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Transcriptome Changes Associated with Protective Immunity in T and B Cell-Deficient *Rag1*$^{-/-}$ Mutant Zebrafish

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Abstract: To elucidate the basis of protective immunity in T and B cell deficient *rag1*$^{-/-}$ mutant zebrafish, we conducted microarray analysis of 15,617 genes from *rag1*$^{-/-}$ mutant zebrafish 48 hours after a primary response and 48 hours after a secondary response. Following primary exposure, the highest fold expression differences (3.8 to 4.95) were genes for serum amyloid A, chemokine CCL-C5a (CCL-19a), signal transducer and activator of transcription (STAT) 1b, interferon regulatory factor 11, and myxovirus resistance A. Strong induction of these genes demonstrated that primary immune responses and innate immune cells were not impaired in T and B cell deficient mutant zebrafish. Following bacterial re-exposure, the highest fold expression differences (2 to 3 fold) were in chemokine CCL-C5a (CCL-19a), myomegalin, bone morphogenetic protein 4, and relaxin 3a. These genes are involved in the immune response and cell proliferation. Genes for cell receptor activation and signal transduction, cell proliferation and cytotoxic functions were also up-regulated. These findings suggest receptor activation and expansion of a cell population. Increased *ifn*γ expression at 48 hpi was associated with both primary and secondary immune responses.

Keywords: *Rag1*$^{-/-}$ Mutant Zebrafish, *Edwardsiella ictaluri,* Protective Immunity, Transcriptome, Cell-Mediated Immunity

1. Introduction

During early stages of life, fish do not have acquired immunity; there is an adaptive component of innate immunity that protects them during this period. Channel catfish do not orchestrate acquired immunity at 1 to 2 weeks post hatch [1, 2], yet fry are frequently vaccinated at that age with varying success. When channel catfish fry were vaccinated with RE-33®, an attenuated live strain of *Edwardsiella ictaluri*, protection lasted from 14 days to 4 months post vaccination [3], or from one month to less than 6 months post vaccination in another study [4]. However, the basis of protective immunity in immunologically immature fish fry is not known.

Rag1$^{-/-}$ mutant zebrafish lack mature T and B cells, as do young fish, making them an excellent model to study the adaptive component of innate immunity in fish [5]. When leucocytes from kidneys of RE-33® vaccinated *rag1*$^{-/-}$ mutant zebrafish were adoptively transferred into naïve *rag1*$^{-/-}$ mutant zebrafish, the naïve fish demonstrated protective immunity following *E. ictaluri* challenge [6]. In the *rag2*$^{-/-}$ mutant mice/murine cytomegalovirus model, NK cells mediated protection in T and B deficient mice [7, 8]. A similar type of response may be occurring in *rag1*$^{-/-}$ mutant zebrafish. Trained macrophages can also provide protective immunity [9]. Another study analyzing global gene expression in channel catfish fry following immersion exposure of RE-33® or wild type [10] *E. ictaluri* was

performed [11], but specific conclusions could not be reached.

The purpose of our study was to identify differentially expressed gene transcripts following a primary exposure (vaccination) and secondary bacteria exposure of WT *E. ictaluri* in *rag1*$^{-/-}$ mutant zebrafish. The results of this study will help us further elucidate mechanisms underlying non-T and B cell-based protective immunity in fish.

2. Materials and Methods

2.1. Animal Source

Rag1$^{-/-}$ mutant zebrafish were produced and reared in the specific pathogen free fish hatchery in the College of Veterinary Medicine following standard operating procedures [5]. The Institutional Animal Care and Use Committee at Mississippi State University approved all propagation, rearing and experimental animal protocols.

2.2. Fish Challenges

During experiments, fish were maintained in 15 L aerated flow-through tanks with charcoal filtered dechlorinated municipal water at 26°C with a water flow rate of 0.5 L/min. Fish were fed twice daily with Zeigler™ Adult Zebrafish Diet (Aquatic Habitats™, Apopka, FL). Adult (6 to 9 month old) *rag1*$^{-/-}$ mutant zebrafish were anesthetized in 110 mg/L buffered tricaine methanesulfonate (MS222). Each fish was administered an IC (intracoelomic) injection on the lateral line above the anal fin. Depending on the treatment schedule, zebrafish were vaccinated with a primary exposure of $1x10^4$ CFU/fish RE-33® (AQUAVAC-ESC Intervet, Inc.), or challenged with $1x10^4$ CFU wild type [10] *E. ictaluri*. The secondary challenge injection tested if the primary vaccination provided protection. Sham treated groups received 10 µl of PBS inoculation per fish. Vaccinated or challenged groups received 10 µl of bacteria-PBS inoculation per fish. The time interval between primary and secondary inoculations was four weeks. Forty-eight hours following vaccination or challenge, hematopoietic tissues of random fish were swabbed with a sterile loop and streaked on BHI plates to confirm *E. ictaluri* presence (or absence for control treatments).

2.3. Preparation of Vaccination and Bacterial Cultures

All primary vaccinations were 10^4 CFU/fish of RE33®, a commercial attenuated *E. ictaluri,* RE-33® (AQUAVAC-ESC Intervet, Inc.), [3]. The WT *E. ictaluri* (#93146) was isolated from fish submitted to the Fish Diagnostic Lab at CVM-MSU. Culture identifications were confirmed by biochemical analysis using the BioMerieux api20E strip (BioMerieux, 69280 Marcy l'Etoile, France). Aliquots (0.5 ml) were stored in 20% glycerol at 28°C until needed for trials, at which time one aliquot was thawed and added into Brain Heart Infusion broth and incubated in a shaker incubator at 30°C overnight. Logarithmic phase cultures were obtained by dilution of the overnight culture 1:10 and grown until the optical density was 0.4 at 540 nm which corresponds to 10^8 colony forming units (CFU) per ml. Culture purities were assessed and bacterial concentrations determined by plating serial dilutions on 5% sheep blood agar plates.

2.4. Experimental Design

The transcriptome study consisted of four treatments that received different combinations of primary exposure to attenuated *E. ictaluri* RE-33® (AQUAVAC-ESC Intervet, Inc.), as a vaccination (E_1) and/or a secondary bacteria exposure of WT *E. ictaluri* (#93146) (E_2). The first treatment was sham vaccinated at day 0 and was challenged with *E. ictaluri* (E_2) four weeks later. This group was designated SE_2 and represents the primary immune response. The second treatment received a primary vaccination at day 0 and a PBS injection at four weeks post-injection. This group was designated E_1S, and represents a persistent primary response. The third treatment was vaccinated at day 0 and challenged four weeks later with *E. ictaluri*. This group was designated E_1E_2 for vaccinated and challenged with bacteria and gene expressions of this group represent the secondary (protective) response. The fourth treatment was the control group was not vaccinated and was not challenged with *E. ictaluri.* This group received PBS injections and was designated SS for sham primary and sham secondary. Fish were euthanized by immersion in 340 mg/L Tricaine Methane Sulfonate (MS-222) (Argent Chemical Laboratories, WA) 48 hours after the secondary inoculation. The kidneys from three fish were collected and pooled for each of three replicates per treatment in the microarray analysis.

2.5. Microarray Analysis

Total RNA was isolated from each of three replicates of pooled kidneys (n=3) from each experimental group by homogenizing the tissue in TRIZOL reagent extraction (Invitrogen) according to the manufacturer's protocol. The quality of each RNA sample was assessed by measuring RNA integration number (RIN) with the Agilent 2100 Bioanalyzer [12]. The RNA samples used in this experiment had RIN values ranging from 7.3 to 9.4, with most being greater than 9.0. For the qPCR, RNA was extracted from individual kidney samples using RNA direct zol kit (Zymo research, USA). The quantity of RNA was determined by NanoDrop ND-1000 and ND-8000 8-Sample Spectrophotometer and stored at -80°C. 100ng cDNA was prepared from RNA by using Super script III VILO™ cDNA Synthesis Kit (Invitrogen).

The transcriptome of each sample was evaluated using the Affymetrix Zebrafish Array (15, 617 probe sets) according to the manufacturer's protocols (Affymetrix™). Briefly, total RNA concentrations of 10µg were used to synthesize double-stranded cDNA followed by its clean up using the GeneChip One-Cycle cDNA Synthesis Kit and Clean Up Module respectively. The resulting cDNA was used in a 16 hours *in vitro* transcription reaction to produce Biotin-labeled cRNA

using IVT Labeling kit and GeneChip clean up module respectively. NanoDrop spectrophotometric analysis was used to measure the final yield of the biotin-labeled cRNA and 20µg of biotin-labeled cRNA was fragmented and then hybridized to the chip and labeled with streptavidin-phycoerythrin using the Affymetrix Fluidic station. Chips were scanned using the Affymetrix scanner and image data for zebrafish. The Genome array was processed using the Affymetrix Microarray Suite version 5.0 software. All gene expression data were evaluated for perfect match and mismatch values and normalized to the median measurement for the genes across all the arrays in the dataset.

2.6. Confirming Selected Gene Expression and Analysis of Selected Genes not Present on the Microarray

Expression patterns of four transcripts that were shown to be differentially expressed using the Affymetrix array (*stat1b, saa, irf1b*, and loc795887) were confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) using the RNA samples used for microarray analysis. The total RNA (2ug) samples were reverse transcribed using super script VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol to generate first strand cDNA. Then qPCR was performed using hydrolysis probe assays (*arp*) or SYBR green assays using Stratagene Mx3000P instrument (Agilent Technologies). Primers and probes were either published sets or were designed using NCBI Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). All qPCR reactions were 20ul and contained cDNA template derived from 5ng RNA and were performed in triplicate. Hydrolysis probe assays were done as previously described [13, 14]. The cycling parameters consisted of 10 min at 95°C then 40 cycles of 30s at 95°C, and 1 min at 61°C. SYBR green assays used EXPRESS SYBR GreenER qPCR supermix kit (Invitrogen) following manufacturer's instructions. The cycling parameters for SYBR green assay are 10 min at 95°C then 40 cycles of 30s at 95°C, 1 min at 57°C, and 15s at 72°C. Melting curve analysis was performed on all SYBR Green assays to confirm that signal was due to the specific amplified product. Pearson correlations of qPCR data with microarray data were performed using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

To determine expression levels of *ifn* , *nitr9* and *t-bet*, adult *rag1*$^{-/-}$ zebrafish were exposed to the following treatments: SS, SE_2, E_1S and E_1E_2 with sample size (n) of 5 for each treatment. Fish were euthanized at 24hpi and 48hpi with MS-222 (Argent Chemical Laboratories, WA), kidneys were taken from each fish and RNA was extracted using TRIZOL reagent (Zymo research, USA) and stored at -80°C. Primers and probes for qRT-PCR were either published sets or were designed using NCBI Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The *t-bet* primers and probes (Table 1) were designed by Primer3 plus software [15], respectively. All primers and probes were purchased from Eurofins MWG, Operon, Huntsville, Alabama, USA. Amplification of the ubiquitously expressed acidic ribosomal phosphoprotein (*arp*) gene was used as a housekeeping gene for normalizing [16].

Table 1. *Oligonucleotides primer and probe used for qRT-PCR to quantify gene expression levels. Housekeeping gene arp was used as a reference gene. All primers and probes without references were designed by Primer3 plus (GraphPad) software.*

Gene	Oligonucleotide sequences (5'–3')	GenBank Accession No.
stat1b	Fwd: TCTCTAGCCATCGTTCGCTTCC Rev: GATCTCTTTTGGCATCGGGTCA	BC044185.1
saa	Fwd: GCAGTGGTATCGCTTCCCAGGAG Rev: AGCTTCATAGTTCCCGCGTGCAT	BI883568
irf11	Fwd: GTGGGCCATTCACACAGGTA Rev: TTCTGCAGACGTGTCCTCAC	BE556864
Loc795887	Fwd: TGGGAAACGCACCATCTGAA Rev: AGTGCCTCCACATGAGTCAACC	AW420565
arp	Fwd: CTGCAAAGATGCCCAGGGA Rev: TTGGAGCCGACATTGTCTGC Probe: [FAM]TTCTGAAAATCATCCAACTGCTGGATGACTACC[BHQ1a] [17]	NM_131580
ifnγ	Fwd: CTTTCCAGGCAAGAGTGCAGA Rev: TCAGCTCAAACAAAGCCTTTCG Probe: [FAM]AACGCTATGGGCGATCAAGGAAAACGAC[BHQ1a] [17]	NM_212864
t-bet	Fwd: GATCAAGCTCTCTCTGTGATAG Rev: GCTAAAGTCACACAGGTCT Probe: [FAM]TTCTGAAGGTCACGGTCACA[BHQ1a]	NM_001170599.1
nitr9	Fwd: GTCAAAGGGACAAGGCTGATAGTT Rev:GTTCAAAACAGTGCATGTAAGACTCA Probe: [FAM]CAAGGTTTGGAAAAGCAC[BHQ1a] [18]	AY570237.1

2.7. Data Analysis

Statistical analysis (Student's t-test) was carried out to identify differentially expressed transcripts. The treatment E_1S was compared to SS and there were no significantly different gene expression changes. The SE_2 (primary) treatment group was compared to SS (control), and genes that were significantly different from SS were evaluated in a pairwise comparison of SE_2 (primary) to the E_1E_2 (secondary). Differentially expressed transcripts were

mapped to UniprotKB and Genbank RefSeq protein accessions. Functional analysis of the differentially expressed transcripts was performed with protein accessions using (pre-existing) GO annotation identification, GO enrichment and pathways and networks. GO annotations of catfish and salmon were identified using Agbase-GOretriever tool [17] and ZFIN GO identified zebrafish genes. GO enrichment analysis was performed using singular enrichment analysis (AgriGO SEA) that computes statistically significant GO term enrichment using Fisher's exact test for differentially expressed transcripts (DET) compared to their background. Pathways and networks analysis was performed using the Ingenuity pathway analysis (IPA) tool, with parameters of $p<0.001$ and $p<0.05$. IPA visualized significant networks and their assigned biological functions from the scientific literature. GO annotations of differentially expressed transcripts compared to the whole array were visualized using the Agbase GOSlim viewer tool [17] with the generic GOSlim set. The percentages of GO terms between the differentially expressed transcripts and the array were compared. GO annotations for the array were obtained from the Affymetrix annotation files. Relative gene expression data was determined using the Delta Delta ct ($\Delta\Delta$ct) analysis method. The data was statistically analyzed by the two-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

3. Results

3.1. Microarray Analysis of Global Gene Expression Following Primary and Secondary E. Ictaluri Infection

There were no significant differences in gene expressions between the SS and E_1S treatment groups. Transcriptional profiling in the kidney of *rag1*$^{-/-}$ mutant zebrafish after the primary exposure (SS and SE_2) demonstrated 129 transcripts that were significantly up-regulated at 95% confidence (Table 1). The differences in increased transcript expression in primary exposed compared to non-exposed fish were 1 to 4.95 fold. The highest fold expression differences (3.8 to 4.95) were SAA, chemokine CCL-C5a, signal transducer and activator of transcription 1b (STAT 1b), interferon regulatory factor 11, and myxovirus resistance A. Gene expressions with 2.1 to 2.7 fold differences were complement components 7 and 1, ceruloplasmin, kappa light polypeptide gene enhancer and inhibitor alpha a, chemokine C-X-C motif receptor 3.1, and calreticulin (like). The majority of the up-regulated transcripts were grouped into acute phase response, complement activation, immune response, response to stimulus, protein degradation and processing, proteasomes and heat shock protein categories. Transcripts that were significantly differentially expressed less than 2.1 fold are shown in the Appendix Table A1.

Table 2. *Log2 changes in expression of zebrafish transcripts that were up-regulated ($p< 0.05$) following primary infection (SE_2) compared to non-infected (SS) controls. The highest fold differences (3.8 to 4.95) of annotated genes are shaded dark gray, while the second highest fold differences (2.1 to 2.7) are shaded light gray. The annotated genes with the highest fold differences are also rated #1 to #12. Zebrafish transcripts that were up-regulated ($p< 0.05$) following primary infection (SE_2) compared to non-infected (SS) controls less than 2.1 fold are listed in Supplemental Table 1.*

Functional classification	Accession number	Putative ID	Log2 difference
Acute phase response			
#1	BI883568	serum amyloid A [15]	4.945338295
#6	AA497156	complement component 7	2.711235993
#7	CD014253	complement component 1, q subcomponent-like 4 like	2.543084809
	BC048037.1	Ceruloplasmin	2.426394913
Immune Response			
#2	BQ479755	chemokine CCL-C5a (CCL-19a)	4.326214098
#3	BC044185.1	stat 1b	4.124193546
#4	BE556864	interferon regulatory factor 11	3.94700698
#8	BC046906.1	calreticulin-like	2.268209135
#10	CD606274	stat 1b	2.220123148
#11	BG985448	calreticulin-like	2.179232528
#12	AW019258	like chemokine (C-X-C motif) receptor 3.1	2.108884501
Response to Stimulus			
#5	AF533769.1	myxovirus (influenza) resistance A (*mxA*)	3.826118133
#9	AW019105	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a	2.250052292

To analyze the secondary response, the gene expressions of E_1E_2 and SE_2 were compared. After disregarding genes identified in the primary exposure, 98 significantly differentially expressed transcripts were identified and associated with protective immunity (Table 2). Forty-six transcripts were up-regulated, and 52 transcripts were down-regulated in E_1E_2 compared to SE_2. In annotated genes, the highest fold expression differences (2 to 3 fold) were C-C like chemokine 19, myomegalin, bone morphogenetic protein 4 and relaxin 3a. These genes are involved in the immune response and cell proliferation. Transcripts that were significantly differentially expressed less than 2 fold are shown in the Appendix, Table A2.

Table 3. *Log2 changes in expression of zebrafish transcripts that were differentially expressed ($p< 0.05$) between the secondary (E_1E_2) and primary (SE_2) exposures. The highest fold differences (2.24 to 3.10) of annotated genes are shaded dark gray. The annotated genes with the highest fold differences are also rated #1 to #4. Zebrafish transcripts that were differentially expressed ($p< 0.05$) between the secondary (E_1E_2) and primary (SE_2) exposures less than 2.1 fold are listed in Supplemental Table 2.*

Functional classification	Accession number	Putative ID	Log2 difference
Immune Response			
#1	BI476419	chemokine CCL-C5a (CCL-C19a)	3.1044014
#4	BI865907	relaxin 3a	2.2479468
Cell proliferation			
#3	D49972.1	bone morphogenetic protein 4	2.2686536
Miscellaneous			
#2	BI982955	myomegalin-like	2.7361798

3.2. ID Mapping

The functional analysis of the differentially expressed transcripts was performed by mapping the transcripts sequence to protein identifiers/accessions of their putative products and were categorized based on the function of the gene product. Of the 98 proteins identified, 64% coded for predicted proteins that had UniProtKB and GenBank RefSeq protein IDs, 46% were up-regulated and 53% were down-regulated. Of the unannotated genes, 26% were expressed sequence tags (ESTs) that did not have connections to predicted known zebrafish genes, and 1% were not listed in NCBI. Annotations for the remaining genes (7%) were not in the NCBI database. However, these genes had UniProtKB and GenBank RefSeq protein accession IDs, so they were included in the analysis along with the 64% predicted proteins. Thus, 71% of the protein identifiers were used in the analysis.

3.3. Functional Analysis

Comparison of the differentially expressed transcripts and the total array transcripts demonstrated that molecular functions such as actin binding, receptor binding, lipid binding and nucleic acid binding were over-represented as 4.75, 4.42, 2.34, and 2.14 fold, respectively. Protein binding, protein kinase activity, and catalytic activity were under-represented by 0.13, 0.25 and 0.48 fold, respectively. Additionally, proteinaceous extracellular matrix, extracellular space, cytoplasmic membrane-bound vesicles, nucleolus, cytoskeleton and chromosome components were over-represented in differentially expressed transcripts by 15.65, 5.6, 3.5, 2.7, 2.38 and 1.92 fold respectively, while, various organelles and cytoplasm sub-categories were under-represented by 0.65 and 0.33 fold respectively. In the biological process category, response to endogenous stimulus, cell-cell signaling, and cell proliferation were over-represented in differentially expressed transcripts by 4.63, 1.98, and 1.90 fold respectively, while protein metabolic process, cellular component organization and transport were under-represented by 0.46, 0.26, and 0.21 fold, respectively.

Out of 71 differentially expressed transcripts that had UniProt IDs, 32 had GO annotations and 21 GO terms associated with these were significant ($p<0.05$). These were in two categories: (i) molecular function: catalytic activity, binding, nucleic acid binding, DNA binding, cation binding, receptor binding, metal ion binding and transition metal ion binding and (ii) cellular component: extracellular region, cell, cell part, intracellular, intracellular part, organelle, intracellular organelle, membrane-bound organelle, intracellular membrane-bound organelle. The molecular functional group had 8 enriched GO (child/secondary) terms. There were three significantly enriched GO terms in the cellular component category: cellular component, molecular function and biological process. None of these were directly connected to each other.

3.4. Confirming Selected Gene Expression and Analysis of Selected Genes not Present on the Microarray

Relative expression values of *stat1b, saa, irf1b, loc795887* from the microarray and qRT-PCR were strongly correlated, with R values >0.95 (Table 4). The analysis of *ifnγ, nitr9* and *t-bet* expressions between SS, SE_1 and E_1E_2 treatments demonstrated significant increases in *ifnγ* expression (Fig. 1 and Supplemental Table 4). Within treatments, *ifnγ* expression was significantly greater at 48 hpi, than at 24 hpi. There were no significant differences in *nitr9* and *t-bet* expressions between treatments.

Table 4. *Correlation of selected genes used for confirmatory qRT-PCR.*

Gene	Accession	Treatment	Microarray relative expression	qRT-PCR relative expression	Correlation [19][19][19][19]
					[19][19][19][19]
					[19][19][86][86]
					[84][84][84][84]
					[84][86][86][86]
					[86][86][87][86]
					[86][85][84][84]
stat1b	BC044185.1	SE_2	6.7	1.72	0.9846
		E_1E_2	6.7	1.64	
		E_1S	2.4	0.31	
		SS	3.3	0.74	

Gene	Accession	Treatment	Microarray relative expression	qRT-PCR relative expression	Correlation [19][19][19][19] [19][19][19][19] [19][19][86][86] [84][84][84][84] [84][86][86][86] [86][86][87][86] [86][85][84][84]
saa	BI883568	SE_2	10.8	1.74	0.9839
		E_1E_2	11.1	2.4	
		E_1S	6.2	0.01	
		SS	6	0.01	
irf1b	BE556864	SE_2	12.2	13.6	0.9563
		E_1E_2	11.9	8.2	
		E_1S	8.2	0.35	
		SS	8.3	0.7	
loc795887	AW420565	SE_2	11	7.42	0.9511
		E_1E_2	11.2	12.2	
		E_1S	6.3	0.4	
		SS	6.6	0.60	

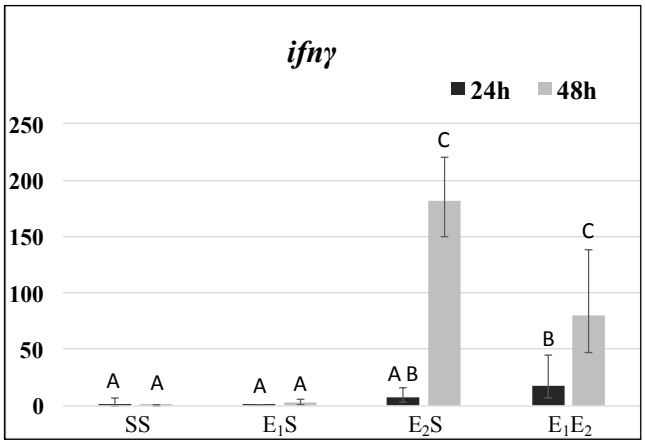

Figure 1. *Fold changes in ifnγ gene expression in kidney 24 and 48 hpi of E. ictaluri, measured by quantitative real-time PCR. Data are presented as mean fold change relative to the PBS control group ± standard deviation based on Log2 data analysis. hpi= hours post injection. *Significant ($p<0.05$) difference in expression between treatments; treatments with the same letter are not different.*

4. Discussion

4.1. Primary Response

There are several studies analyzing the gene responses of catfish to *E. ictaluri* infection. Differences in responsive genes in blue catfish [20] and channel catfish [11] demonstrate there are species specific responses to the same bacteria. There have not been any studies performed analyzing the gene responses of zebrafish to *E. ictaluri*. In our study, transcriptome analysis comparing the primary response to non-exposed controls revealed 129 functionally known genes that were significantly up-regulated. These genes were involved in acute phase response, complement activation, immune response, response to stimulus, proteasomes, protein degradation, chaperons, processing and heat shock protein categories. These are normal components of the innate response and cellular injury and indicate activation of the innate immune system. The highest fold expression differences (3.8 to 4.9) were SAA, chemokine CCL-C5a (also named CCL-C19a), signal transducer and activator of transcription 1b (STAT 1b), interferon regulatory factor 11, and myxovirus resistance A. Gene expressions with 2.1 to 2.7 fold differences were complement components 7 and 1, ceruloplasmin, kappa light polypeptide gene enhancer and inhibitor alpha a, chemokine C-X-C motif receptor 3.1, and calreticulin (like).

SAA has multiple isoforms that are expressed during the initial stages of inflammation, and affect cell adhesion, proliferation and migration. Serum amyloid A is also an innate immune opsonin, and binds to some Gram-negative bacteria [21], with the outer membrane protein A [22] being the major ligand. *Edwardsiella ictaluri* is a gram-negative bacteria, and in our study, SAA could be acting as a pattern recognition protein for the OmpA of *E. ictaluri.* In rainbow trout, SAA was upregulated 72 and 96 hours post bacterial injection [23]. Another heat shock protein, Hsp 60, was up-regulated in primary exposed fish compared to non-exposed fish. Hsp60 in humans is associated with functional TLR-4 and is involved in ATP-dependent protein folding. Hsp 90 functions as a chaperone and is involved in housekeeping functions such as protein folding and unfolding [24].

Chemokine CCL-C5A (also known as CCL-19 or 19a) was another of the primary response genes that were in the highest up-regulated group. The CCL-C5a gene was expressed in zebrafish embryos at 8 hpi of *Salmonella enterica* serovar *Typhimurium* [25]. The zebrafish genome has over 100 chemokine genes, but the functions have not been well studied [26]. CCL-C5a (CCL-19) was the highest up-regulated gene in the secondary response, and is discussed more later.

Chemokines are expressed by various cell types in response to inflammatory stimuli. Chemokines also induce various biological activities such as effects on degranulation, cell division, cell activation and secretion of cytokines in both leukocytic and non-leukocytic cell types [27]. In our study, the presence of cytokines was supported by the up-

regulated expression of 19 chemokine (C-C motif)-like molecules that induce cytokine secretion from leukocytes as well as provides pro-adhesive and migratory signals. CC chemokines promote chemotaxis of anti-tumor NK cells [28]. Zebrafish have increased number of chemokines due to duplication events. Subfamilies such as CXC, CC, XC and CX were found in zebrafish. CX is a novel subfamily found only in zebrafish. It is speculated that these novel chemokine genes are involved specifically in zebrafish development. To cope with environmental challenges, each species has species-specific chemokines during their evolution [26]. Zebrafish have an extensive chemokine system and a well established CC chemokine family [29]. To understand this complex network of molecules further research needs be carried out to in zebrafish [30], with loss of stat3 function resulting in immune disorders in zebrafish [31]. Among the immune response related transcripts, suppressor of cytokine signaling 1, present in multiple forms in fish, is up-regulated in response to infection.

Signal transducer and activator of transcription 1b, or STAT1b, was in the highest up-regulated group of the primary response genes. STAT proteins have important roles in immune cell-cell communication. *Stat1, stat3* and *stat5* have been identified in zebrafish [32]. *Stat1b* expression was significantly up-regulated following infection in zebrafish [33]. The up-regulation we observed in our study could have also resulted from increased ifnγ production. Another study suggested that *stat1b* promotes myeloid development in zebrafish [34].

Interferon regulatory factors (IRFs) are a large family of transcription factors involved in host immune response, haemotopoietic differentiation and immunomodulation [35], [32]. Interferon regulatory factors were identified originally as transcription factors in the regulation of interferon expression [36]. There are nine IRF orthologs in mammals, and all of these have been identified in fish, with zebrafish having additional factors: IRF 11 and IRF 12 [37].

MX GTPAses play key roles in viral immunity, and myxovirus resistance A genes are up-regulated by *ifnγ* signaling [38], as are *stat1a* and *stat1b*. Vertebrate *Mx* were compared, and similarities grouped them into fish *mx*, avian *mx*, human *mx2*-like, and human *mx1*-like [39]. Diverse *mx* proteins are found in fish [40]. In our study, up-regulated *mx* probably resulted from increased *ifnγ* production.

Other genes encoding acute phase proteins that were up-regulated in response to primary infection were ceruloplasmin and major acute phase reactant apolipoprotein of the HDL complex. Ceruloplasmin is involved in iron binding, homeostasis and transport. One important innate defense is the sequestering of iron to limit the availability of this critical nutrient to the invading bacteria.

Nearly 35 transcripts were up-regulated which were associated with proteasomes, protein degradation and processing. Proteasomes are involved in non-lysosomal intracellular protein degradation [41], cell cycle regulation as well as various cellular processes such as proliferation, differentiation, apoptosis and response to external stimuli [42]. Some of the up-regulated transcripts have roles in protein processing and folding such as dolichyl-diphospho oligosaccharide-protein glycosyltransferase, glycosyltransferase-like domain containing 1 and Dnajb 11 protein. The antigenic peptides presented on MHC I molecules are produced by proteolytic degradation in the cytosol by proteasome, transported to endoplasmic reticulum, and loaded onto MHC I molecules with the help of several other proteins. The upregulation of the ER chaperone calreticulin which is present in various forms, further support the MHC I mediated immune response. Calreticulin is unique in its ability to bind to peptides that are suitable to be loaded on MHC I molecules [43].

At least 6 of the up-regulated transcripts encoded complement components including C1q like genes, C3b, factor B, C7 and C9, indicating the involvement of the complement systems in response to infection. The teleost fish complement system exhibits conserved roles such as sensing and clearing the invading pathogens [44]. The expression of complement system components has been shown to be responsive to infection in other fish. Analysis of complement protein indicated the key involvement of the C7 gene in tissue specificity and pathogen responses [45]. The C7 responses in grass carp were sensitive and rapid in response to a pathogenic bacterial infection and indicates the involvement of C7 in innate immune responses [45]. Complement component C1q like gene is involved in the classical pathway [46].

Fibroblast growth factor (FGF) and FGF receptor (FGFR) gene families in the human and mouse comprise 22 and 4 members, respectively. In zebrafish, the FGF gene family comprises 27 members. The co-evolution of FGF and FGFR gene families enabled the FGF signaling system to acquire functional diversity. This has allowed the involvement of FGF signaling in many physiological and developmental processes. FGF knockout and mutation studies in mice and zebrafish respectively indicated the crucial role of FGFs in various developmental processes [47]. FGF-2 is involved in cytokine interaction networks for positive regulation of hematopoiesis and in the regulation of pathological and physiological hematopoiesis, granulopoiesis, and megakryocytopoiesis. Granulopoiesis is mediated by FGF-2 though secondary cytokine production, stimulation of granulocytic progenitor growth and differentiation. FGF-2 stimulates proliferation, enhances cytokine secretion and prevents apoptosis. It is also involved in proliferation and/or survival of hematopoietic progenitors [48]. FGF-2 is expressed in stromal cells, macrophages and leukemic cell lines and is involved in physiological and pathological hematopoiesis [48]. FGF4 is vital for the development of visceral organs and is transcriptionally regulated by lymphoid enhancer factor-1[49] belonging to subfamily of HMG proteins [50]. In our study FGF4 was down regulated in the immunized fish.

Myeloid/lymphoid mixed-lineage leukemia protein (MLL) which is a Drosophila trithorax (trx) G homolog, plays an important role in hematopoietic stem cell (HSC) development in embryos [51]. Embryonic stem cells deficient

in MLL failed to differentiate into any of HSC types in fetal liver or in adult animals [52]. Germline loss-of-function studies have demonstrated that MLL is essential for both development and maintenance of HSC [51, 52]. MLL is maternally supplied, expressed in the adults and is an important transcriptional regulator during the entire lifespan of zebrafish [53].

Alpha-melanin concentrating hormone (MCH) plays an important role in host defense. Alpha-MCH is an ancient anti-inflammatory peptide produced by phagocytes and keratinocytes. Increased expression of α -MCH in the blood indicates infectious and inflammatory disorders. Elevated levels of α-MCH in human plasma have antimicrobial functions [54]. Under inflammatory conditions, MCH receptor (MCHR1) expression was up-regulated on human colonic epithelial cells [49]. In our study fish hematopoietic tissue may have been inflamed due to the injection of *E. ictaluri,* resulting in up-regulated MCHR1 expression in kidney epithelial cells. In the present study MCH receptor 1 was up-regulated in immunized fish, suggesting that the innate immune system is providing enhanced protection for the immunized fish compared to the non-immunized fish.

4.2. Secondary Response

Transcriptome analysis comparing the E_1E_2 (secondary response) and SE_2 (primary response) treatment groups demonstrated 98 significantly differentially expressed transcripts that were uniquely associated with the secondary response, and protective immunity. In annotated genes, the highest fold expression differences (2 to 3 fold) were C-C like chemokine 19 (CCL-5a), myomegalin, bone morphogenetic protein 4, and relaxin 3a.

The gene for chemokine CCL-5a (CCL-C19) had the highest differential expression (3.1 fold) following the secondary response. This gene was the second highest differentially expressed gene in the primary response (4.3 fold), emphasizing its importance in the immune responses of *rag1*$^{-/-}$ mutant zebrafish. Inflammatory chemokine genes are expressed after an immune stimulus, and result in the re-location of leukocytes to the site of inflammation [21], but their functions are not well studied [26]. The CC chemokines have two cysteine residues bound directly to each other and are the largest sub-family of chemokines. One study stated zebrafish have 46 CC chemokine genes [55], and another reported 63 chemokine genes [29]. In our study, up-regulation of CCL-5a suggests significant cell trafficking in the secondary response. In rainbow trout, C5a was shown to enhance antibody response to a viral protein [56].

Myomegalin is also known as phosphodiesterase 4D-interacting protein. Four genes encode over 20 isoforms of this protein, and they are involved in intracellular signaling [57]. Intracellular signaling and cross-talk occurs between cells and between pathways, and between tissues. Pathway interactions operate in multiple directions. The cAMP phosphodiesterases are required for cell signaling and cross-talk [57]. Certain isoforms of myomegalin are necessary for centrosomal microtubule formation [58] and protein trafficking between Golgi and endoplasmic reticulum [59]. These findings suggest heightened cell signaling and pathway cross-talk.

Bone morphogenic proteins (BMP) are signaling cytokines belonging to the superfamily of TGF-βs and are involved in the regulation of cell proliferation, differentiation, apoptosis and morphogenesis [60-62]. Function and development of specific hematopoietic lineages are mediated by individual BMP's [63]. They are also involved in blood vessel formation [64].

In our study, bone morphogenetic protein 4 (BMP4) was one of the highest up-regulated genes in the secondary response. In mammals, it is involved in embryonic hematopoiesis [65]. BMP endothelial cell precursor derived regulator (BMPER), is an extracellular BMP modulator that plays an important role in BMP4 function in endothelial cells [66, 67]. Both BMP and BMPER are necessary for endothelial cells to deliver pro-BMP signals [66]. BMPER is also involved in endothelial cell migration [66] by modulating the expression of adhesion molecules [68]. Zebrafish BMP4 shares 68% identity and 80% similarity to that of human, mouse and frog BMP4 [69]. Its expression is associated with the developing pronephric mesoderm in normal zebrafish.

Relaxins (RLN) are a pleotropic hormone group with a wide range of biological and pathological activities in various tissues and organs in various physiological and pathological conditions [70]. Relaxins are hormones that regulate the migration of leukocyte to sites of inflammation, and increases substrate adhesion [71]. Teleost RLN3a and RLN3b paralogues display similarities in evolution and expression to the mammalian counterparts [72]. Relaxins regulate vasodilation and the movement of macrophages to the site of infection in response to cytokines. Relaxins are involved in wound healing, fibrosis, allergic responses [73] regulation of appetite and feeding in rats [74]. RLN3 acts as a neurotransmitter. Relaxins act on inactivation of contractile machinery leading to cell relaxation. It is also involved in vasodilation in several organs and tissues [70]. Dilation of the blood vessels is a result of the movement of tissue macrophage derived cytokines to the site of injection and/or bacterial presence, which in turn leads to the movement of leucocytes such as neutrophils and monocytes to the site of bacterial infection [75]. Up-regulated expression of RLN3 in immunized fish compared to non-immunized fish suggests enhanced leukocyte migration and adhesion during the secondary memory response.

Go functional analysis demonstrated the over represented transcripts included genes coding molecular processes such as actin binding, receptor binding, lipid binding, nucleic acid binding, proteinaceous extracellular matrix, extracellular space, cytoplasmic membrane bound vesicles, nucleolus, cytoskeleton and chromosome components, response to endogenous stimulus, cell-cell signaling and cell proliferation. The underrepresented categories were comprised of transcripts coding for protein binding, protein kinase activity, catalytic activity, organelles and cytoplasm

sub-categories, protein metabolic process, cellular component organization and cellular transport. AgriGO:GO enrichment analysis revealed pancreas specific transcription factor 1a (*ptf1a*), fibroblast growth factor 2, bone morphogenetic protein 4, fibroblast growth factor 4, BMP binding endothelial regulator, spondin 2b, extracellular matrix protein, high-mobility group protein (*hmgp*) isoforms I and Y, nuclear receptor subfamily 6, myosin-10-like, collagen triple helix repeat containing 1b, type I collagen, alpha 2 collagen, type XI alpha-2 collagen, 19 (chemokine (C-C motif)-like) and novel immune-type receptor 1(*nitr1*).

Different categories and GO terms that were over represented in the secondary response compared to the primary response are consistent with a cell mediated protection for vaccinated *rag1*$^{-/-}$ mutant zebrafish. Cell activation is evidenced by the over representation of cell communication, signal transduction and receptor binding categories. Activated cells were believed to be involved in secreting pro-inflammatory cytokine, effector cytokines and undergoing clonal proliferation, which was evidenced by up-regulated expression of *ifn*γ and C-C chemokine, and over representation of the cell proliferation category respectively in E_1E_2 (secondary) compared to SE_2 (primary). Activation of leukocytes is a cell differentiation process. Cell differentiation is suggested by the over representation of transport, structural morphogenesis, intracellular membrane bound organelles and cellular metabolic process categories. Functional analysis of differentially expressed transcripts between E_1E_2 and SE_2 associated with specific secondary immune responses corroborate potential heightened and more rapid responses of cells involved in the secondary response.

Over representation of cell communication, signal transduction and receptor binding categories demonstrates receptor activation and its communication with downstream signaling molecules. Upregulation of *ptf1a* suggests the occurrence of signal transduction because of receptor mediated cellular activation. The function of clonal proliferation is supported by the over representation of the category "cell proliferation" as well as the transcripts such as fibroblast growth fator-2 (*fgf-2*), *fgf-4*, bone morphogenetic protein– 4 (*bmp-4*), BMP binding endothelial regulator protein (*bmprp*), *hmgi/y*, and *ptf1a* which regulate proliferation. *Hmgi/y* proteins participate in a wide variety of cellular processes including transcriptional regulation and inducing changes in chromatin structure during cell proliferation [76]. Increased expression of *hmgi/y* occurs during rapid proliferation of certain cells from rat embryos and from undifferentiated cells of young rat thymi [77]. HMGI/Y binds specifically to A-T rich regions on the double stranded DNA [78], affecting chromatin conformation to regulate gene expression by facilitating the binding of transcription factors to dsDNA [79, 80]. In our experiment, *hmgi/y* expression may be associated with rapid expansion of the 'memory' cell population following secondary exposure. The rate of transcription of large proportions of immune response related genes such as *ifnβ*, *e-selectin*, *tnf-β*, *il-2* and granulocyte macrophage colony stimulating factor (*gm-csf*) and certain chemokines are correlated to the presence of *hmgi/y* protein [81]. This protein binds with transcription factors and affects its binding to DNA by introducing bends in the DNA [81]. In our study HMGI/Y up-regulation correlates with the over representation of binding, and certain GO terms such as sequence-specific DNA binding transcription factor activity.

To perform cytotoxic functions, cells undergo cytoskeletal remodeling. These functions are suggested by the over representation of the "structural morphogenesis" category and differential expression of the transcripts myosin 10, envoplakin, collagen triple helix repeat containing 1b, collagen type I, collagen type alpha 2, collagen type XI alpha-2 and resistance to inhibitors of cholinesterase 8 proteins (ric-8) that perform structural morphogenesis. Another up-regulated functional group is "proteinaceous extracellular matrix". Cytoskeletal rearrangement is further supported by up-regulated expression of *spondin 2*. *Spondin 2* (mindin) like lectin is an extracellular matrix (ECM) protein that plays essential roles in innate immunity [82]. *Spondin 2* recognizes intracellular pathogens [82]. It acts as a unique pattern recognition moiety [83] for macrophages by direct interaction with LPS components on pathogenic microbes [82] and interacts directly with receptors on neutrophils [84]. *E. ictaluri* is a facultative intracellular pathogen, and *spondin 2* may be playing an important role in recognizing *E. ictaluri* when they are localized in intracellular compartments. *Spondin 2* may also enhance macrophage phagocytosis of *E. ictaluri* when they are located in extracellular compartments. The extracellular space sub-category was up-regulated 5 fold. The genes included in extracellular space are bone morphogenic protein - 4 (*bmp-4*), collagen - 2 (*coll-2*), fibroblast growth factor - 4 (*fgf-4*), myosin heavy chain14 (*myhc14*), and *spondin2*.

Using the GOSlim Viewer resulted in three categories of GO annotations: cellular components, molecular functions and biological process. The over represented sub-categories from the cellular component category are (i) cell part, (ii) cell organelle, (iii) intracellular, (iv) plasma membrane, (v) cellular component in general and (vi) protein complex. In our study a sub-category of cell part, cytoplasmic membrane bound vesicles, was over represented. These genes are involved in transportation of macromolecules to their cellular destinations. Macromolecules are exchanged between endoplasmic reticulum, golgi apparatus, lysosomes and plasma membrane through vesicular transport [85]. In addition, sub-category "intracellular" is also overrepresented which may be due to efforts to eliminate the *E. ictaluri,* an intracellular pathogen. This idea is further substantiated by the over representation of the sub-category 'transporter activity' from 'molecular function' category and 'transport' from 'biological process' category.

FGF2 is involved in granulopoiesis in response to bacterial infection. Up-regulated expression of FGF2 and bone morphogenetic protein BMP4 suggests increased hematopoiesis.

Neuropilin (NP) 1 is a receptor expressed on endothelial

cells that selectively binds to vascular endothelial growth factor (VEGF) [86]. NP-1 supports the protective mechanisms of VEGF on glomerular endothelial cells, preventing damage and apoptosis. NP-1 expression in glomeruli is correlated with damage [87]. It was also reported that NP-1 is involved in the initiation of the primary immune response [88]. Expression of NP1 in the immunized fish was low compared to non-immunized fish, suggesting that the immune system protected kidney tubules from damage by the bacteria. MLL was down-regulated, while FGF2 and BMP4 were up-regulated in immunized fish compared to the non-immunized fish after bacterial challenge, suggesting dynamic regulation of hematopoiesis in the vaccinated fish.

RIC-8 is a unique non-receptor [89] guanine nucleotide exchange factor that enhances the exchange of GDP-GTP in the absence of receptor binding to the membrane [90] and is involved in PGDFR mediated actin cytoskeletal rearrangements [91]. Upregulation of RIC-8A in the immunized fish suggests involvement in cell differentiation.

The signals that are involved in the induction of immune responses often suppress other processes. The immune response in zebrafish had increased expression of cytokines and interferon induced genes and dynamic regulation of factors that control hematopoiesis. Other factors that are more vegetative in nature were significantly down-regulated, which include nuclear receptor subfamily 6, group A, member 1 (NR6A1), envoplakin, collagen triple helix containing-1, collagen I and collagen XI, myosin binding protein C, Myosin 10, A-kinase anchoring proteins, synaptotagmin, pancreatic transcription factor 1a, ceramide synthases proteins (TLC domain containing 1 and $Na^{(+)}$, $K^{(+)}$ ATPase), and genes involved in gonadal development (doublesex- and mab-3-related transcription factor 1). Further, cellular migration is supported by the differential expression of *spondin 2*, *bmp-4* and *fgf-2*.

Another large functional category with up-regulated transcripts is the "immune response" category. Some of these included up-regulated transcripts such as chemokine CCL-C5a, signal transducer and activator of transcription 1b (STAT1b), interferon regulatory factor 11, colony stimulating factor 1 receptor alpha, TNF receptor-associated factor, TNF ligand superfamily member 10, TNF receptor-associated factor 2a, coagulation factor V, lipopolysaccharide-induced TNF factor, interleukin enhancer binding factor 2 and nuclear factor kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.

Ifnγ expression was significantly greater in exposed than control fish. In both the primary and secondary responses, *ifnγ* expression was significantly greater at 48 hpi than 24 hpi. However, *ifnγ* expression was the same in the primary and the secondary responses.

5. Conclusion

Our findings suggest the primary immune response and innate immune cells are not impaired in T and B cell deficient mutant zebrafish. Acute phase proteins play the predominate role in the primary response, and cell trafficking proteins play a dominant role.

In the secondary response, cell trafficking proteins play the predominate role. Up-regulation of genes involved in cell signaling and cell cross-talk suggest receptor recognition and activation. Cell proliferation and cytotoxic functions were significantly up-regulated, suggesting expansion of cell populations. Up-regulation of genes involved in structural morphogenesis, intracellular transport and cellular metabolic processes suggest cell functions are occurring at a heightened level.

Significantly increased *ifnγ* expression is associated with primary and secondary protective responses in *rag1*$^{-/-}$ mutant zebrafish. This expression is significantly greater at 48 hpi than 24 hpi, but is the same in primary and secondary responses.

Appendix Supplementary Data

Table A1. *Log2 changes in expression of zebrafish transcripts that were up-regulated ($p < 0.05$) less than 2 fold differences following primary infection (SE_2) compared to non-infected (SS) controls.*

Functional classification	Accession number	Putative ID	Log2 difference
Acute phase response			
	NM_131338.1	complement factor B zgc:153240	1.941949535
	BQ284848	complement component 9	1.941177194
	BM778002	complement component 9	1.896008966
	BI878414	complement component c3b	1.891921911
	BI845861	CXC chemokine 46	1.585600317
	BI845737	C1q and tnf related protein 4	1.379582099
Immune Response			
	BG985448	calreticulin-like	2.059551719
	BC049424.1	interferon regulatory factor 7	1.863780175
	BG302583	calreticulin, like 2	1.83177963
	BM095893	interferon regulatory factor 9	1.81895559
	BI845861	CXC chemokine 46	1.585600317
	BG985449	calreticulin-like	1.54697111
	NM_131672.1	colony stimulating factor 1 receptor, a	1.482921894
	BM082447	TNF receptor-associated factor 7	1.451537876

Functional classification	Accession number	Putative ID	Log2 difference
Acute phase response			
	Z46776.1	MHC class I gene	1.453299417
	BM775009	tnf (ligand) superfamily, member 10 like 4	1.444936285
	BI983290	calreticulin, like 2	1.377430169
	CA474845	Tnf receptor-associated factor 2a	1.328678832
	AF515275.1	coagulation factor V	1.328445833
	AW232141	LPS-induced TNF factor	1.318596853
	AW232141	LPS-induced TNF factor	1.318596853
	NM_131047.1	calreticulin	1.262957707
	BM102177	like CC chemokine SCYA103	1.063116385
Response to Stimulus			
	AF510108.1	HSP 90, beta (*grp94*), member 1	1.839962141
	NM_153657.1	prostaglandin-endoperoxide synthase 2a	1.829305794
	NM_131157.1	crystallin, alpha B, a	1.779110338
	AW232570	glutathione peroxidase 1b	1.753479161
	BI474294	ras homolog gene family, memberGb	1.548966166
Protein degradation			
	AI878703	proteasome (prosome, macropain) 26S subunit, non-ATPase, 12	1.882078969
	NM_131678.1	proteasome (prosome, macropain) subunit, beta type, 9b	1.547871834
	AW420599	proteasome (prosome, macropain) subunit, alpha type, 2	1.537063194
	NM_131795.1	proteasome (prosome, macropain) subunit, alpha type, 6b	1.465710587
	BM776726	proteasome (prosome, macropain) subunit, alpha type,5	1.46080581
	NM_131375.1	proteasome activator subunit 1	1.456107519
	BC049010.1	proteasome (prosome, macropain) subunit, beta type, 3	1.361401934
	NM_153655.1	proteasome (prosome, macropain) subunit, alpha type, 6a	1.289222347
	BI534099	proteasome (prosome, macropain) subunit, beta type, 2	1.209112514
	BM037579	proteasome (prosome, macropain) subunit, beta type, 1	1.200739227
	AI477254	proteasome (prosome, macropain) 26S subunit, ATPase, 3	1.167589576
	AA658796	proteasome (prosome, macropain) subunit, alpha type, 8	1.157853387
	BI867867	proteasome (prosome, macropain) assembly chaperone 1	1.155346818
	BC044358.1	proteasome (prosome, macropain) 26S subunit, non-ATPase, 7	1.117427693
	BM859971	proteasome (prosome, macropain) subunit, beta type, 4	1.111095754
	BG305906	proteasome (prosome, macropain) 26S subunit, ATPase, 1b	1.110038362
	BC049471.1	proteasome (prosome, macropain) 26S subunit, ATPase, 1a	1.083532561
	AI943154	proteasome (prosome, macropain) 26S subunit, ATPase, 6	1.036215752
	BM102205	proteasome (prosome, macropain) 26S subunit, non-ATPase, 3	1.03255884
	BC045970.1	proteasome (prosome, macropain) subunit, alpha type, 4	1.024666414
	BI867479	proteasome (prosome, macropain) 26S subunit, ATPase, 4	1.022923298
	BC042325.1	proteasome (prosome, macropain) 26S subunit, non-ATPase, 12	1.015459136
Miscellaneous			
	CA472784	ubiquitin carboxyl-terminal hydrolase L5	1.22927282
	BI672243	translocase of inner mitochondrial membrane 8 homolog A (yeast)	1.228679678
	AW171078	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	1.247599938
	AI965054	NSFL1 (p97) cofactor (p47)	1.225614645
	AL925726	fatty acid binding protein 1b-like	1.243418738
	AL925726	fatty acid binding protein 1b-like	1.243418738
	BQ450267	IMP4, U3 small nucleolar ribonucleoprotein, homolog (yeast)	1.242436608
	BI865765	CDP-diacylglycerol-inositol 3-phosphatidyltransferase (phosphatidylinositol synthase)	1.242263428
	BM186551	protein O-fucosyltransferase 2	1.236996416
	BC053310.1	iroquois homeobox protein 4a	1.235529787
	CD605135	NHa-ras Harvey rat sarcoma viral oncogene homolog b	1.233934081
	BG305942	Novel protein like vertebrate cyclic nucleotide gated channel protein family	1.231240895
	AW171596	centrosomal protein 55 like	1.219244028
	AW777876	tyrosyl-tRNA synthetase	1.210944877
	BC049319.1	vaccinia related kinase 2	1.208703546
	BI881888	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	1.20805041
	CD283149	asparagine synthetase	1.20485812
	BI983167	calcineurin-like phosphoesterase domain containing 1	1.201518532
Unannotated			
	AW174559	wu:fj05f05	4.730217037
	AI496754	---	4.373735771
	AI496738	wu:fb64b08	3.866031518
	BQ616817	--	3.619344146
	AL725462	---	3.039173573

Functional classification	Accession number	Putative ID	Log2 difference
Acute phase response			
	BM186508	zgc:152945	2.830896542
	BI672165	---	2.697690393
	BI878415	---	2.601745091
	AI617721	---	2.530152336
	BI864822	zgc:158271	2.516612853
	BQ075086	si:rp71-1c23.2	2.482899493
	CD605001	---	2.466878713
	BI865858	---	2.438501226
	BM777312	si:ch211-20b12.2	2.430396283
	BI864002	zgc:92903	2.377660001
	BI878750	si:dkey-53p21.1	2.185374681
	AI974163	si:ch1073-126c3.2	2.185000536
	AI331661	wu:fa99f01	2.180037946
	AI584672	wu:fb82a05	2.174633072
	AI397316	wu:fb09h07	2.167623349
	AI384591	wu:fb10g08	2.122730272
	AI477673	zgc:103710	2.057605963
	BM277076	si:dkey-27i16.2	2.050685063
	CD015330	zgc:152809	2.046307942
	AW232318	wu:fj17f10	2.040389333
	BM777295	Zgc:172136	2.020805737

Table A2. *Log2 changes in expression of zebrafish transcripts that were differentially expressed ($p < 0.05$) between the secondary (E_1E_2) and primary (SE_2) exposures less than 2.24 fold differences.*

Functional classification	Accession number	Putative ID	Log2 difference
Immune Response			
	NM_131385.1	recombination activating gene 2	1.5715873
	BQ450131	Myeloid/lymphoid or mixed-lineage leukemia 3a	-1.325213
Cell proliferation			
	AY269790.1	fibroblast growth factor 2	1.2784336
	BG985627	BMP binding endothelial regulator	-1.0152304
	NM_131635.1	fibroblast growth factor 4	-1.9751336
Receptor Binding			
	AF318394.1	novel immune-type receptor 1k*	1.533402
Signal Transduction			
	AY245546.1	pancreas specific transcription factor, 1a	1.4248564
Intracellular			
	NM_131008.1	spondin 2b, extracellular matrix protein	1.2243558
Cellular Metabolic process			
	AL715408	High-mobility group protein isoforms I and Y	1.0482427
	NM_131256.1	nuclear receptor subfamily 6, group A, member 1a	-1.9057999
Structural Morphogenesis			
	AI331605	collagen, type I, alpha 2	-1.0141198
	AL672176	collagen type XI alpha-2	-1.0306758
	AL922076	collagen triple helix repeat containing 1b	-2.1751793
	AL723844	myosin-10-like	-2.8897076
Miscellaneous			
	BC051151.1	like mucin	1.491553
	BG305271	resistance to inhibitors of cholinesterase 8 homolog A	1.4264007
	NM_131669.1	ATPase, Na+/K+ transporting, beta 2a polypeptide	1.2700753
	AY161857.1	melanin-concentrating hormone receptor 1a	1.2491393
	AB097825.1	trophoblast glycoprotein-like	1.2355278
	BG884560	Zinc finger protein 347-like	1.2162185
	AL724232	LSM14 homolog A (SCD6, S. cerevisiae)	1.0996213
	BQ132362	like MGC107856 protein	1.0679448
	BM777899	like MGC107856 protein	-1.0957169
	BI842004	synaptotagmin IV	-1.1001618
	AJ286843	hypothetical protein LOC100331174	-1.1235053
	AI397227	envoplakin	-1.1430245
	BQ078258	like CG14142-PA	-1.1469466
	AF495875.1	estrogen-related receptor gamma a	-1.3828564
	BI845673	protein kinase (cAMP-dependent) inhibitor beta	-1.5030164
	NM_181497.2	Neuropilin 1a-like /// neuropilin 1a	-1.578668
	NM_131287.1	SRY-box containing gene 17	-1.584243
	AI331287	TLC domain containing 1	-1.6717343

Functional classification	Accession number	Putative ID	Log2 difference
Immune Response			
	BI983629	mCG142610-like	-1.8298359
	BG303134	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	-2.0876298
	BQ074821	doublesex and mab-3 related transcription factor	-2.3731684
Unannotated	CD606487	SET domain, bifurcated 2	-2.8538423
	AI793605	wu:fc49d07	2.6504161
	BI710320	---	2.3408558
	BM187461	zgc:92035	2.2996033
	AW281782	---	2.2427615
	BG883314	---	2.2250069
	AW018957	---	2.2219393
	AW059176	---	2.1737237
	BI883324	---	2.1613121
	BQ419619	---	2.0832168
	BI867354	si:ch211-147m20.1	1.9192022
	AA497170	---	1.8085751
	BG303757	si:dkey-4c15.6	1.5619177
	BI709723	zgc:165508	1.4714972
	CD606304	---	1.4665004
	BI891762	zgc:158366	1.4638643
	BQ092536	---	1.4309018
	BM005167	---	1.3862775
	AI544667	wu:fb77d09	1.3832316
	AL731009	---	1.3729446
	BM186516	---	1.3577334
	BI864110	---	1.3536182
	AL719663	Wu:fc11a05	1.2914202
	AL913138	---	1.2142156
	BI877608	zgc:152863	1.1424723
	AW233702	wu:fj40e09	1.1144797
	AFFX-Dr-pAsRed2	---	1.0867881
	BG728511	---	1.0407045
	CD283215	wu:fb81c07	-1.0332313
	BI318519	---	-1.0924965
	BI845653	---	-1.1600338
	BI981210	---	-1.1832179
	AW279902	si:ch73-46j18.5	-1.3152468
	BI847022	---	-1.5061064
	BM186526	---	-1.5448244
	BM571195	si:ch211-266a5.1	-1.6606248
	AI959658	wu:fd12e04	-1.6830505
	BM005010	---	-1.7118405
	BE605275	wu:fb15e04	-1.7141458
	BG305300	---	-1.8103463
	AL719266	zgc:110283	-1.8852786
	AI444465	wu:fb39e08	-1.9695472
	BE201957	zgc:194138	-1.9698771
	BI673395	---	-1.9842097
	BI982878	---	-1.9922919
	BI671488	---	-2.0579275
	AW280155	wu:fj51e11	-2.1208847
	AL927596	---	-2.2341451
	AI721504	wu:fc44h05	-2.3347106
	AL722000	---	-2.4008245
	AI794137	hypothetical protein LOC100332904	-2.4113868
	BM154625	wu:fb12c09	-2.439278
	AI878410	wu:fc57f08	-2.4814408
	BM025943	Si:ch211-261c8.5	-2.5580451
	BI979237	---	-2.5911717
	AI667492	---	-2.6889276
	AL724042	---	-2.8792889
	BI882036	zgc:64003	-3.3085005

*Mammalian ortholog

Table A3. *Comparison of ifnγ gene expression between treatments at 24 hpi and 48hpi. hpi= hours post injection. *Significance ($p<0.05$) and ns=no significance.*

Gene	Treatment	Time	Adjusted P Value	Significance
ifnγ	SS vs. SE_2	24 hpi	0.100	ns
ifnγ	SS vs. E_1E_2	24 hpi	0.0045	**
ifnγ	SE_2 vs. E_1E_2	24 hpi	0.5988	ns
ifnγ	SS vs. SE_2	48 hpi	<0.0001	****
ifnγ	SS vs. E_1E_2	48 hpi	<0.0001	****
ifnγ	SE_2 vs. E_1E_2	48 hpi	0.4722	ns
ifnγ	SS vs SS	24 hpi vs 48 hpi	0.4206	ns
ifnγ	SE_2 vs SE_2	24 hpi vs 48 hpi	0.0079	**
ifnγ	E_1E_2 vs E_1E_2	24 hpi vs 48 hpi	0.0317	*

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