modulation of Mϕ-FV3 susceptibility experiments, bone marrow derived CSF-1- and IL-34-Mϕs (10^4 cells/well) from 5 individual animals ($N = 5$) were stimulated overnight (16 h) with select PAMPs at concentrations listed above and infected with FV3 the following day at a multiplicity of infection of 0.5 plaque forming units (PFU) per cell for an additional 24 h. Subsequently, the cells were collected by centrifugation and DNA was isolated towards quantification of FV3 DNA viral loads, as described above.

2.8. Statistical analysis

For the experiments involving PRR agonist stimulations, multiple comparison analyses were performed using one-way analysis of variance (ANOVA) with a post-hoc Dunnett's multiple comparisons test. Statistical comparisons of the baseline IL-34- and CSF-1-Mϕ gene expression were conducted using paired Student's T tests. All statistical analyses were performed using GraphPad Prism 7.0 software, with statistical significance deemed at P values less than 0.05.

3. Results

3.1. Analyses of baseline toll-like receptor gene expression in CSF-1- and IL-34-Mϕs

We previously showed that expanding the frog IL-34-Mϕs *in vivo* confers greater anti-FV3 resistance whereas expanding the frog CSF-1-Mϕs renders these animals more susceptible to this pathogen (Grayfer and Robert, 2015, 2014; Yaparla et al., 2018). To discern the pathogen-recognition capacities of these FV3-resistant and susceptible Mϕ populations, we examined the expression of endosomal toll-like receptor genes (*tlrs 3*, *7*, *8* and *9*), which are important to the detection of intracellular pathogens such as viruses. Consistent with their antiviral nature, the frog IL-34-differentiated Mϕs exhibited significantly greater gene expression of these *tlr* genes than the CSF-1-Mϕs (Fig. 1A).

To expand upon these findings, we next examined the expression of genes encoding plasma membrane-bound toll-like receptors (*tlrs 1*, *2*, *4*, *5* and *6*) by CSF-1- and IL-34-differentiated Mϕs. Compared to the CSF-1-Mϕs, the IL-34-Mϕs also possessed significantly greater transcript levels of *tlrs 2* and *4* (Fig. 1B), the mammalian counterparts of which are implicated in the recognition of bacterial cell wall lipopolypeptides and lipopolysaccharides, respectively (Hajjar et al., 2002; Vasselon et al., 2004). The two Mϕ populations possessed comparable mRNA levels of *tlrs 1*, *5* and *6* (Fig. 1B).

To gain a comprehensive understanding of the baseline *tlr* gene expression by these frog Mϕ subsets, we also analyzed the IL-34- and CSF-1-differentiated Mϕs for their mRNA levels of toll-like receptors that are absent in humans but are expressed by other vertebrates (*tlr12*, *13*, *21* and *22*). Notably, the mouse TLR12 is involved in the recognition of *Toxoplasma gondii* (Koblansky et al., 2013) while the murine and marsupial TLR13 recognizes bacterial ribosomal RNA (Oldenburg et al., 2012) and the teleost fish and avian TLRs 21 and 22 detect double-stranded DNA and RNA, respectively (Keestra et al., 2010; Matsuo et al., 2008; Roach et al., 2005). Intriguingly, the CSF-1-Mϕs exhibited significantly greater expression of the *tlrs 12, 13* and *21* genes than the IL-34-Mϕs (Fig. 1C). The two Mϕ populations did not significantly differ in their expression of *tlr22* (Fig. 1C).

3.2. Analyses of CSF-1- and IL-34-Mϕ baseline expression of genes encoding toll-like receptor downstream signaling components

Many of the TLR signaling pathways converge on the major downstream signaling components, myeloid differentiation primary response 88 (MyD88) and TNF receptor-associated factor 6 (TRAF6). To get insight into the TLR downstream signaling capacities of the CSF-1- and IL-34-Mϕs, we next examined their transcript levels of the *myd88* and *traf6* genes (Fig. 2A). Notably and in contrast to the disparate *tlr* gene expression by these Mϕ populations (Fig. 1), the CSF-1- and IL-34-Mϕs possessed comparable expression of both *myd88* and *traf6* genes (Fig. 2A).

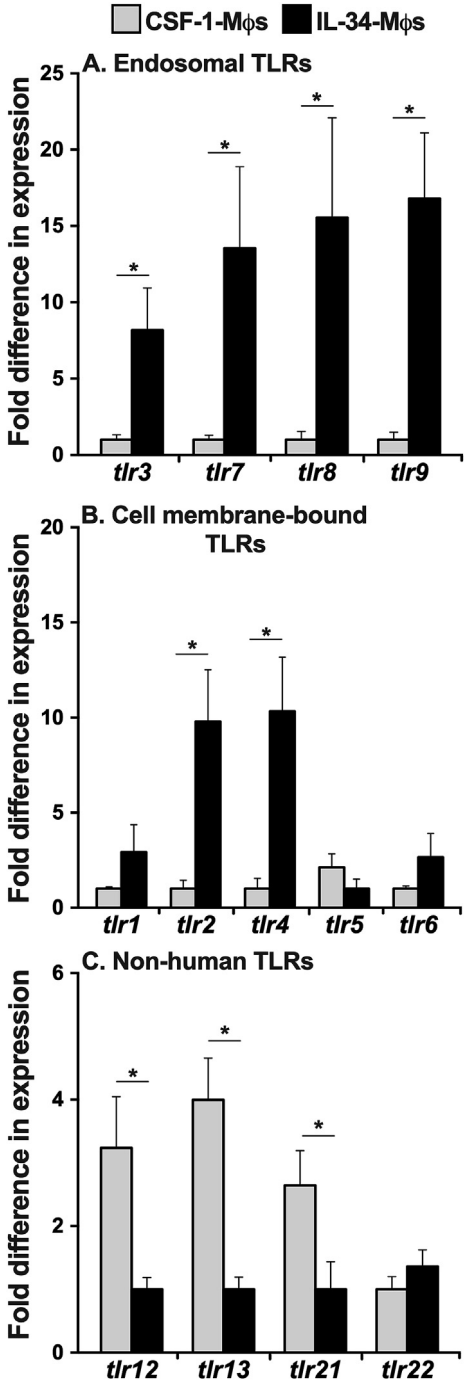

Fig. 1. Quantitative analyses of *tlr* gene expression by *X. laevis* CSF-1- and IL-34-Mϕs. (A) IL-34-Mϕs exhibit greater transcript levels of endosomal *tlrs*, compared to that of CSF-1-Mϕs. (B) Mϕ expression of plasma-membrane-bound *tlr* genes. (C) Mϕ expression of *tlrs* that are absent in humans, but present in other vertebrates. All gene expression was quantified relative to the *gapdh* endogenous control and normalized against the lowest observed expression. Results are means ± SEM of cells derived from 5 individual frogs ($N = 5$) and (*) overhead of horizontal lines denote statistical significance, assessed using paired Student's T test ($P < 0.05$).

In addition to the major propagators of TLR signaling (MyD88, TRAF6), there are several notable negative regulators of TLR signaling. We next examined the gene expression of a panel of such factors. Of these, the *yy1* gene product is a transcriptional repressor of interferon genes (Siednienko et al., 2011); the *socs1* and *triad3A* gene products induce the degradation of an adaptor proteins in TLR signaling

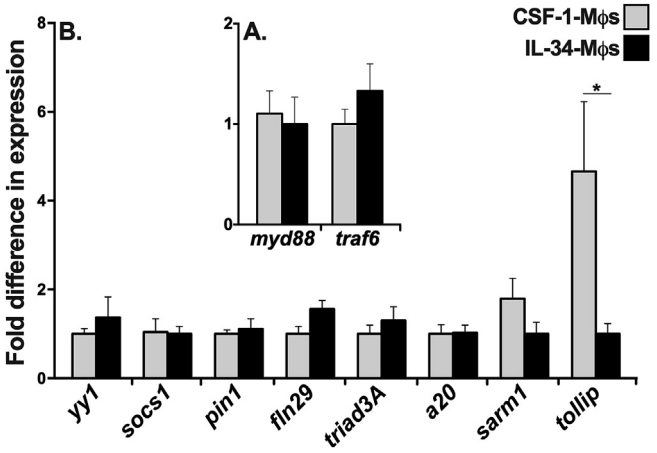

Fig. 2. CSF-1- and IL-34-Mϕs possess comparable expression levels of most TLR-signaling genes. Expression analyses of (A) TLR signaling components and (B) negative regulators of TLR signaling. All gene expression was quantified relative to the *gapdh* endogenous control and normalized against the lowest observed expression. Results are means ± SEM of cells derived from 5 individual frogs ($N = 5$) and (*) overhead of horizontal lines denote statistical significance, assessed using paired Student's T test ($P < 0.05$).

(Fujimoto and Naka, 2010; Guven-Maiorov et al., 2015; Mansell et al., 2006); the *pin1* gene product elicits the degradation of interferon regulatory factor 3 (IRF3), thus suppressing antiviral interferon responses (Kondo et al., 2012); *fln29* and *a20* negatively regulate TLR signaling by interacting with TRAF6 (Guven-Maiorov et al., 2015; Mashima et al., 2005) and *tollip* interacts with the other TLR downstream signaling components to negatively regulate TLR signaling (Zhang and Ghosh, 2002) (Fig. 2B). From these genes, *tollip* expression was significantly more abundant in CSF-1-Mϕs compared to the IL-34-Mϕs (Fig. 2B), while the expression of all other examined signaling genes did not significantly differ between the two Mϕ populations (Fig. 2B).

3.3. Analysis of baseline cytosolic PRR gene expression in CSF-1- and IL-34-Mϕs

Viral pathogens often infiltrate their infected cell cytoplasms (Gruenberg and Goot, 2006; Sodeik, 2000) and the FV3 dsDNA virus is a great example of this (Goorha, 1982). Because the frog CSF-1- and IL-34-Mϕs exhibit distinct FV3 sensitivities (Grayfer and Robert, 2015, 2014; Yaparla et al., 2018), we next examined their expression of cytosolic RNA and DNA sensor genes, whose products detect such pathogenic threats. While the IL-34-Mϕs possessed greater baseline transcript levels of the cytosolic RNA sensor retinoic acid inducible gene I (*rigi*; Pichlmair et al., 2006; Saito et al., 2007) than the CSF-1-Mϕs, the two Mϕ subsets displayed no significant differences in the baseline expression of all other examined cytosolic DNA sensor (CDS) genes: *ddx41* (Stavrou et al., 2018), *dhx36* (Kim et al., 2010), *dhx58* (Saito et al., 2007), *zbp1* (DeFilippis et al., 2010; Sannula et al., 2018) (Fig. 3).

3.4. CSF-1- and IL-34-Mϕs exhibit distinct responses to PAMP stimulation

The activation of PRRs culminates in increased expression of proinflammatory and antiviral genes for products such as tumor necrosis factor-alpha and type I interferon cytokines, respectively (Kawasaki and Kawai, 2014; Takeda and Akira, 2005). Moreover, we recently demonstrated that the anti-FV3 capacities of IL-34-Mϕs are in part attributed to their robust expression of the frog-specific antiviral interferon cytokines (Yaparla et al., 2018). Accordingly, to discern the downstream consequences of CSF-1- and IL-34-Mϕ PRR activation, we stimulated these Mϕ subsets with a panel of hallmark PRR agonists and examined their gene expression of a *type I interferon* (*ifn7;* a well

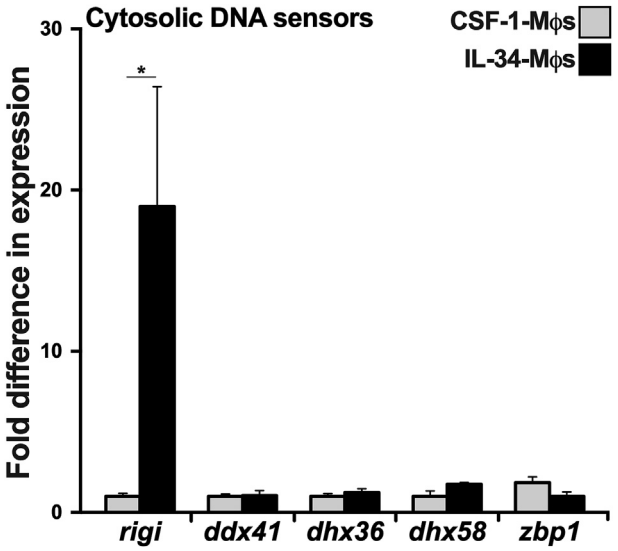

Fig. 3. Analysis of CSF-1- and IL-34-Mϕs cytosolic PRR gene expression. All expression was quantified relative to the *gapdh* endogenous control and normalized against the lowest observed expression. Results are means ± SEM of cells derived from 5 individual frogs ($N = 5$) and (*) overhead of horizontal lines denote statistical significance, assessed using paired Student's T test ($P < 0.05$).

characterized *X. laevis* antiviral IFN; Wendel et al., 2017) and tumor necrosis factor-alpha (*tnfa*) genes. As previously reported (Yaparla et al., 2018), the IL-34-Mϕs possessed significantly greater baseline expression of *ifn7* than the CSF-1-Mϕs (Fig. 4A) and this IL-34-Mϕ *ifn7* expression was significantly elevated following the stimulation of these cells with the heat-killed *Listeria monocytogenes* (TLR2 agonist) and CpG DNA (TLR9 agonist; Fig. 4B). By contrast, the CSF-1-Mϕs exhibited significant increases in their *ifn7* gene expression following activation with LPS (TLR4 agonist) and imiquimod (TLR7 agonist; Fig. 4B). Both CSF-1- and IL-34-Mϕs increased their *ifn7* expression following ssRNA stimulation (TLR8 agonist), albeit not significantly so (Fig. 4B). With the exception of low molecular weight dsRNA and ssRNA treatments, IL-34-Mϕ possessed significantly greater *ifn7* transcript levels than seen in the respectively stimulated CSF-1-Mϕs (Suppl. Fig. 1A).

At baseline, the IL-34-Mϕs also possessed significantly greater *tnfa* transcript levels than the CSF-1-Mϕs (Fig. 4C) and this IL-34-Mϕ expression of *tnfa* was significantly elevated following stimulation with the heat-killed *Listeria monocytogenes* (TLR2 agonist) and CpG DNA (TLR9 agonist; Fig. 4D). Interestingly, the stimulation of the IL-34-Mϕs with high and low molecular weight dsRNA (Poly I:C, TLR3 agonists), diacylated lipoprotein (TLR2/6 agonist) and imiquimod (TLR7 agonist) significantly reduced their *tnfa* gene expression (Fig. 4D). Similarly, CSF-1-Mϕs stimulated with triacylated lipopeptide (TLR1/2 agonist) and imiquimod (TLR7 agonist), likewise down-regulated the *tnfa* mRNA levels within these cells (Fig. 4D). The CSF-1-Mϕ gene expression of *tnfa* only exceeded the IL-34-Mϕs expression of this gene following the low molecular weight dsRNA stimulation of the two subsets, which resulted in decreased *tnfa* expression by IL-34-Mϕs and increased expression in CSF-1-Mϕs (Suppl. Fig. 1B).

3.5. IL-34-Mϕs are more responsive to stimulation with cytosolic DNA sensor agonists

As previously reported (Yaparla et al., 2018), IL-34-Mϕs possessed greater baseline gene expression of *ifn7* than seen in CSF-1-Mϕs (Fig. 5A). We next analyzed the IL-34- and CSF-1-Mϕ *ifn7* transcriptional responses to stimulation with select CDS agonists including herpes simplex virus 1 genome-derived DNA (HSV-DNA), vaccinia virus genome-derived DNA (VACV-DNA) and non-CpG DNA (aka interferon

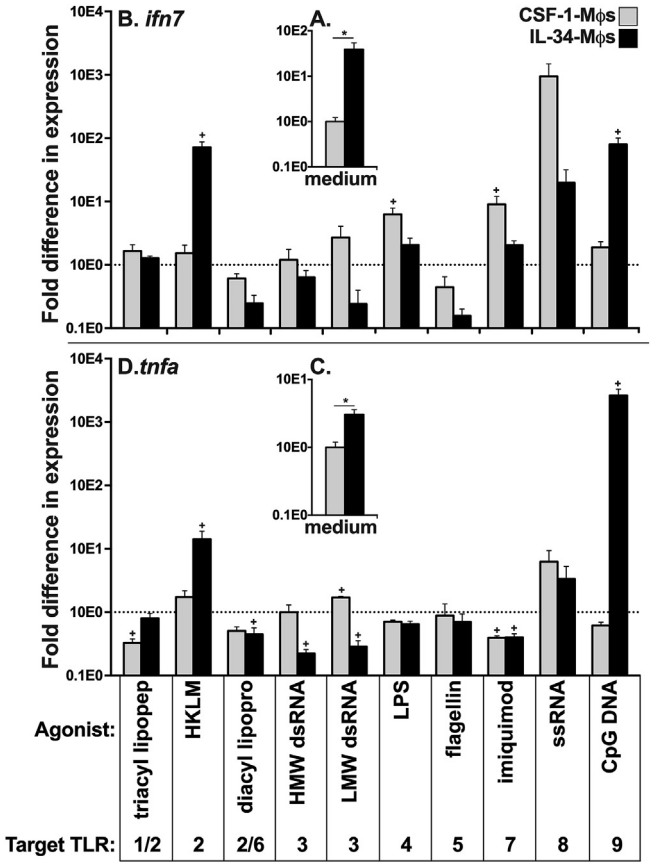

Fig. 4. CSF-1- and IL-34-Mϕs exhibit distinct responses to PRR agonist stimulation. (A) *ifn7* and (C) *tnfa* gene expression by unstimulated CSF-1- and IL-34-Mϕs. Fold change in expression (normalized against the respective unstimulated Mϕ subsets; dashed lines) of Mϕ (B) *ifn7* and (D) *tnfa* following PAMP stimulation. All expression was quantified relative to the *gapdh* endogenous control. Results are means ± SEM of cells derived from 5 individual frogs ($N = 5$) and (*) overhead of horizontal lines denote statistical significance between the two Mϕ subsets, assessed by paired Student's T test ($P < 0.05$) and (+) denotes statistical differences between the respective control and PAMP-stimulated Mϕ subsets, assessed by one-way ANOVA with Dunnett's test ($P < 0.05$).

stimulatory DNA, ISD; Fig. 5, Suppl. Fig. 2), which are known to culminate in *type I ifn* gene expression in a TLR-independent manner (Stetson and Medzhitov, 2006; Unterholzner et al., 2010). While HSV-— and VACV-DNAs did not elicit significant effects on the Mϕ *ifn7* expression, both CSF-1- and IL-34-Mϕs significantly increased their *ifn7* gene expression following stimulation with non-CpG DNA (Fig. 5B). Notably, the CSF-1-Mϕs increased their *ifn7* expression by a significantly greater magnitude (approximately 100 fold) than the IL-34-Mϕs in response to non-CpG DNA (Fig. 5B).

We also examined the consequences of CDS agonist stimulation on the Mϕ gene expression of *tnfa*, the baseline expression of which was significantly greater in the IL-34-Mϕs than CSF-1-Mϕs (Fig. 5C). The *tnfa* transcript levels were significantly upregulated following VACV-DNA stimulation in both the Mϕ subsets, but were not altered in either population in response to HSV-DNA (Fig. 5D). The non-CpG DNA stimulation of both Mϕ subsets resulted in their greater gene expression of *tnfa*, although only significantly so for IL-34-Mϕs (Fig. 5D).

None of the examined CDS agonists elevated the CSF-1-Mϕ expression of the *ifn7* or *tnfa* genes above the respective mRNA levels detected in the IL-34-Mϕs (Suppl. Fig. 2).

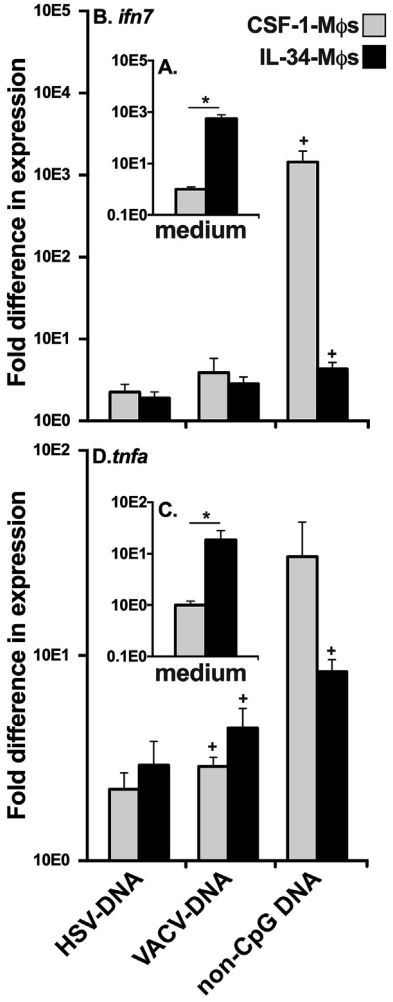

Fig. 5. IL-34-Mϕs are more responsive to stimulation with CDS agonists. (A) *ifn7* and (C) *tnfa* gene expression by unstimulated CSF-1- and IL-34-Mϕs. Fold change in expression (normalized against the respective unstimulated Mϕ subsets) of Mϕ (B) *ifn7* and (D) *tnfa* following CDS-agonist stimulation. All expression was quantified relative to the *gapdh* endogenous control. Results are means ± SEM of cells derived from 5 individual frogs ($N = 5$) and (*) overhead of horizontal lines denote statistical significance between the two Mϕ subsets, assessed by paired Student's T test ($P < 0.05$) and (+) denotes statistical differences between the respective control and PAMP-stimulated Mϕ subsets, assessed by one-way ANOVA with Dunnett's test ($P < 0.05$).

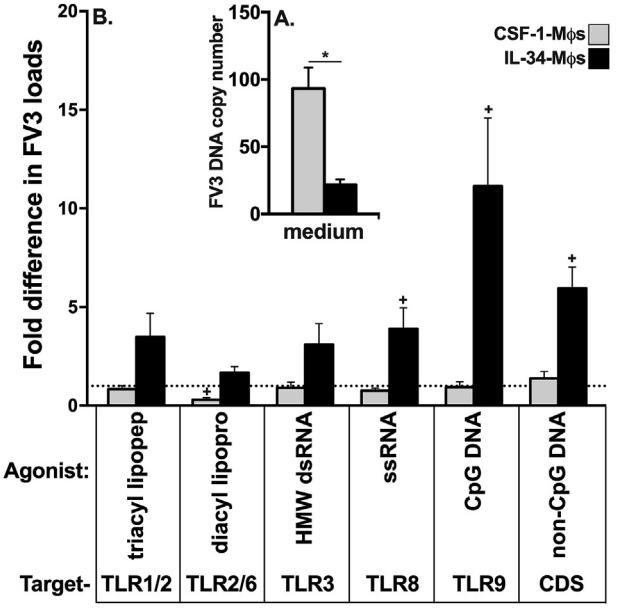

Fig. 6. TLR agonist stimulation alters the frog CSF-1- and IL-34-Mϕ susceptibility and resistance to FV3. (A) FV3 DNA loads in control CSF-1- and IL-34-Mϕs. (B) Fold change in the FV3 DNA loads (normalized against the respective unstimulated Mϕ subsets; dashed lines) following PRR agonist stimulation. Results are means ± SEM of cells derived from 6 individual frogs ($N = 6$) and (*) overhead of horizontal lines denote statistical significance between the two Mϕ subsets, assessed by paired Student's T test ($P < 0.05$) and (+) denotes statistical differences between the control Mϕ subset and its corresponding agonist stimulation, calculated by one-way ANOVA with Dunnett's test ($P < 0.05$).

3.6. Consequences of PRR agonist stimulation on frog Mϕ susceptibility to FV3

Our past work indicates the IL-34-Mϕ resistance to FV3 depends at least in part on their *ifn* gene expression while our present studies indicate that certain PAMPs may modulate the IL-34- and CSF-1-Mϕ expression of *ifn7* and *tnfa*. Amphibians reside in pathogen-rich environments and thus, gaining insights into the consequences of frog Mϕ pathogen-recognition and PRR activation on the functionality of these cells is crucial to understanding the functional plasticity of these distinct Mϕ subsets. Accordingly and to gain greater understanding of the frog Mϕ susceptibility and resistance to FV3 in the context of PRR activation, we stimulated CSF-1- and IL-34-Mϕs with those PAMPs that enhanced or abrogated their *ifn7* and *tnfa* gene expression and subsequently challenged the PAMP-stimulated Mϕs with FV3. As previously reported (Grayfer and Robert, 2015, 2014), unstimulated CSF-1-Mϕs possessed significantly greater FV3 loads than IL-34-Mϕs (Fig. 6A). While most PAMP stimulations of CSF-1-Mϕs had no effects on their FV3 sensitivity, the stimulation of the CSF-1-Mϕs with the diacylated lipoprotein (TLR 2/6 agonist) prior to viral challenge significantly reduced their FV3 loads following infection (Fig. 6B, Suppl. Fig. 3). Interestingly, stimulation of IL-34-Mϕs with ssRNA, CpG DNA and non-CpG DNA increased the susceptibility of these cells to FV3 (Fig. 6B, Suppl. Fig. 3). This was surprising considering that these PAMPs also resulted in increased expression of *ifn7* and/or *tnfa* by these cells (Figs. 4 and 5).

To discern the molecular mechanisms dictating the PAMP-elicited changes in the Mϕ sensitivity to FV3, we examined the diacylated lipoprotein (TLR2/6 agonist; increased CSF-Mϕ FV3 resistance; Fig. 6B) and CpG DNA (TLR9 agonist; decreased IL-34-Mϕ resistance to FV3; Fig. 6B)-treated CSF-1- and IL-34-Mϕs for their expression of candidate antiviral and antimicrobial genes, including those encoding type I and III IFNs, antiviral restriction factors and Mϕ antimicrobial enzymes. Notably, the majority of these genes were more broadly expressed by unstimulated IL-34-Mϕs (Fig. 7A, Suppl. Fig. 4). We previously reported that the CSF-1-Mϕ susceptibility to FV3 coincided with their increased expression of *ifnx20* and here we found that the increased resistance of CSF-1-Mϕs to FV3 following their stimulation with diacylated lipoprotein (Fig. 6B) coincided with decreased *ifnx20* expression by this Mϕ subset (Fig. 7B).

Following diacylated lipoprotein stimulation, the IL-34-Mϕs significantly downregulated their *ifnx2* expression and upon CpG DNA stimulation, these cells displayed significant downregulation of *ifn1*, *ifnx2* and *ifnlx1/2* expression (Fig. 7B&C, Suppl. Fig. 4), suggesting that these cytokines may be important to the anti-FV3 capacities of this Mϕ subset. Moreover, both the *inos* and *apobec2* restriction factor gene transcripts were significantly diminished in the diacylated lipoprotein and CpG DNA-treated IL-34-Mϕs (Fig. 7B&C, Suppl. Fig. 4), suggesting that the products of these genes may likewise contribute to the antiviral immunity of newly differentiated IL-34-Mϕs.

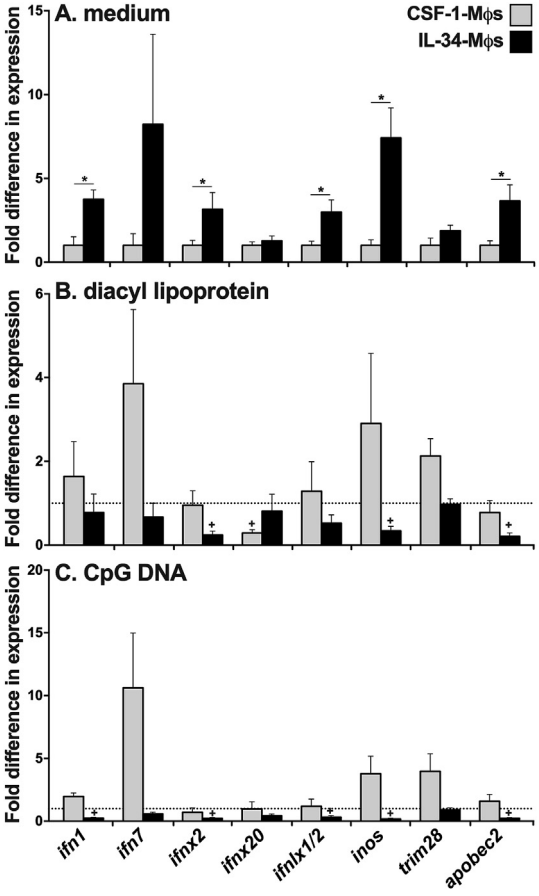

Fig. 7. Gene expression analyses of antiviral and antimicrobial genes in PAMP-stimulated CSF-1- and IL-34-Mϕs. (A) Immune gene expression by unstimulated CSF-1- and IL-34-Mϕs (B) Fold change in the CSF-1- and IL-34-Mϕ immune gene expression (normalized against the respective unstimulated Mϕ subsets; dashed lines) following (B) diacylated lipoprotein (TLR2/6 agonist) and (C) CpG DNA (TLR9 agonist) stimulation. All expression was quantified relative to *gapdh* endogenous control. Results are means ± SEM of cells derived from 5 individual frogs ($N = 5$). The (*) overhead of horizontal lines denote statistical significance between the two Mϕ subsets, assessed by paired Student's T test ($P < 0.05$) and (+) denotes statistical differences between the control and PAMP-stimulated Mϕ subset, assessed by one-way ANOVA with Dunnett's test ($P < 0.05$).

4. Discussion

The mammalian CSF-1 and IL-34 cytokines exhibit distinct gene expression patterns, are associated with different biological and immune processes and are thought to give rise to functionally disparate macrophage subsets (Chemel et al., 2012; Paquin-Proulx et al., 2018; Zwicker et al., 2015). Interestingly, this functional Mϕ dichotomy appears to be evolutionarily conserved as our past studies indicate that the frog CSF-1- and IL-34-Mϕs likewise possess distinct morphologies, immune gene expression profiles and functionalities (Grayfer and Robert, 2015, 2014). Perhaps most notably, our ongoing work indicates that the frog IL-34-Mϕs possess considerably greater antiviral capacities than CSF-1-Mϕs, which appears to be at least in part due to their robust production of type I and type III IFNs and their expression of antiviral restriction factors (Yaparla et al., 2018). Moreover, we recently demonstrated that the human peripheral blood monocyte-derived IL-34-Mϕs are likewise more resistant to HIV-1 than the counterpart CSF-1-Mϕs (Paquin-Proulx et al., 2018), suggesting that the respective susceptibility and resistance of CSF-1- and IL-34-Mϕs to viral pathogens may also have been evolutionarily conserved.

Presently, we show that compared to the frog CSF-1-Mϕs, their IL-34-Mϕs also possess significantly greater transcript levels for *tlrs* that recognize intracellular PAMPs as well as the retinoic acid inducible gene I (*rigi*), whose product recognizes viral 5' triphosphate uncapped double-stranded or single-stranded RNA (Pichlmair et al., 2006). This further corroborates the antiviral nature of the IL-34-Mϕs. Notably, the frog IL-34-Mϕs also possessed significantly greater transcripts for *tlrs 2* and *4*, which are thought to recognize bacterial lipopeptides and lipopolysaccharides, respectively (Hajjar et al., 2002; Vasselon et al., 2004). We previously showed that CSF-1-Mϕs rather than IL-34-Mϕs possess greater capacities to kill *E. coli* (Grayfer and Robert, 2015), so it is somewhat surprising that the IL-34-Mϕs possessed greater expression of PRRs that would recognize extracellular bacteria. Possibly, the CSF-1-Mϕs function more like conventional tissue Mϕs and may upregulate their PRR gene expression under inflammatory conditions. This notion may be supported by our observation that CSF-1-Mϕs exhibited a significant *ifn7* gene expression response to LPS, a hallmark inflammatory stimulus (of higher vertebrates). Conversely, the IL-34-Mϕs may be likened to mammalian plasmacytoid dendritic cells (Asselin-Paturel and Trinchieri, 2005), as both cell types are highly antiviral, produce IFNs and exhibit heightened expression of PRRs that recognize intracellular pathogens.

Alternatively and considering that amphibians reside in very distinct, pathogen rich environments to those that shaped higher vertebrate immunity, it is possible that the frog CSF1- and IL-34-derived Mϕs have evolved to mediate very disparate sentinel roles, not easily reconciled from our broader knowledge of mammalian immune systems. Indeed, it is striking that while the IL-34-Mϕs expressed greater levels of most examined TLR genes, the CSF-1-Mϕs displayed broader expression of those *tlrs* that are absent from the human genome (*tlrs 12*, *13*, *21* and *22*). Much remains to be learned regarding the tissue localization and functionalities of these amphibian Mϕ subsets, but undoubtedly these expression differences hint at further differences in the respective immunological roles of CSF-1- and IL-34-Mϕs. Possibly, the frog CSF-1-Mϕs are polarized to detect distinct intracellular pathogens or may be better equipped at recognizing chronic Mϕ intracellular pathogens. In support of this notion, the CSF-1-Mϕs possessed comparable gene expression levels of cytosolic DNA sensors to IL-34-Mϕs and exhibited robust responses to the *Listeria monocytogenes*-derived DNA and to vaccinia virus derived-DNA.

The expression of the *tollip* gene, the product of which dampens TLR2 and TLR4 signaling (Shah et al., 2012), was more broadly expressed by the CSF-1-Mϕs and this, together with the more robust *tlr2* expression by IL-34-Mϕs, possibly explains why the IL-34-Mϕs had significant responses to the heat-killed *Listeria monocytogenes* (TLR2 agonist) stimulation whereas the CSF-1-Mϕs did not. Similarly, CpG DNA (TLR9 agonist) stimulation resulted in significant *ifn7* and *tnfa* responses in IL-34-Mϕs, which expressed substantially greater levels of *tlr9* than the CSF-1-Mϕs. Indeed, IFN production is an archetypal TLR9-mediated response to numerous dsDNA viruses (Guggemoos et al., 2008; Zannetti et al., 2014) and because IFN production appears to be important to the IL-34-Mϕ anti-FV3 effector function (Yaparla et al., 2018), presumably TLR9 activation is likewise important to this IL-34-Mϕ-mediated production of IFNs during antiviral responses.

The CSF-1- and IL-34-Mϕ *tlr* gene expression reported here did not always reflect the capacities of the respective Mϕ subsets to respond to the corresponding PRR agonist stimulation. This included Mϕ stimulation with low and high molecular weight dsRNA (TLR3 agonist), LPS (TLR4 agonist), imiquimod (TLR7 agonist) and CpG DNA (TLR9 and TLR21 agonist). Notably, gene expression does not always correlate with the respective protein production (Edfors et al., 2016), possibly accounting for these discrepancies in macrophage PRR gene expression and PAMP recognition capacities. Unfortunately, the lack of antibodies against the *X. laevis* PRRs has precluded us from examining the CSF-1- and IL-34- Mϕ surface PRR levels.

Another plausible explanation for these discrepancies may be that some of the amphibian TLRs require greater thresholds of activation

than achieved with the respective concentrations of PAMPs used in the present studies. This may also explain the non-responsiveness of both CSF-1- and IL-34-Mϕs to flagellin (TLR5 agonist) and ssRNA (TLR8 agonist) stimulation. In this respect, it is noteworthy that aquatic vertebrates are notoriously resistant to LPS stimulation *in vivo* (Berczi et al., 1966) and likewise, CSF-1- and IL-34-Mϕs may be desensitized to certain PAMPs. In contradiction of this notion, we did observe increased CSF-1-Mϕ expression of *ifn7* following their LPS stimulation, suggesting that these cells are not compromised for their LPS detection and downstream activation. Again, this may reflect a biological role of the frog CSF-1-Mϕs that corresponds to these animals' unique physiologies and environmental pressures. Indeed, it is possible that the aquatic vertebrate recognition and subsequent responses to those disparate PAMPs that these animals are constantly in contact with through their environments involves more complex regulation strategies to those seen in mammals, thus precluding our speculation.

It is compelling that the susceptibility/resistance of frog Mϕs to an ecologically relevant amphibian pathogen such as FV3 may be altered by PAMP stimulation, especially considering the pathogen-rich environments in which amphibians reside. In particular, it is notable that the FV3-resistant IL-34-Mϕs were rendered more susceptible to this virus following stimulation with the HIV-1 long terminal repeat U-rich ssRNA (TLR8 agonist), CpG DNA (TLR9 agonist) and the *L. monocytogenes*-derived non-CpG DNA (CDS agonist). By contrast, the FV3-susceptible CSF-1-Mϕs were granted greater resistance against FV3 following their treatment with a diacylated lipoprotein (TLR2/6 agonist). As new insights into amphibian susceptibility and resistance against this etiological agent are crucial to the development of counteractive measures, such changes in frog immune cell susceptibility/resistance to FV3 may hold the key to developing viable counteractive/preventative measures. For example, we previously demonstrated that the IL-34-Mϕ resistance to FV3 reflects their IFN production (Yaparla et al., 2018) and here, we show that the decreased resistance/increased susceptibility of IL-34-Mϕs to FV3 following CpG DNA (TLR9 agonist) and diacylated lipoprotein (TLR2/6 agonist) treatments parallels their decreased expression of specific *ifns*, antiviral restriction factors and antimicrobial genes (*ifnx2, ifnlx1/2, inos, apobec2*), the products of which are presumably important to the IL-34-Mϕ antiviral resistance. Concurrently, we previously reported that the FV3-susceptible CSF-1-Mϕs upregulate *ifnx20* expression during FV3 challenge (Yaparla et al., 2018), while the decreased susceptibility/increased FV3 resistance of these cells following diacylated lipoprotein (TLR2/6 agonist) stimulation correlates with decreased *ifnx20* gene expression. Thus, we speculate that IFNX20 may be a factor that exacerbates, rather than alleviating FV3 infections.

Our past and present work clearly indicates that the *X. laevis* CSF-1- and IL-34-Mϕs possess disparate immune capacities, presumably as the result of their distinct differentiation pathways. Unfortunately, the lack of *X. laevis*-specific reagents has prevented us from addressing the differences in the CSF-1R signaling that may contribute to the distinct PRR gene expression and PAMP-recognition capacities of these respective Mϕ populations. With the development of more refined *X. laevis*-targeted molecular approaches, we hope the address some of these questions.

Our results indicate that compared to CSF-1-Mϕs, the frog IL-34-Mϕs express significantly greater levels of the retinoic acid inducible gene I (*rigi*), the product of which recognizes cytosolic short uncapped double stranded, or single stranded RNAs (Pichlmair et al., 2006; Saito et al., 2007). As we have to date primarily studied the *X. laevis* CSF-1- and IL-34-Mϕs in the context of a dsDNA virus (FV3) infections, the present studies focused on the frog Mϕ detection of cytosolic dsDNAs (CDS agonists) specifically, and were restricted to the frog Mϕ recognition of distinct TLR agonists. It will be interesting to learn through future studies whether the robust IL-34-Mϕ *rigi* gene expression correlates with greater responsiveness of this Mϕ subset to RIG-I agonists.

As Mϕ-lineage cells represent indispensable sentinels of innate immunity, it is interesting that in CSF-1- and IL-34-Mϕs, frogs possess two Mϕ subtypes with what appear to be very distinct pathogen recognition and antimicrobial response capacities. We believe that gaining further insights into the mechanisms by which these two immune cell types differ in their pathogen recognition and subsequent responses will aid in understanding of the resistance and susceptibility of amphibians to emerging pathogens such as FV3, concurrently yielding a clearer understanding of the evolution of vertebrate Mϕ-pathogen interactions.

Disclosures

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2019.04.011.

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