Title: Beta-glucan enhances the response to SVCV infection in zebrafish

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Abstract

The antiviral effects of beta-glucan, an immunostimulatory agent were studied in zebrafish both *in vitro* and *in vivo*. Here we show that zebrafish ZF4 cells as well as whole fish primed with yeast β-glucan zymosan exhibited increased cytokine expression and elevated response to spring viremia of carp virus (SVCV) infection. *In vitro*, previous treatment of β-glucan enhanced ZF4 cell viability against SVCV infection which is associated to the activation of interferon signaling pathway and inflammatory cytokines gene expression. *In vivo*, the SVCV-infected fish primed with β-glucan had a higher survival rate (≈73%) than the control SVCV-infected group (≈33%). Additionally, up-regulation of the expression of a set of genes involved in innate immune response was detected in zebrafish intraperitoneally injected of β-glucan: *il1b*, *il6*, *il8*, *il10* and *tnfa* transcripts showed increased expression that appear to be rapid (2 days) but not long-lived (less than 2 weeks).

The present study is, to our knowledge, the first to combine cell culture and in vivo approaches to describe host response to β-glucan stimulation and viral infection in zebrafish.

Keywords: beta-glucan, zebrafish, SVCV, immunostimulant, antiviral

1. Introduction

Beta-glucans (β-glucans) are glucose polymers found in the wall of yeast cells. In mammals, β-glucans have been shown to activate innate immunity through Toll-like receptors TLR2/TLR6 heterodimers (Roeder, 2004) and though recognition by the small membrane receptor dectin-1 (Brown and Gordon, 2001). Previous studies have demonstrated that β-glucans have also immunostimulatory properties in teleost fish. *In vivo* administration of β-glucan was shown to boost protection against parasitic, bacterial and viral pathogens (Beaulaurier, 2012; Guselle, 2006; Petit and Wiegertjes, 2016; Rodriguez 2016). Oral administration in the fish diet is the most common way of supplementing β-glucans to fish. There are a number of reports examining the expression of immune response genes in salmon and other fish species where β-glucan enriched diets increased the levels of transcripts of a number of key genes of both innate and adaptive immune response (Douxfils, 2017; Kiron, 2016; Pionnier, 2014; Rodríguez, 2009). However, there are a limited number of studies examining the influence of β-glucan on a subsequent virus challenge of fish (Beaulaurier, 2012; La Patra, 1998). Pacific herring maintained on a diet containing β-glucan showed enhanced resistance to viral hemorrhagic virus (VHSV) challenge (Beaulaurier, 2012). Another way of glucan application, single dose injection, has also been used to enhance the immune response to vaccines as an effective mean to increase disease resistance to virus in fish (La Patra, 1998).

Spring viremia of carp virus (SVCV) belongs to the Rhabdoviridae family and has ssRNA genome. Disease caused by SVCV is a threat to carp and other cyprinid species in aquaculture industry. Adult zebrafish (*Danio rerio*) is susceptible to SVCV infection, showing clinical signs and experiencing mortality (Encinas, 2013; Sanders, 2003).The availability of the whole genome sequence as well as established cell lines susceptible to viral infection (Wang, 2015) have made zebrafish, also a cyprinid fish, an attractive experimental model for studies of infectious disease and immunity in teleosts (Varela, 2017).

In the present study the effect of β-glucan was investigated both *in vitro* and *in vivo*. Zymosan is a component of the cell wall of *Saccharomyces cerevisiae* consisting of repeating

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units of glucose linked by β-glycosidic bonds. Zebrafish cells stimulated with β-glucan prior to viral infection with SVCV displayed an increased cellular response to β-glucan exposure associated to an enhanced protection against SVCV infection. The antiviral potential of β-glucan was also investigated *in vivo*: adult zebrafish were intraperitoneally injected with a dose of β-glucan and subsequently challenged with SVCV. In both cases the results showed that β-glucan treated group exhibited enhanced the immune response and survival against virus infection demonstrating the immunostimulatory capacities of β-glucan and therefore supporting the potential applicative value of β-glucans as antiviral therapeutic agents.

2. Materials and Methods

2.1. Cell culture and virus

The cells used in this work were zebrafish embryonic fibroblast ZF4 cells (Wang, 2015) purchased from the American Type Culture Collection (ATCC number CRL-2050). The cells were maintained under RPMI-1640 Dutch modified culture medium (Gibco, Invitrogen Corporation, UK) at 28ºC in a 5% CO_2 containing 10% fetal calf serum (Sigma, St Louis, USA), 1mM pyruvate, 2mM glutamine, 50μg/mL gentamicin and 2μg/mL fungizone.

The SVCV strain 56/70 isolated from carp (Fijan, 1971) was propagated in epithelioma papulosum cyprini (EPC) cells (Winton, 2010) at 22ºC. Supernatant from SVCV infected cells monolayers were clarified by centrifugation al 4000g for 30 min. and virus aliquots were stored at -80ºC.

2.2. Viral cytopathic effect (CPE) inhibition assay.

ZF4 cell monolayers were detached by trypsin treatment (Tryple Select, Gibco) and resuspended in fresh RPMI medium supplemented with 10% FCS. 15000 cells were seeded into 96-well cell culture plates. After 24h the cells were treated with β-glucan Zymosan (Sigma) at various concentrations. The plates were incubated at 28ºC for 24h and then MTT (Sigma) was added on the medium followed by a 3 hour incubation period. The medium was removed and

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100μL DMSO was added to each well before reading for optical density (570nm) in a Biotek

Eon plate reader. Readings were converted to percentages of uninfected controls and normalized

to virus control: $OD_{\text{non-infected cells}} - OD_{\text{SVCV-infected cells}} = 100\%$ CPE.

2.3. *In vitro* antiviral assay.

ZF4 cells were treated with β-glucan (10μg/ml for 24h) prior to infection with SVCV at a

multiplicity of infection (moi) of $1x\ 10^{-3}$ (Espín-Palazón, 2016) in a final volume of 100μL of

culture medium supplemented with 2% FCS at 22ºC for 90 minutes. Infected cells monolayers

were then washed, fresh medium added, and plates further incubated until the end of the

experiment. At 24 hours post-infection (hpi) SVCV replication in ZF4 cells was evaluated by

quantitative real-time RT-PCR (RT-qPCR, described in 2.5.) using specific primers for the gene

encoding the protein N of SVCV (Table 1). Cells infected with SVCV but not treated with

zymosan were used as reference.

2.4. *In vivo* β-glucan administration and virus challenge of zebrafish.

Zebrafish (8 months, average weight 600 mg) were maintained at 28ºC in 30 liter tanks

equipped with a re-circulating dechlorinated water systems. Fish were fed daily. Prior to the

experiment fish were acclimatized to 22ºC for 2weeks. All the experimental procedures with

live zebrafish were performed using protocols approved by the European Union Council

Guideline (86/609/EU). Briefly, fish were anaesthetized by immersion in 50μg/mL buffered

MS-222 (Sigma) prior to handling. Groups were intraperitoneally immunized with one of the

following: 10μL of PBS (non-immunized or control fish or 10μL of PBS containing 5μg of β-

glucan Zymosan. At day 14 post-stimulation fish were challenged with SVCV by bath

immersion: fish were introduced in 1 liter of water containing 10^4 PFU/mL SVCV for 90 min at

22ºC. The fish were transferred to tanks with fresh dechlorinated water and the flow was

restored. Mortality was recorded daily and the percentage of survival was calculated using the

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formula: %survival = (number of remaining fish in the tank/ initial number of fish in the tank) x 100.

2.5. RNA isolation and gene expression analysis by RT-qPCR.

For total RNA extraction the E.Z.N.A. HP total RNA kit (Omega Bio-tek, Norcross, GA, USA) and E.Z.N.A. HP Tissue RNA kit (for cell RNA and tissue RNA isolation, respectively) were used in accordance with the manufacturer´s instructions. RNA samples were stored at -80ºC until use. One microgram of RNA, as estimated by a Nanodrop 1000 spectrophotometer (Thermo-Fisher Scientific, Inc), was added to the cDNA synthesis reaction with Maloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). Quantitative PCR was performed using the ABI PRISM 7300 System (Applied Biosystem, NJ) Primers used for quantitative real-time PCR analysis are listed in Table 1. The reaction was performed in a total volume of 20μL, comprising 2 μL cDNA reaction mixture, 900nM each primer, 10μL of SYBR Green master mix (Applied Biosystem, NJ) and 6μL H_2O, the cycling conditions were 50 ºC for 2 min, and 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s, and 60 ºC for 1 min.

Expression of gene transcripts was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmitten, 2001) where ΔCt is determined by subtracting the Ct value from the eukaryotic translation elongation 1 alpha (*ef1a*) gene, used as endogenous control, to the target Ct value. Every sample was run in triplicate.

2.6. Interleukin-1β immunofluorescence microscopy.

To detect the presence of IL-1β in response to cells exposure to β-glucan, ZF4 cells monolayers, grown in 96-well plates at 28 ºC, were treated with β-glucan (10μg/mL) for 24h. Then the cell monolayers were fixed for 15 minutes with a 4% paraformaldehyde solution followed by 15 minutes in cold methanol. For IL-1β detection, fixed ZF4 cells were incubated overnight with a mouse polyclonal antibody against rainbow trout IL1B (Nombela, 2017) kindly donated by Dr. Luis Mercado (Universidad Católica de Valparaiso, Chile) diluted 100-fold in

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PBS with 0.3% Triton X-100 at 4ºC. The anti-IL1B antibody has been tested against ZF4 cells samples by ELISA (Fig.S3). After primary antibody incubation the cells were washed with PBS and incubated for 45 min with FITC labeled goat anti-mouse antibody (Sigma, USA) diluted 1:300 with 0.3% triton x100. Finally, the cells were then washed 3 times with PBS. To visualize the nuclei of the cells, the monolayers were counterstained with 4´-6-diamidino-phenylin-indole (DAPI) for 10 min. The cells were observed and photographed with an IN Cell Analyzer 6000 image system (GE Healthcare).

2.7. Statistical analysis.

Statistical analysis was carried out using the Graph Pad Prism 5 software. All data are shown as means ±SD. Difference between groups were evaluated by using analysis of variance (ANOVA) followed by a post-hoc Tukey´s test. Statistical significance was defined by p-values <0.05.

3. Results

3.1. β-glucan treatment enhances ZF4 cell response to SVCV infection.

Treatment of ZF4 cells for 24h with β-glucan before infection prevented SVCV-induced cytopathic effect as measured by a MTT cell viability assay. Good protection values were obtained in the 2.5-10μg/ml range (Fig.1), while the highest dose of glucan (20μg/ml) exhibited a lesser protective effect on SVCV-infected cells.

To get an insight into the cell immune response to β-glucan, changes in the expression of innate immune genes were analyzed by RT-qPCR. For *il1b, il6* and *tnfa*, a higher level of gene expression was observed in the β-glucan/SVCV group over the SVCV group (Fig.2). We observed a stimulatory effect on the inflammatory cytokine genes in ZF4 cells after β-glucan administration which usually is further augmented by SVCV infection. β-glucan treatment induced significantly higher transcription levels of all tested genes than in the non-treated

SVCV-infected cells, with *il8* gene expression having the greatest increase after SVCV infection (13.6-fold increase).

Interleukin 1β (IL-1β) protein expression in cells treated with beta-glucan was further checked by immunofluorescence assay. Increased IL-1β expression was detected in ZF4 cells stimulated with β-glucan. Basal expression of IL-1β yields a diffuse pattern, whereas in the β-glucan treated cells there is a shift to a more intense and distinct cytoplasmic pattern of IL-1β expression (Fig.3).

3.2. *In vivo* up-regulation of innate immune signaling pathways by β-glucan.

To evaluate the fish response to β-glucan intraperitoneal (i.p.) injection was used and the relative expression levels of innate immune genes in internal organs of adult zebrafish were analyzed by RT-qPCR amplification at different times post-treatment (2, 14 and 22 days). All studied genes except *tnfa* showed similar kinetics: they were rapidly induced, with highest expression at 2 dpi. and declined after a 2-week period (Fig.4). In contrast, the peak expression of *tnfa* gene was observed at the 14 days post β-glucan injection, but also dropped at 22 days.

3.3. β-glucan-induced protection against SVCV disease *in vivo*.

We further investigated the capacity of β-glucan to establish an antiviral state *in vivo* by injecting adult zebrafish with β-glucan and challenging 14 days later with SVCV (Fig.5A). Mortality was recorded over a 17 day period (Fig.5B). The survival rate greatly improved in the groups of fish treated with beta-glucan: 73% survival compared to 33% survival in the non-treated group was obtained. Viral load determined as the abundance of SVCV N gene RNA in the infected fish also reflected an inhibition of viral replication in the group of fish that received β-glucan (Fig.5C). SVCV RNA was not detected in non-infected control fish.

3.4. Innate immune response in SVCV-infected zebrafish after β-glucan administration.

A parallel trial following the same procedure (β-glucan i.p. injection and SVCV challenge 14 days later, Fig.S4) was set to investigate changes in gene expression profiles. RT-qPCR was performed on total RNA extracted from a pool of internal organs (kidney, liver, spleen) to detect the induction of selected genes related to innate immune response. I.p. administration of β-glucan before SVCV challenge led to a higher expression of immune-related genes than SVCV infection alone, including several highly up-regulated genes: *il1b, il6, il8, il10* and *gig2* (Fig.6). For those genes with a rapid response (*tlr2*, *nod2*, *il1b*, *il6*, *il8*, *il10*, *gig2*) β-glucan injection enhanced gene expression in SVCV-infected fish over the non-stimulated SVCV group. The expression of the *ifnphi1* gene displayed a different profile: at 2 dpi. the previous treatment with β-glucan does not enhance *ifnphi1* activation over the SVCV-infected control fish. At longer times of infection (7 dpi) β-glucan treatment led to *ifnphi1* overexpression at higher levels than in the non-treated SVCV-infected group.

4. Discussion

Yeast β-glucans are potential immunostimulants for fish. Enhanced resistance to parasitic, bacterial or viral diseases has been observed either by injection or by dietary administration of β-glucan (Diao, 2013; Guselle, 2016; LaPatra, 1998; Rodriguez, 2009). In the present study we investigated the antiviral protective potential of β-glucan in zebrafish. We chose the zebrafish/SVCV model which allows a combined *in vitro* plus *in vivo* approach. Cell culture has the benefit of being a more controlled system. Fish trials, on the other hand, although closer to the real situation are often hampered by the great variability of the data. Zebrafish presents a suitable model due to the availability of cell lines and the possibility of performing *in vivo* challenge with SVCV (Varela, 2017).

In vitro examination of β-glucan effect revealed that it triggers a response in ZF4 cells by activating the transcription of a number of genes involved in innate immune system. In this study we found that β-glucan administration enhanced the survival and viability of ZF4 cells after SVCV infection. SVCV is considered as a good inducer of the innate immune response in

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infected cells. In the zebrafish ZFL cell line, SVCV rapidly activates *ifnphi1* and *ifnphi3* expression from 12 to 24 hpi (Feng, 2016) although times of SVCV infection later than 24 hpi were not examined in that study.

In vivo, intraperitoneal injection of a single dose of zymosan β-glucan resulted in enhanced activation of a set of inflammatory genes expression 2 days post-treatment. Although oral administration is a more practical route, for time course studies of gene expression a single dose was a more appropriate approach. I.p. injection has been used successfully before (Kim, 2009; Rodriguez, 2009) and allows for a more accurate determination of the post-treatment time-course profile of the immune-modulating effects of β-glucan. Our data show that most of the studied cytokines appeared to have a short induction period, with a marked decline in the expression levels from 2 to 14 days post-infection. A similar pattern of gene expression has been reported in mice where the stimulatory effect of β-glucan seemed to decline 2-3 weeks after administration (García-Valtanen, 2017).

In the SVCV-challenged adult zebrafish, the virus could be detected in internal organs at 2 dpi establishing an infection that progresses within the following days with SVCV N gene RNA levels increasing from day 2 to day 7 post-challenge. The reduced viral loads found in β-glucan treated fish suggested that an antiviral response might be occurring. Although the enhancement of the immune response induced by β-glucan did not completely inhibit virus replication it appeared to be sufficient to reduce mortality/morbidity since fish immunized with β-glucan had significantly higher survival rates (73%) than the SVCV-infected control group (33%) after 17 days. In rainbow trout, i.p. administration of β-glucan led to 48-63% relative survival values after exposure to IHNV (LaPatra, 1998).

Enhanced protection to virus disease is likely connected to the stimulation of the host immune response. Thus, variations in gene expression levels were assessed. Amongst genes that showed β-glucan-induced transcription we found several *ifn*-related genes: *gig2, tlr2, ifnphi1* and the inflammatory cytokines *il1b, il6, il8, il10* and *tnfa*. Increased levels of *il1b, il6, il8* and *tnfα* were observed at 48h after β-glucan injection, followed by a decline over the days. Our

present findings showed that *il1b*, *il6*, *il8* and *il10* gene transcription in SVCV-infected fish could be induced 16 days after i.p. injection of β-glucan at higher levels than SVCV infection alone. This is in agreement with other authors´observation that β-glucan injection of fish results in sustained levels of antiviral gene expression for longer times after virus challenge (Kim, 2009).

Interleukin 1 beta (Il-1β) is a potent pro-inflammatory cytokine that participates in the regulation of hundreds of other molecules including other cytokines and toll-like receptors (TLRs) (Weber, 2010). In cyprinid fish the up-regulation of *il1b* gene expression 4 days after intraperitoneal injection with zymosan has been observed (Chadzinska, 2008). Interleukin 8 (Il-8) is another cytokine with a key role in inflammatory processes. In salmon, *il8* transcription has been found to be stimulated after viral challenge (Collet, 2014; Xu, 2012). Increased levels of both *il1b* and *il8* gene transcription were observed after stimulation of macrophage-like cultures of eel with zymosan (Callol, 2013). Thus, the coordinated activation of *il1b* and *il8* gene transcription appears to be a common feature of the antiviral response in fish (Carballo, 2017). Interleukin 6 (*il6*) is also associated to response to β-glucan in fish (Diao, 2013) and plays an active role in antiviral response in conjunction with other cytokines (Overgard, 2013). Tumor necrosis factor alpha (TNFα) has a key role in regulating inflammation at the early stages of virus infection in fish where it functions together with IL-1β, IL-6 and IL-8 (Carballo, 2017).

Contrary to the aforementioned cytokines, interleukin 10 (Il-10) has been described primarily as an anti-inflammatory cytokine. Thus, *il10* activation might reflect an attempt of the host to counteract the inflammatory response caused by the pathogen. Simultaneous activation of pro-inflammatory (*il1b, il6, il8*) and anti-inflammatory genes (*il10*) has been reported before in fish stimulated with poly I:C (Du, 2017; Fierro-Castro, 2013; Seppola, 2008) as well as in common carp infected with cyprinid herpesvirus (Rakus, 2012). In SVCV-infected zebrafish, significantly higher *il1b*, *il10* mRNA levels were observed in symptomatic fish (Rodriguez, 2009).

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A novel contribution of this study is the identification of *gig2* gene being involved in immunomodulatory effect of β-glucan in zebrafish. Grass carp reovirus (GCRV)-induced gene 2 (*gig2*) is considered by some authors as an interferon-stimulated gene, not found in mammals (Poynter and DeWitte-Orr, 2016). Inducible expression of *gig2* gene in response to poly I:C or to SVCV infection has been reported in zebrafish ZFL cells (Li, 2012). Gig proteins have been found overexpressed in the cytoplasm of GCRV-infected carp cells (Sun, 2014). *In vivo*, there is evidence of *gig2* playing a role in the host antiviral response (Valenzuela-Miranda, 2015; Wang, 2017). Here we have shown that β-glucan is an even stronger inducer of zebrafish *gig2* than SVCV infection.

In teleost fish nucleotide oligomerization domain 2 (NOD2) can trigger the production of interferon by sensing bacterial and viral pathogen-associated molecular patterns, leading to the activation of NFkβ and then the transcription of inflammatory genes (Zou, 2016). We found very small stimulation of *nod2* expression in ZF4 cells exposed to β-glucan which is in agreement with the lack of responsiveness of *nod2* to β-glucan reported by others, in contrast to the strong response of *nod2* to bacterial peptidoglycan (Zou, 2016).

In summary, we found that β-glucan treated zebrafish exhibit higher responsiveness and protection to SVCV infection with an increased transcription of cytokine genes after challenge with SVCV. Moreover, the enhanced immune response is maintained at later times (7 days) post challenge. This higher and longer lasting stimulation of innate immune genes is associated to improved resistance and better survival to SVCV disease.

One attractive feature of β-glucans is the possibility that their effects can last during a relatively long period of time. There are several indications in the literature on this respect, showing increased protection against pathogen challenge 15 days (LaPatra, 1998), 21 days (Guselle, 2006) and 30 days (Diao, 2013) post-administration. In our hands β-glucan treatment of fish has protective effects against SVCV over a 31 day period (14 days before plus 17 days after virus challenge). An explanation of this longer than previously observed duration of the effects of β-glucan may be found in the trained immunity phenomenon, as suggested by others

(Petit and Wiegertjes, 2016). Trained immunity implies a sort of non-specific memory of innate immune response that results in a better host response to a secondary stimulus. Based on our finding that the effect of β-glucan is maintained 14 days after administration to zebrafish we can speculate that β-glucan treatment may induce some epigenetic changes in the primed cells/fish resulting in increased levels (elevated production) of cytokines (*il1b, il8, tnfa*) after a subsequent challenge with a viral pathogen. Ongoing studies in our lab are aimed to address this issue.

5. Conclusions

Overall, by means of a combined *in vitro* and *in vivo* study examining times post-treatment longer than what had been previously done we have been able to highlight the potential use of β-glucan immunostimulatory properties to develop antiviral strategies. The present work demonstrates for the first time the immune-stimulating capacities of β-glucan in zebrafish associated to increasing resistance to a subsequent challenge with a viral pathogen. The successful application of β-glucan as a treatment against fish diseases will rely on determining the most effective dose and timing of administration for each species.

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Competing interest statement

The authors declare that there are no potential competing interests that may influence this

publication.

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Figure captions

Figure 1: Inhibition of viral-induced CPE in ZF4 cells. ZF4 cells were stimulated with increasing concentrations of β-glucan for 24h and 7 days later infected with SVCV (moi = 10^{-3}) for 24 hours and subjected to a MTT cell viability assay. Optical density readings (570nm) were converted to percentages and normalized to virus control (100% CPE). The experiment was performed in triplicate.

Figure 2: Cytokine expression levels in β-glucan -treated ZF4 cells (Bgc =10 µg/ml for 24h) and infected with SVCV (moi = 10^{-3}) seven days after treatment. At 24 hpi RNA samples were collected for RNA extraction and real-time PCR. Data represent mean ±SD of two experiments each one performed in duplicate. Results are expressed relative to the control (untreated cells); Significant differences are indicated as follows: * p<0.05, ** p<0.01 and ***p< 0.001.

Figure 3: Cytoplasmic IL1β expression is induced in Zf4 cells by β-glucan. Zf4 cells were pretreated with 10µg/mL β-glucan for 24h. Polyclonal anti-IL1β antibody was used to determine the location and pattern of IL1β staining, and DAPI (blue) for nuclei labelling.

Figure 4: Expression levels of inflammatory cytokine genes and interferon stimulated genes (ISGs) in zebrafish treated with β-glucan. Fish were i.p. injected with β-glucan (5µg/fish). Samples from internal organs were collected at 2, 14, 22 days after stimulation for RT-qPCR analysis of the relative expression compared to control fish. Data represent mean ±SD of 3 samples.

Figure 5: Bath immersion challenge of zebrafish with SVCV. A. Experimental design. B) Cumulative percent survival. βGlc (▲): fish injected with 5µg β-glucan 14 days before SVCV infection. C) Relative quantification of N-SVCV gene by RT-qPCR (n = 3 /time point). * p<0.05.

Figure 6: Difference in the expression of zebrafish innate immune genes after SVCV challenge. BGluc: fish injected with β-glucan (5µg/fish) and harvested at 2 days post-treatment. SVCV: 7 days post β-glucan injection the fish were challenged with SVCV. Samples were harvested at 2dpi (white) and 7dpi (dark). *ef1a* was used as a endogenous reference. Data are presented as mean expression relative to non-treated control fish (n=3).

Supplementary Material

Figure S1. Experimental design of β-glucan treatment of ZF4 cells. ZF4 cells were incubated uneder medium containing 10µg/ml β-glucan for 24 hours. Six days after β-glucan removal the cells were infected with SVCV. Times of RNA extraction are indicated.
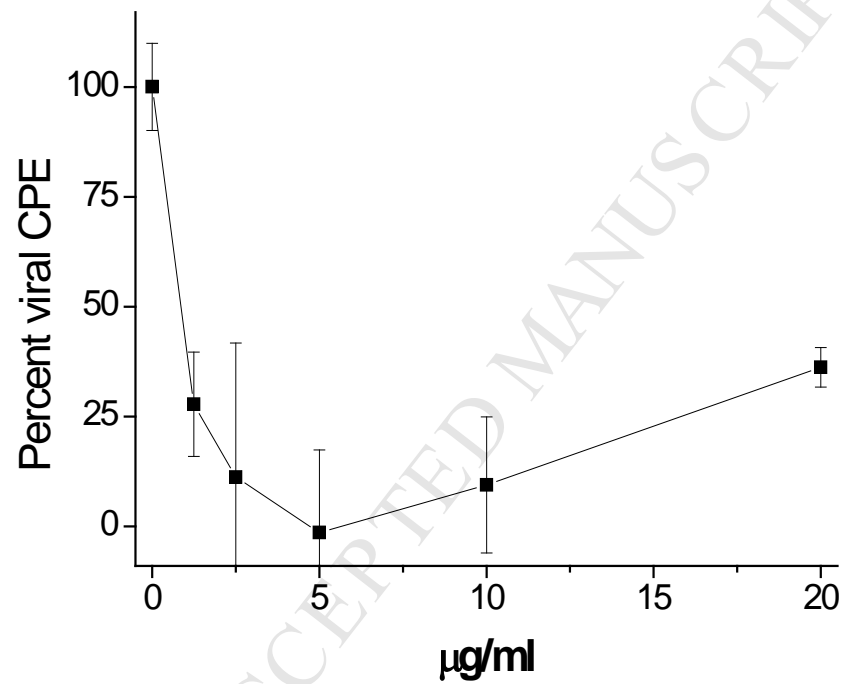
Figure S2. Images of ZF4 cells treated with fluorescent zymosan after medium removal followed by one washing step. ZF4 cells were seeded in 96-well plates and treated with 10µg/ml Zymosan-AlexaFluor488 (ThermoFisher) for 24 hours. Then the medium was removed and the cells were washed with fresh medium. Pictures of the cells were taken with an INCell Analyzer 6000 image system (60X).

Figure S3. ELISA testing of the anti-IL1B antibody. RTS11 (macrophage-like cells from rainbow trout, *Oncorhynchus mykiss*) and ZF4 cells, were stimulated with 10µg/ml zymosan for 24h. A) Mean OD 450nm values ±SD (n=3) of samples from cell supernatans (ss) or from cell lysates (cells). B) A standard curve was prepared using purified trout recombinant IL1B. Average OD values of the cell lysate samples are also plotted.

Figure S4. Schematic diagram of the β-glucan administration and SVCV challenge of zebrafish.

Gene	Sequence	Access number
ef1α	Fw: 5´-CCACGTCGACTCCGGAAA-3´ Rv: 5´-CGATTCCACCGCATTTGTAGA-3´	AY422992.1
il1b	Fw: 5´-GAACAGAATGAAGCACATCAAACC-3´ Rv: 5´-ACGGCACTGAATCCACCAC-3´	NM_212844
nod2	Fw: 5´-TTTAGCGGTGACGTCCAGAA-3´ Rv: 5´-GCCTCATAGCCCAGTCACAA-3´	NM_001328044.1
il8	Fw: 5´- GTCGCTGCATTGAAACAGAA -3´ Rv: 5´- CTTAACCCATGGAGCAGAGG -3´	XM_009306855.3
il10	Fw: 5´-ATTTGTGGAGGGCTTTCCTT-3´ Rv: 5´-AGAGCTGTTGGCAGAATGGT-3´	NM_001020785
infphi1	Fw: 5´-AGTTGTGAAAAGCCACCTTCAGA-3´ Rv: 5´-CATGTGTGACACTCAAGGATTGAC-3´	NM_207640
tnfa	Fw: 5´-AAGCCACTTTTCAGTGCAATCC-3´ Rv: 5´-AGCGCCGAGGTAAATAGTGTTG-3´	BC167066.1
gig2l	Fw: 5´-GGGGTTTTGCCAGTCTAAGGA-3´ Rv: 5´-GCCAGGTTTTCTGCAGTGGA-3´	NM_001245989.1
SVCV-N	Fw: 5´-AGCTTGCATTTGAGATCGACATT-3´ Rv: 5´-GCATTATGCCGCTCCAAGAG-3´	U18101

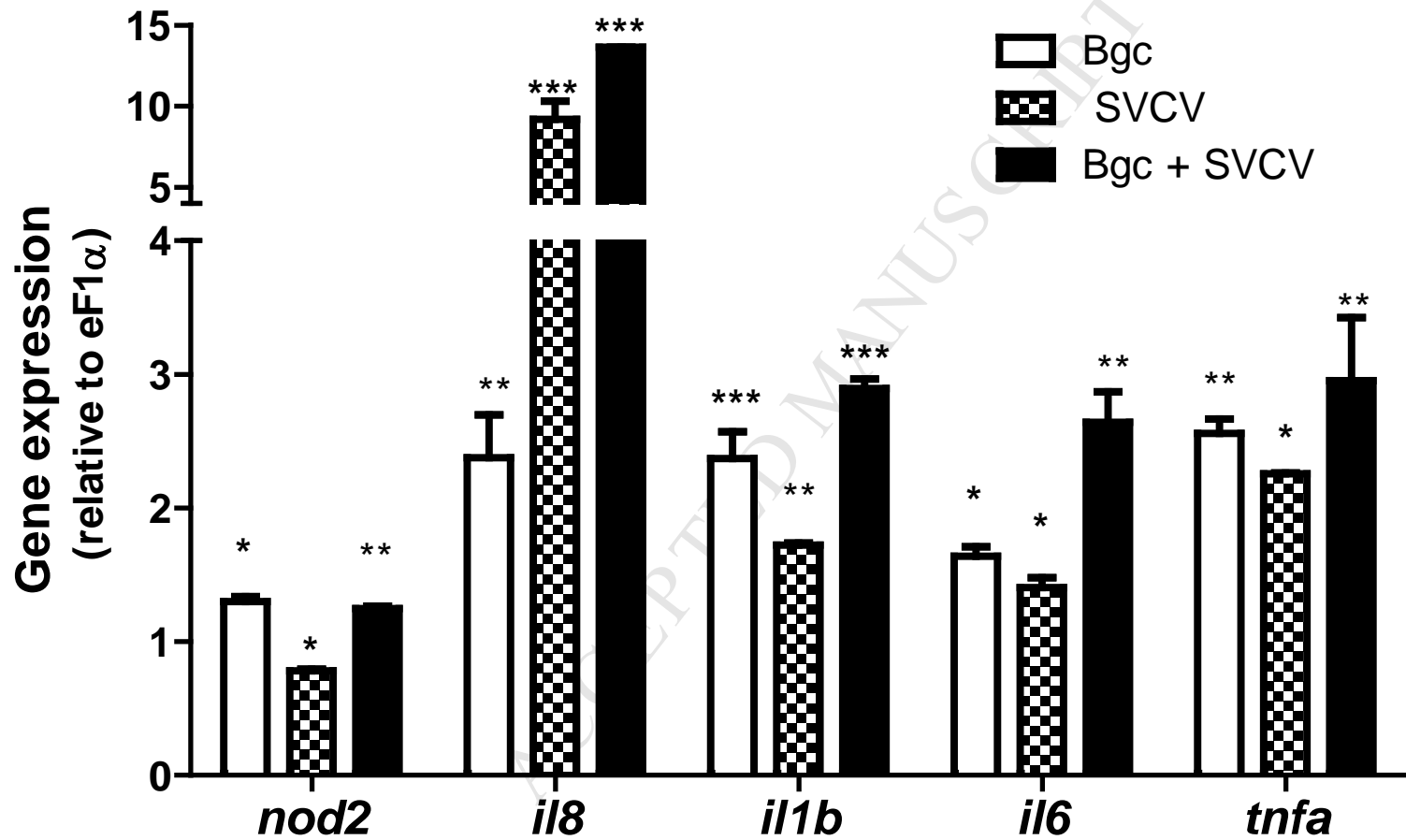**Table 1.** Primers used for quantitative reverse-transcription PCR.
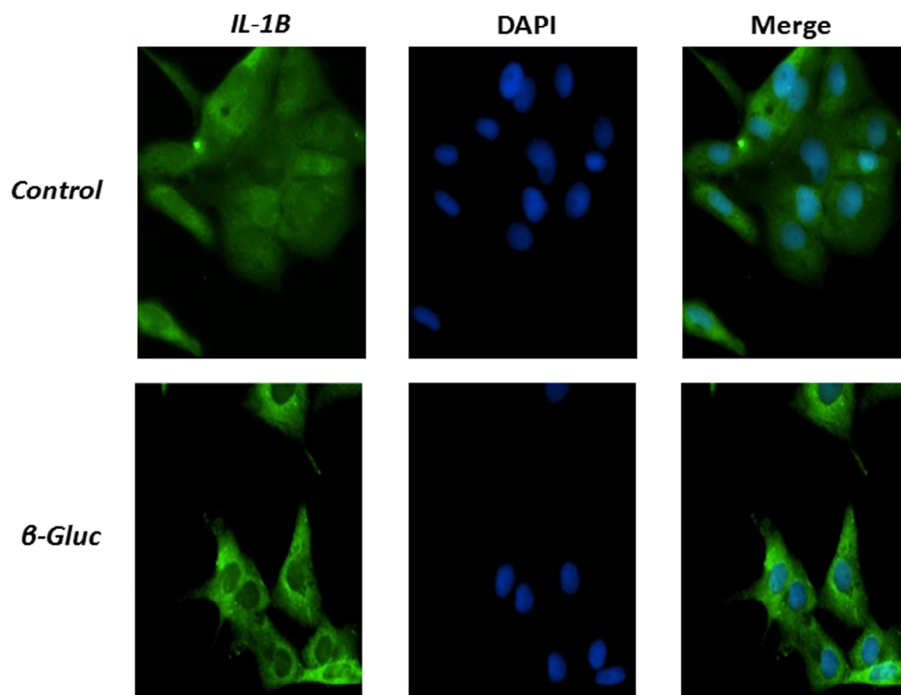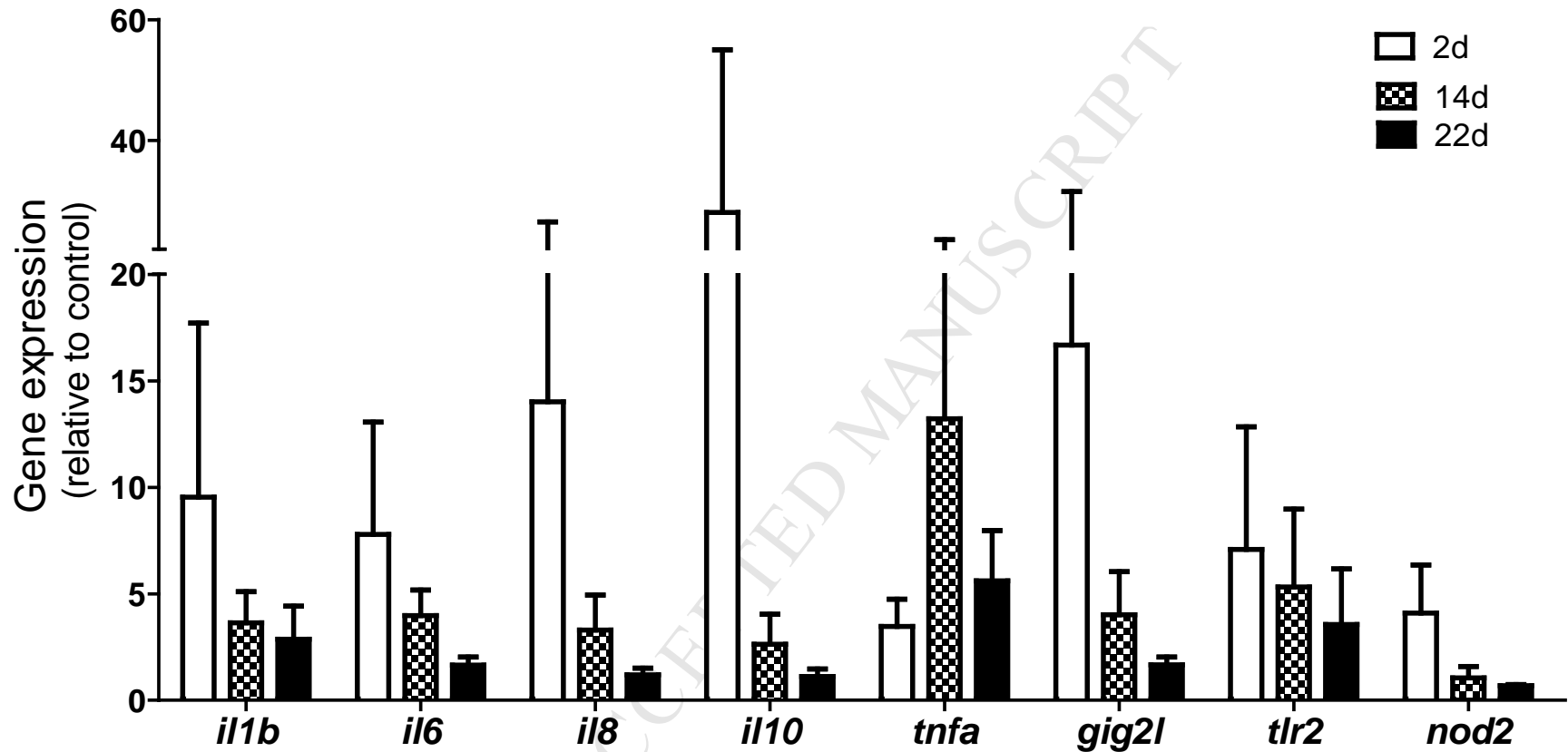
Figure 1

Figure 2

	IL-1B	DAPI	Merge
Control			
ß-Gluc			

Figure 3

Figure 4

Figure 6

A

1st stimulus
β-Gluc

Time | 2 d | 14d | 2 dpi | 7 dpi

RNA ext

2nd stimulus
SVCV

RNA ext

RNA ext

B

% Survival vs dpi

- control
- SVCV
- BGlc+SVCV

C

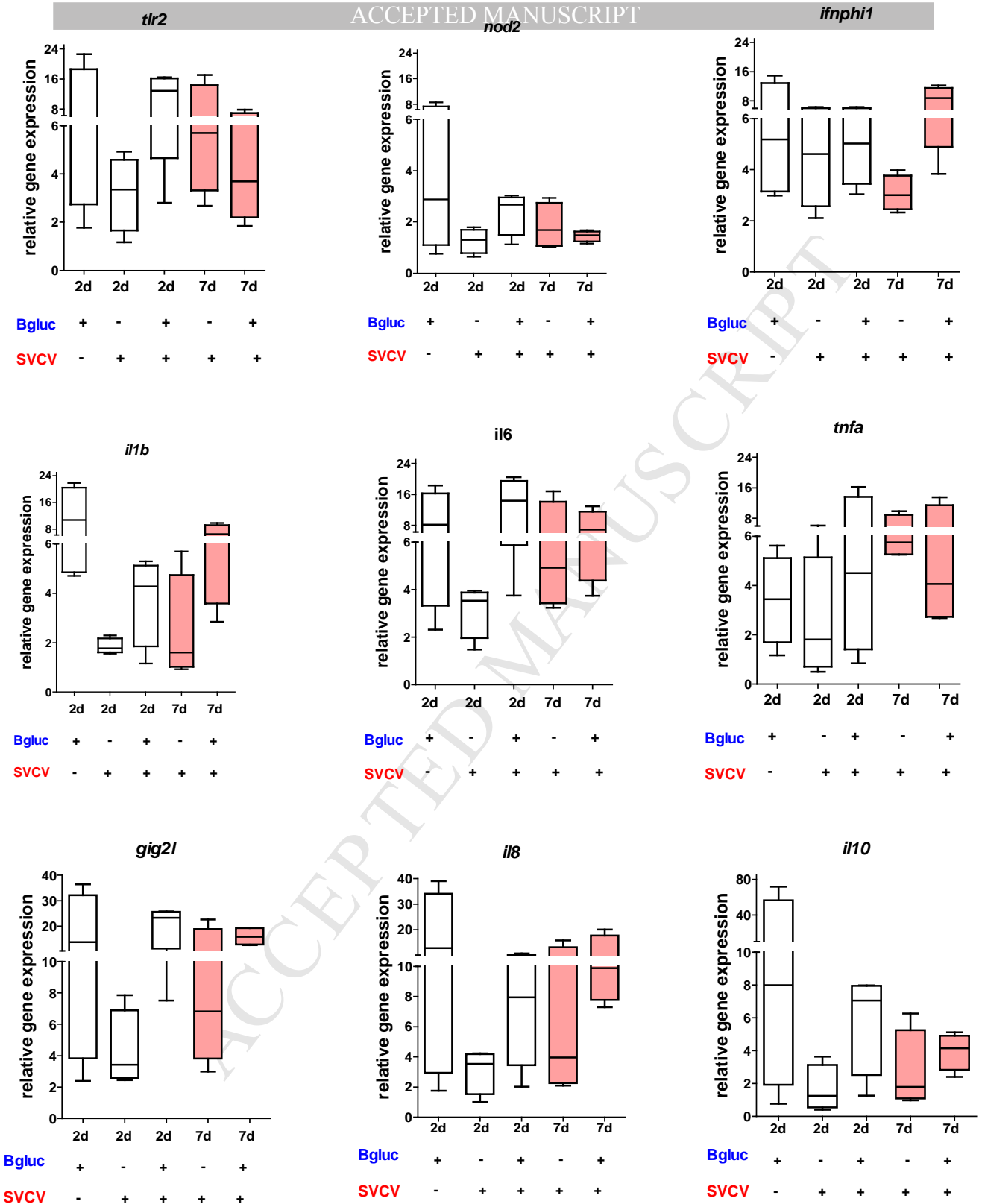SVCV-N gene expression
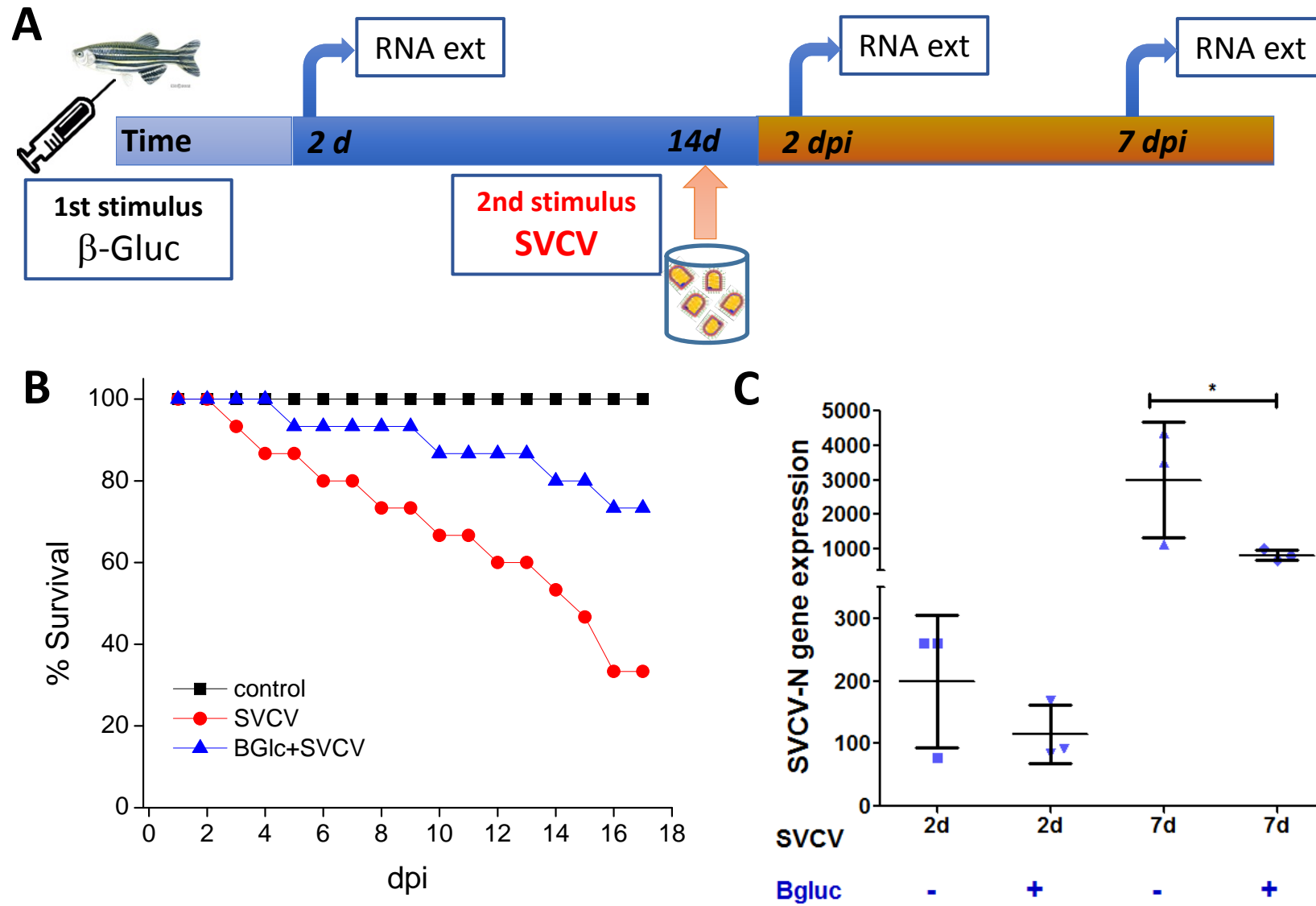
SVCV: 2d, 2d, 7d, 7d
Bgluc: -, +, -, +

*

Figure 5

Highlights

- Yeast β-glucan zymosan is a potent immunostimulant in *D. rerio* both *in vitro* and *in vivo*.

- ZF4 cells exposed to β-glucan exhibited protection against SVCV infection.

- β-glucan intraperitoneal injection into adult zebrafish enhances resistance to SVCV challenge two weeks after the glucan administration.

- Up-regulation of host antiviral genes expression (*gig2*, *ifnphi1*, *il1b*, *il6*, *il8* and *il10*) was observed in zebrafish administered with β-glucan.