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Characterization of immune response against *Mycobacterium marinum* infection in the main hematopoietic organ of adult zebrafish (*Danio rerio*)

Sanna-Kaisa E. Harjula[a], Anni K. Saralahti[a], Markus J.T. Ojanen[a,b], Tommi Rantapero[c], Meri I.E. Uusi-Mäkelä[a], Matti Nykter[c], Olli Lohi[d], Mataleena Parikka[e,f], Mika Rämet[a,g,h,i,*]

[a] Laboratory of Experimental Immunology, BioMediTech, Faculty of Medicine and Health Technology, FI-33014, Tampere University, Finland
[b] Laboratory of Immunoregulation, BioMediTech, Faculty of Medicine and Health Technology, FI-33014, Tampere University, Finland
[c] Laboratory of Computational Biology, BioMediTech, Faculty of Medicine and Health Technology, FI-33014, Tampere University, Finland
[d] Tampere Center for Child Health Research, Tampere University and Tays Cancer Center, Tampere University Hospital, FI-33014, Tampere University, Finland
[e] Laboratory of Infection Biology, BioMediTech, Faculty of Medicine and Health Technology, FI-33014, Tampere University, Finland
[f] Oral and Maxillofacial Unit, Tampere University Hospital, P.O. Box 2000, FI-33521, Tampere, Finland
[g] Department of Pediatrics, Tampere University Hospital, P.O. Box 2000, FI-33521, Tampere, Finland
[h] PEDEGO Research Unit, Medical Research Center Oulu, P.O. Box 8000, FI-90014, University of Oulu, Finland
[i] Department of Children and Adolescents, Oulu University Hospital, P.O. Box 10, FI-90029, OYS, Finland

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ABSTRACT

Tuberculosis remains a major global health challenge. To gain information about genes important for defense against tuberculosis, we used a well-established tuberculosis model; *Mycobacterium marinum* infection in adult zebrafish. To characterize the immunological response to mycobacterial infection at 14 days post infection, we performed a whole-genome level transcriptome analysis using cells from kidney, the main hematopoietic organ of adult zebrafish. Among the upregulated genes, those associated with immune signaling and regulation formed the largest category, whereas the largest group of downregulated genes had a metabolic role. We also performed a forward genetic screen in adult zebrafish and identified a fish line with severely impaired survival during chronic mycobacterial infection. Based on transcriptome analysis, these fish have decreased expression of several immunological genes. Taken together, these results give new information about the genes involved in the defense against mycobacterial infection in zebrafish.

1. Introduction

In 2017, approximately 10 million people worldwide developed tuberculosis, a pulmonary or disseminated infection caused by *Mycobacterium tuberculosis* (World Health Organization, 2018). Although *M. tuberculosis* infection can be cleared by innate immunity (Verrall et al., 2014), it more typically leads to a latent phase. According to current estimations, 23% of the world's population are asymptomatic carriers of the bacteria (World Health Organization, 2018). The currently available Bacillus Calmette-Guérin (BCG) tuberculosis vaccine protects infants from disseminated tuberculosis but is less effective against pulmonary disease in adults. This vaccine is also unable to prevent the reactivation of latent disease (Mangtani et al., 2014; Tang et al., 2016). Thus, BCG vaccination cannot prevent the spread of tuberculosis.

Based on genome-wide association studies (GWAS) and candidate gene studies in various human populations, as well as on animal studies, host genetics contributes to the susceptibility of developing active tuberculosis (Berg et al., 2016; Cooper et al., 1993, 1997; Filipe-Santos et al., 2006; Harjula et al., 2018a; Sullivan et al., 2005; Tobin et al., 2010; Yim and Selvaraj, 2010). Human polymorphisms in genes encoding human leukocyte antigens (HLA) (Dallmann-Sauer et al., 2018; Yim and Selvaraj, 2010), Interferon (IFN) gamma, Interleukin10 and various chemokines and their receptors (Yim and Selvaraj, 2010), to mention but a few, have been associated with tuberculosis susceptibility. For some of these genes, there is also experimental evidence

Abbreviations: BCG, Bacille Calmette-Guérin; CFU, colony forming unit; dpf, days post fertilization; dpi, days post infection; GWAS, genome-wide association study; hpi, hours post infection; RIN, RNA integrity number; TL, *Tüpfel long fin*; wpi, weeks post infection; WT, wild type

* Corresponding author. Faculty of Medicine and Health Technology, FI-33014, Tampere University, Finland.
E-mail addresses: sanna.harjula@tuni.fi (S.-K.E. Harjula), anni.saralahti@tuni.fi (A.K. Saralahti), markus.ojanen@tuni.fi (M.J.T. Ojanen), tommi.rantapero@tuni.fi (T. Rantapero), meri.uusi-makela@tuni.fi (M.I.E. Uusi-Mäkelä), matti.nykter@tuni.fi (M. Nykter), olli.lohi@tuni.fi (O. Lohi), mataleena.parikka@tuni.fi (M. Parikka), mika.ramet@tuni.fi (M. Rämet).

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from animal models on their importance in disease resistance (Beamer et al., 2008; Cooper et al., 1993; Cyktor et al., 2013; Flynn et al., 1993; Harjula et al., 2018a; Higgins et al., 2009; Peters et al., 2001; Torraca et al., 2015).

During the last couple of decades, *Mycobacterium marinum* infection in both zebrafish embryos/larvae and adult zebrafish has become a widely used model of tuberculosis (Myllymäki et al., 2015, 2016). *M. marinum* is a natural zebrafish pathogen and, similarly to its close relative *M. tuberculosis* (Stinear et al., 2008), it infects macrophages (Barker et al., 1997; El-Etr et al., 2001) and can cause a latent or slowly progressive infection, which can be reactivated by immunosuppression (Harjula et al., 2018a; Myllymäki et al., 2018; Parikka et al., 2012). Also comparable to tuberculosis, *M. marinum* infection both in zebrafish larvae and adult zebrafish results in granuloma formation (Davis et al., 2002; Myllymäki et al., 2018; Parikka et al., 2012; Swaim et al., 2006). In fact, the notion that granuloma not only benefits the host but also gives the bacteria a good environment in which to survive and spread the disease, originates from zebrafish studies (Davis and Ramakrishnan, 2009; Volkman et al., 2004).

The host response to the *M. marinum* infection in zebrafish has been studied in several transcriptome analyses (Benard et al., 2016; Hegedüs et al., 2009; Kenyon et al., 2017; Meijer et al., 2005; Ojanen et al., 2019; Rotman et al., 2011; Rougeot et al., 2014, 2019; van der Sar et al., 2009; van der Vaart et al., 2012; Veneman et al., 2015). These studies show that the gene expression profile of *M. marinum* infected zebrafish changes as the infection progresses. Consequently, in zebrafish larvae infected at 1 day post fertilization (dpf), three stages of the transcriptional response have been recognized: early, mid and late phase (Benard et al., 2016). Characteristic of the early phase is a transcriptional response, including upregulation of genes involved in response to bacterium, proteolysis and cellular macromolecular complex assembly and a downregulation of protein folding-associated genes at 2 h post infection (hpi), concurrent with phagocytosis of the bacteria (Benard et al., 2014). During mid-phase, starting at 6 hpi, the number of the differentially regulated genes is low. This is followed by an increase in differentially expressed genes during late phase (4 and 5 days post infection, dpi) (Benard et al., 2016). According to the aforementioned studies, the exact response not only depends on the stage of the infection but also on the virulence of the *M. marinum* strain (Rotman et al., 2011; van der Sar et al., 2009).

In general for adult zebrafish, genes encoding for zinc-finger proteins, immune-related transcription factors and proteins related to apoptosis, among others, are upregulated at the early stages of the *M. marinum* infection (van der Sar et al., 2009). At later stages, genes encoding for immune-related transcription factors, cytokines, chemokine receptors, complement components, matrix metalloproteases and lysosomal proton transporters, among others, are induced independently of the virulence of the bacteria in the adult zebrafish (Meijer et al., 2005; van der Sar et al., 2009).

In the present study, we performed a genome-wide transcriptome analysis using cells from kidney, the main hematopoietic organ in zebrafish, at 14 days post *M. marinum* infection. In this manner, we focused the analysis predominantly on immune cells. Furthermore, the immune response of wild type (WT) zebrafish was compared to the immune response of a susceptible mutant fish line, identified from an ongoing forward genetic screen.

2. Materials and methods

2.1. Zebrafish lines and maintenance

3 to 15 month-old WT AB and TL (*Tüpfel long fin*, *gja5b*$^{t1/t1}$, *lof* $^{dt2/dt2}$) zebrafish (*Danio rerio*) lines from the Tampere Zebrafish Core Facility were used for the experiments. The *rag*$^{hu1999/hu1999}$ mutant fish obtained from the Zebrafish International Resource Center (ZIRC, University of Oregon, Eugene, Oregon, USA) at the age of 6–8 months were used as a positive control in the infection experiments.

The zebrafish were maintained according to standard protocols (Nüsslein-Volhard and Dahm, 2002). Briefly, the unchallenged zebrafish were kept in a standard flow-through system (Aquatic Habitats, Apopka, Florida, USA) with a light/dark cycle of 14/10 h and fed once a day with SDS 400 (Special Diet Services, Witham, Essex, UK) and once a day with in-house cultured *Artemia nauplia*. Alternatively, fish were fed once a day with GEMMA Micro 500 (Skretting, Stavanger, Norway). The *M. marinum* infected fish were kept in a standard flow-through unit (Aqua Schwarz GmbH, Göttingen, Germany) with the same light/dark cycle as the unchallenged fish and fed twice a day with SDS 400 or once a day with GEMMA Micro 500. Zebrafish embryos/larvae were maintained in embryonic medium (5 mM NaCL, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 10% methylene blue at 28.5 °C, fed with SDS100 (Special Diet Services) or GEMMA Micro 75 (Skretting) starting at 5 dpf and transferred to the flow-through system at 6 dpf.

The well-being of the fish was monitored daily and the humane endpoint criteria determined in the animal experiment permits were used. The Animal Experiment Board has approved the housing and care of the zebrafish and all the conducted experiments (permits ESAVI/4234/04.10.03/2012, ESAVI/6403/04.10.03/2012, ESAVI/10079/04.10.06/2015, ESAVI/2776/2019, ESAVI/10539/2019, LSLH-2007-7254/Ym-23 and ESAVI/10366/04.10.07/2016). Furthermore, the Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013) as well as the EU Directive on the Protection of Animals Used for Scientific Purposes (2010/63/EU) were applied during this study.

2.2. The gene-breaking Tol2 transposon based mutagenesis in zebrafish embryos

The gene-breaking transposon pGBT-RP2-1 (RP2) vector (Addgene plasmid # 31828; http://n2t.net/addgene:31828; RRID:Addgene_31828) and pT3TS-Tol2 vector (Addgene plasmid # 31831; http://n2t.net/addgene:31831; RRID:Addgene_31831) for the production of *tol2* mRNA were received as a generous gift from Professor Stephen C. Ekker's laboratory (Mayo Clinic, Rochester, Minnesota, USA) (Balciunas et al., 2006; Clark et al., 2011). Both plasmids were transformed into *E. coli* One Shot TOP10 cells (Invitrogen™, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Plasmid DNA was extracted with QIAGEN Plasmid Plus Maxi Kit (Qiagen, Hilden, Germany) and sequenced to confirm successful transformation. In order to produce *tol2* mRNA, pT3TS-Tol2 plasmid was first linearized by digestion with FastDigest BamHI (Thermo Scientific, Thermo Fisher Scientific). Then, the linearized plasmid was used as a template for *in vitro* transcription performed with mMESSAGE mMACHINE Transcription Kit, (Invitrogen™, Thermo Fisher Scientific) according to the manufacturer's instructions. The zebrafish mutagenesis was conducted as previously described (Clark et al., 2011). Briefly, 12.5 pg of RP2 plasmid and *tol2* mRNA in 1x PBS with 0.6% phenol red (Sigma-Aldrich, Saint Louis, Missouri, USA) were injected into the cell of 1-cell stage WT AB zebrafish embryos with a borosilicate capillary needle (Sutter Instrument Co., Novato, California, USA) using a PV830 Pneumatic PicoPump (World Precision Instruments, Sarasota, Florida, USA) and a micromanipulator (Narishige International, London, UK). If the mutagenesis was successful, RP2 was randomly inserted into the zebrafish genome causing one or several mutations per fish (Clark et al., 2011). Mutation carrying embryos (the founder fish in the genetic screen, designated as F0) were selected based on the expression of *Green fluorescent protein* (*GFP*) under a Lumar V.12 fluorescence stereomicroscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) or Nikon AZ100 Fluorescence Macroscope (Nikon, Minato, Tokyo, Japan) (Clark et al., 2011).

2.3. Generation of the mutant zebrafish lines

In order to produce zebrafish lines carrying unknown mutations, F0 founder fish were first crossed to TL. The fish in the resulting F1 generation carried various sets of mutations. These fish were named with a running number (also the final name of the subsequent resultant mutant zebrafish line) and crossed to TL. This resulted in an F2 generation consisting of fish heterozygous for the mutations. The F2 generation was then incrossed to produce an F3 generation with a theoretical Mendelian ratio of WT, heterozygous and homozygous fish for each mutation. Further fish generations were produced by incrossing the previous generation (until F5). In some cases, in order to maintain the line, the fish were again crossed to TL. For the *M. marinum* infection experiments on embryos, the F7 generation was utilized. Mutation carrying progeny in each generation were selected for based on *GFP* expression.

2.4. Experimental M. marinum infections

The culture of *M. marinum* strain ATCC 927 and the inoculation into adult zebrafish were done as described previously (Harjula et al., 2018a; Parikka et al., 2012). For these infections, the zebrafish were anesthetized with 0.02% 3-amino benzoic acid ethyl ester (Sigma-Aldrich). Following this, 5 μl of *M. marinum* suspended in 1x PBS with 0.3 mg/ml phenol red (Sigma-Aldrich) was injected into the abdominal cavity of the fish using a 30 gauge Omnican 100 insulin needle (Braun, Melsungen, Germany). The survival of the fish was followed for 12–14 weeks. During the screen, 2–10 mutant lines were included in one experiment together with the WT line and occasionally $rag^{hu1999/hu1999}$ mutants. For the lines showing increased or decreased susceptibility, the survival assay was performed at least three times in total.

For the zebrafish embryo infections, *M. marinum* was suspended in 1x PBS with 0.3 mg/ml phenol red (Sigma-Aldrich). At 1 dpf, zebrafish embryos were anesthetized with 0.02% 3-amino benzoic acid ethyl ester (Sigma-Aldrich) and 2 nl of bacterial solution was injected into the caudal vein with a borosilicate capillary needle using a PV830 Pneumatic PicoPump (World Precision Instruments) and a micromanipulator (Narishige International). The survival of the embryos was monitored once a day for seven days. The infection dose (colony forming units, CFU) in both adult and embryo survival assays was verified by plating injected bacterial suspension on 7H10 agar (Becton Dickinson and Company, Franklin Lakes, New Jersey, USA) plates.

2.5. Experimental Streptococcus pneumoniae infections

The culture of *Streptococcus pneumoniae* WT strain TIGR4 (T4), of serotype 4 and sequence type 205, and the inoculation into zebrafish embryos were done as described previously (Aaberge et al., 1995; Rounioja et al., 2012). Briefly, 5% lamb blood agar plates (Tammer-Tutkan maljat Oy, Tampere, Finland) were used to grow T4 in 37 °C and 5% CO_2 overnight. From the plate, T4 was suspended in 5 ml of Todd Hewitt broth (Becton, Dickinson and Company) and grown from OD_{620} 0.1 to OD_{620} of 0.4. After this, bacteria were suspended in 0.2 M KCl with 1% of 70 kDa Rhodamine Dextran (Invitrogen™, Thermo Fisher Scientific). At 2 dpf, zebrafish embryos were anesthetized with 0.02% 3-amino benzoic acid ethyl ester (Sigma-Aldrich) and 2 nl of the bacterial solution was microinjected into the blood circulation valley with a borosilicate capillary needle (Sutter Instrument Co.) using a PV830 Pneumatic PicoPump (World Precision Instruments) and a micromanipulator (Narishige International). The survival was followed once a day for five days. The infection dose (CFU) was verified by plating injected bacterial suspension on 5% lamb blood agar plates (Tammer-Tutkan maljat Oy).

2.6. RNA extraction and quality control

For the transcriptome analysis, the RNA was extracted from zebrafish kidney with the Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The removal of genomic DNA from the samples was done with the RapidOut DNA Removal Kit (Thermo Scientific, Thermo Fisher Scientific). The purity of the samples was checked with a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Thermo Fisher Scientific) and the concentration was measured with a Qubit™ RNA BR Assay Kit (Invitrogen™, Thermo Fisher Scientific). The integrity of RNA was checked with a Fragment Analyzer (Advanced Analytical Technologies, Iowa, USA) using the Standard Sensitivity RNA Analysis Kit (Advanced Analytical Technologies) and the PROSize® 2.0 Data Analysis Software (Advanced Analytical Technologies). The samples with an RNA integrity number (RIN) ≥ 7.8 were chosen for the RNA sequencing.

2.7. Whole genome transcription analysis by RNA sequencing

The preparation of the cDNA library and the RNA sequencing were performed at Novogene, Hong Kong. The cDNA library of 250–300 bp was prepared with the 150 bp paired-end sequencing on the Illumina platform yielding > 20 million reads/sample.

2.8. RNA sequencing data analysis

The quality of the reads was inspected using FastQC (Andrews, 2010). The reads were aligned against the GRCz10 reference genome with STAR using default parameters (Dobin et al., 2013). The expressions of genes were quantified with FeatureCounts (Liao et al., 2014) using Ensembl GRCz10.91 as the reference gene set (Hubbard et al., 2002). The normalization of the raw expressions and the differential gene expression analysis were both conducted using R-package DESeq2 (Love et al., 2014).

To analyze the transcriptome data further, we included genes which were expressed ≥ |3|-fold between the groups and also had medians of ≥ |3|-fold. Gene ontology enrichment analysis was performed with The Gene Ontology Enrichment Analysis (GO Ontology Database released 1st of January 2019 and Panther Overrepresentation Test released 13th of November 2018) (Ashburner et al., 2000; Mi et al., 2017; The Gene Ontology Consortium, 2019) using the unranked list of upregulated or downregulated genes as the target list and the list of all the protein coding genes present in the RNA sequencing data as a background list. Classification of upregulated genes was based on the data available from Ensembl genome browser (Zerbino et al., 2018) versions 91 (version used for the data analysis) and 95, Reactome (Fabregat et al., 2018), The Zebrafish Information Network ZFIN (Howe et al., 2013), InterPro (Mitchell et al., 2019), The PROSITE database (Sigrist et al., 2013), Pfam 32.0 (El-Gebali et al., 2019), the NCBI Gene database (Gene, 2004), the NCBI BioSystems database (Geer et al., 2010) and the literature. Downregulated genes were classified according to the gene ontology data available in Ensembl, ZFIN and NCBI gene databases. If the gene did not have an official name/description, the preferred name of the gene from the NCBI database was used if available.

2.9. qPCR

Reverse transcription of the RNA samples was done using the SensiFAST™ cDNA synthesis kit (BioLine, London, UK). cDNA was then used for determining the relative gene expression levels of the target genes with quantitative PCR (qPCR) using PowerUp™ SYBR® master mix (Applied Biosystems™, Thermo Fisher Scientific). Supplementary Table 1 gives the sequences of the qPCR primers used and the Ensembl gene identification codes for the analyzed genes. $2^{-\Delta Ct}$ method was used for calculating the expression levels of target genes relative to the expression of *eef1a1l1* (Tang et al., 2007). *M. marinum* burden (CFU)

was quantified from the total DNA of the infected fish with qPCR using SensiFAST™ SYBR® No-ROX (Bioline) as previously described (Parikka et al., 2012), considering 100 CFU as the detection limit. DNA for the analysis was extracted from the abdominal organ blocks (excluding kidney) with TRI Reagent® (Molecular Research Center, Cincinnati, Ohio, USA) according to manufacturer's protocol. A CFX96 qPCR machine (Bio-Rad, California, USA) was used to perform qPCR and the Bio-Rad CFX Manager software v3.1 (Bio-Rad) to analyze the data. Random RNA samples with no reverse transcription and non-template controls were used in qPCR to control for genomic DNA and other contamination. A melt curve analysis and 1.5% TAE agarose gel electrophoresis was used to validate the specificity of the qPCR products. PCR products below the detection limit or the ones with incorrect melt curves were given a Ct value of 40 when the gene expression analysis was performed.

2.10. Statistical analysis and power calculations

Sample size calculations were conducted as described earlier (Harjula et al., 2018a). As for the *M. marinum* quantification, based on the high mortality of mutant463 zebrafish and our previous results (Myllymäki et al., 2018; Parikka et al., 2012), the difference between the groups was this time estimated to be 0.75 unit on the log10 scale resulting in the group size minimum of 7.

In the gene ontology enrichment analyses *P* values were calculated with Fisher's exact test and Bonferroni correction was used for multiple testing. Statistical analyses for other results were conducted with Prism, version 5.02 (GraphPad Software, Inc, California, USA). For the survival analyses a log-rank (Mantel-Cox) test was used. To test whether the bacterial count is increased in the mutant463 line compared to WT and whether the differences in gene expression detected by RNA sequencing are repeated when analyzed with qPCR, a nonparametric one-tailed Mann-Whitney test was used. *P* values of < 0.05 were considered significant.

2.11. Data management

The RNA-sequencing data discussed in this publication (Harjula et al., 2018b, 2018c) have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession numbers GSE118288 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118288) for the WT data and GSE118350 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118350) for the mutant463 data.

3. Results

3.1. A low-dose infection with M. marinum leads to induction of immune response and changes in expression of genes regulating metabolism in kidney derived cells in adult zebrafish

We have previously shown that a low-dose *M. marinum* infection into the abdominal cavity of adult zebrafish leads to a latent or slowly progressive infection, which can be used to model different phases of tuberculosis (Harjula et al., 2018a; Myllymäki et al., 2018; Parikka et al., 2012). In order to characterize the immune response to low-dose *M. marinum* infection in WT zebrafish, we infected 5–6 month-old zebrafish with 5–9 CFU of *M. marinum* and performed a whole-genome level transcriptome analysis from their kidneys. Since the kidney is the main hematopoietic organ in adult zebrafish (Davidson and Zon, 2004), it was selected to characterize the immunological response to the infection, particularly in immune cells. At 7, 14 and 28 days post infection (dpi), we collected kidneys for RNA extraction. From the fish collected at 14 dpi, we selected four males for each group for RNA sequencing. As a negative control, we used four 7 month-old unchallenged WT male fish. The yield from RNA sequencing was 23–28 million reads per sample and 90% of the reads were successfully mapped to the transcript database Ensembl GRCz10.91.

Table 1
Enriched processes from gene ontology analysis of the upregulated protein coding genes. The Gene Ontology Consortium Enrichment analysis was performed using upregulated genes as a target list and all the protein coding genes in the RNA sequencing data as a background list. *P* values were calculated with Fisher's exact test and Bonferroni correction was used for multiple testing. $P < 0.05$ was considered significant.

GO term	Description	*P* value
GO:0006952	defense response	1.05E-06
GO:0006955	immune response	1.48E-06
GO:1990266	neutrophil migration	1.53E-06
GO:0097530	granulocyte migration	2.57E-06
GO:0097529	myeloid leukocyte migration	4.58E-06
GO:0070098	chemokine-mediated signaling pathway	7.44E-06
GO:0050900	leukocyte migration	7.85E-06
GO:1990869	cellular response to chemokine	9.50E-06
GO:1990868	response to chemokine	9.50E-06
GO:0030593	neutrophil chemotaxis	2.86E-05
GO:0006954	inflammatory response	4.02E-05
GO:0071621	granulocyte chemotaxis	4.67E-05
GO:0060326	cell chemotaxis	8.22E-05
GO:0030595	leukocyte chemotaxis	8.75E-05
GO:0002376	immune system process	1.44E-04
GO:0009617	response to bacterium	1.72E-04
GO:0006959	humoral immune response	4.57E-04
GO:0019221	cytokine-mediated signaling pathway	1.15E-03
GO:0071345	cellular response to cytokine stimulus	1.37E-03
GO:0043207	response to external biotic stimulus	2.06E-03
GO:0051707	response to other organism	2.06E-03
GO:0009607	response to biotic stimulus	2.13E-03
GO:0061844	antimicrobial humoral immune response mediated by antimicrobial peptide	2.42E-03
GO:0034097	response to cytokine	5.04E-03
GO:0006950	response to stress	6.42E-03
GO:0019730	antimicrobial humoral response	6.53E-03
GO:0002440	production of molecular mediator of immune response	1.07E-02
GO:0002377	immunoglobulin production	1.07E-02
GO:0051704	multi-organism process	1.10E-02
GO:0006935	chemotaxis	1.54E-02
GO:0042330	taxis	2.26E-02
GO:0009605	response to external stimulus	2.33E-02
GO:0071222	cellular response to lipopolysaccharide	3.91E-02
GO:0071219	cellular response to molecule of bacterial origin	3.91E-02
GO:0071216	cellular response to biotic stimulus	4.36E-02

In transcriptome analysis, we found 201 protein coding genes to be differentially expressed at 14 days post a low-dose *M. marinum* infection compared to the unchallenged WT fish (Tables 1–3, Supplementary Tables 2–3). More specifically, 96 of the differentially expressed protein coding genes were upregulated and 105 downregulated. In addition, we identified 21 upregulated and 30 downregulated noncoding RNAs (Supplementary Tables 4 and 5). We performed Gene Ontology Consortium enrichment analysis for both the upregulated and downregulated protein coding genes. According to this analysis, all the significantly enriched biological processes ($P < 0.05$), among the analyzed 86 upregulated genes recognized by the tool, were related to immune response (Table 1). This emphasizes the feasibility of using kidney tissue to characterize the immune response to infection. Among the most enriched processes were inflammatory response, response to bacterium and chemotaxis (Table 1). Next, we divided all the upregulated genes into subgroups based on the data available the databases listed in Chapter 2.8., as well as on the existing literature (Fig. 1A, Tables 2 and 3). The categories included immune response (43 genes), lipid binding and metabolism (9 genes), other metabolic role (13 genes), other role (27 genes) and unknown role (4 genes) (Fig. 1A, Tables 2 and 3).

Downregulated protein coding genes are shown in Fig. 1B and Supplementary Tables 2–3. Among the analyzed 92 downregulated genes, many of the significantly ($P < 0.05$) enriched processes were

Table 2
Immune response -related protein coding genes induced in mycobacterial infection in adult zebrafish kidney at 14 dpi (days post infection). The table shows the fold change in expression in *M. marinum* infected zebrafish compared to the unchallenged fish (n = 4 in both groups). Fold change represents the fold change between the group medians. The table includes only the genes with at least two samples with ≥ 20 normalized reads after infection and the genes whose expression was induced at least 3.0-fold (calculated both with the DEseq-tool and from the medians).

Gene symbol	Gene name/description	Ensembl gene ID	Fold change (median)	Reference
Acute phase response and antimicrobial activity				
saa	*serum amyloid A*	ENSDARG00000045999	21.8	
hamp	*hepcidin antimicrobial peptide*	ENSDARG00000102175	20.5	
lygl2	*lysozyme g-like 2*	ENSDARG00000099562	7.4	Mohapatra et al., 2019
leap2	*liver-expressed antimicrobial peptide 2*	ENSDARG00000104654	3.3	
Neutrophil degranulation				
SERPINB8 (1 of many)	*zgc:173729*	ENSDARG00000057263	8.7	
plaub	*plasminogen activator, urokinase b*	ENSDARG00000039145	4.2	
cst14b.1	*cystatin 14b, tandem duplicate 1*	ENSDARG00000045980	3.2	
serpinb1l4	*serpin peptidase inhibitor, clade B (ovalbumin), member 1, like 4*	ENSDARG00000096888	3.1	
krt5	*keratin 5*	ENSDARG00000058371	3.0	
Inflammasome				
si:ch211-236p5.3	*NACHT, LRR and PYD domains-containing protein 3-like*	ENSDARG00000086418	13.3	Hu et al., 2017
si:ch211-233m11.1	*NACHT, LRR and PYD domains-containing protein 12*	ENSDARG00000074653	6.8	
fosab	*v-fos FBJ murine osteosarcoma viral oncogene homolog Ab*	ENSDARG00000031683	3.4	Chinenov et al., 2001; van Dam et al., 2001; Malik et al., 2017
Immune signaling and regulation				
chad	*chondroadherin*	ENSDARG00000045071	7.5	
ccl39.1	*chemokine (C–C motif) ligand 39, duplicate 1*	ENSDARG00000101041	7.3	
cxcl11.1	*chemokine (C-X-C motif) ligand 11, duplicate 1*	ENSDARG00000100662	6.0	
cxcl8a	*chemokine (C-X-C motif) ligand 8a*	ENSDARG00000104795	5.3	
si:dkey-117a8.4	*c3a anaphylatoxin chemotactic receptor-like*	ENSDARG00000097698	5.0	
ccl34b.8	*chemokine (C–C motif) ligand 34b, duplicate 8*	ENSDARG00000093098	4.0	
rxfp1	*relaxin family peptide receptor 1*	ENSDARG00000090071	3.9	Figueiredo et al., 2006
CABZ01001434.1		ENSDARG00000098602	3.8	
BX323596.1	*C-X-C motif chemokine 11-6-like*	ENSDARG00000101138	3.8	
cxcl11.8	*chemokine (C-X-C motif) ligand 11, duplicate 8*	ENSDARG00000095747	3.8	
bmp5	*bone morphogenetic protein 5*	ENSDARG00000101701	3.7	Shih et al., 2017; Rosendahl et al., 2002
selenou1b	*selenoprotein U1b*	ENSDARG00000087059	3.6	Guo et al., 2015; Avery et al., 2018
sox2	*SRY (sex determining region Y)-box 2*	ENSDARG00000070913	3.4	
egr3	*early growth response 3*	ENSDARG00000089156	3.3	Kenyon et al., 2017
Other role in innate immune response				
si:dkey-21e2.15	*mast cell protease 1A*	ENSDARG00000092788	5.9	
foxq1a	*forkhead box Q1a*	ENSDARG00000030896	5.5	Earley et al., 2018
p2ry11	*purinergic receptor P2Y, G-protein coupled, 11*	ENSDARG00000014929	3.8	Berchtold et al., 1999; Adrian et al., 2000
cyp21a2	*cytochrome P450, family 21, subfamily A, polypeptide 2*	ENSDARG00000037550	3.5	Poliani et al., 2010
MFAP4 (1 of many)	*microfibril-associated glycoprotein 4-like*	ENSDARG00000089667	3.1	Walton et al., 2015
Adaptive immune response				
RAC1	*ras-related C3 botulinum toxin substrate 1-like*	ENSDARG00000099506	64.5	
BX649608.2		ENSDARG00000102940	16.5	Mandel et al., 2012
ighv5-5	*immunoglobulin heavy variable 5-5*	ENSDARG00000096342	10.1	
ighv4-1	*immunoglobulin heavy variable 4-1*	ENSDARG00000096259	9.8	
CU896602.3		ENSDARG00000074999	7.0	
tmem176l.3b	*transmembrane protein 176l.3b*	ENSDARG00000096874	6.0	Louvet et al., 2005; Zuccolo et al., 2010
egr1	*early growth response 1*	ENSDARG00000037421	5.7	Gomez-Martin et al., 2010
si:dkey-234i14.12		ENSDARG00000097228	5.4	
unc119b	*unc-119 homolog b (C. elegans)*	ENSDARG00000044362	5.2	Gorska et al., 2004, 2009; Gorska and Alam 2012 Stephen et al., 2018
ighv13-2	*immunoglobulin heavy variable 13-2*	ENSDARG00000096372	5.1	
zgc:153659		ENSDARG00000039801	5.0	
igl3v1	*immunoglobulin light 3 variable 1*	ENSDARG00000093258	4.0	

associated with metabolism, in particular with lipid metabolism (Supplementary Table 2). Since the gene ontology tool did not recognize all the downregulated protein coding genes, they were also manually classified into different biological processes, based on the gene ontology data available. The resultant downregulated subgroups were: immune system (4 genes), lipid metabolism and transport (10 genes), other metabolic process (the largest category with 36 genes), other localization process (8 genes), signaling and regulation (9 genes), other process (10 genes) and an unknown process (28 genes) (Fig. 1B, Supplementary Table 3).

Of note, there was a clear expression of several blood cell and hemoglobin related genes in addition to known kidney expressed genes (Elmonem et al., 2018; Song et al., 2004; Walters et al., 2010) such as *chemokine (CXC motif), receptor 4b* (*cxcr4b*), *aminoleculinate, delta-,*

Table 3
Metabolic and other non-immunological protein coding genes induced in mycobacterial infection in adult zebrafish kidney at 14 dpi (days post infection). The table shows the fold change in expression in *M. marinum* infected zebrafish compared to unchallenged fish ($n = 4$ in both groups). Fold change represents the fold change between the group medians. The table includes only the genes with at least two samples with ≥ 20 normalized reads after infection and the genes whose expression was induced at least 3.0-fold (calculated both with the DEseq-tool and from the medians). The fold changes are presented as the quotient of medians when the divisor is zero.

Gene symbol	Gene name/description	Ensembl gene ID	Fold change (median)
Lipid binding and metabolism			
fabp6	*fatty acid binding protein 6, ileal (gastrotropin)*	ENSDARG00000044566	131.5
dpep1	*dipeptidase 1*	ENSDARG00000068181	16.8
scd	*stearoyl-CoA desaturase (delta-9-desaturase)*	ENSDARG00000033662	6.3
abhd5b	*abhydrolase domain containing 5b*	ENSDARG00000100388	5.3
alox5b.2	*arachidonate 5-lipoxygenase b, tandem duplicate 2*	ENSDARG00000043089	4.4
nceh1a	*neutral cholesterol ester hydrolase 1a*	ENSDARG00000020427	3.6
crabp1b	*cellular retinoic acid binding protein 1b*	ENSDARG00000035904	3.4
g0s2	*G0/G1 switch 2*	ENSDARG00000078859	3.3
rbp7b	*retinol binding protein 7b, cellular*	ENSDARG00000070486	3.0
Other metabolic role			
CR559945.2	*probable N-acetyltransferase CML5*	ENSDARG00000106491	13:0
TPO	*thyroid peroxidase*	ENSDARG00000033280	43.0
ca4b	*carbonic anhydrase IV b*	ENSDARG00000042293	11.5
tgm5l	*transglutaminase 5, like*	ENSDARG00000098837	10.5
arid6	*AT-rich interaction domain 6*	ENSDARG00000069988	6.3
tgm8	*transglutaminase 8*	ENSDARG00000097651	6.0
ca9	*carbonic anhydrase IX*	ENSDARG00000102300	5.8
lum	*lumican*	ENSDARG00000045580	5.6
hoxb5b	*homeobox B5b*	ENSDARG00000054030	4.1
si:dkey-203a12.9		ENSDARG00000104721	3.9
paplna	*papilin a, proteoglycan-like sulfated glycoprotein*	ENSDARG00000027867	3.7
si:ch211-243a20.3	*si:ch211-243a20.3*	ENSDARG00000092240	3.1
prrx1b	*paired related homeobox 1b*	ENSDARG00000042027	3.0
Other role			
si:dkey-7i4.24	*myosin heavy chain, clone 203*	ENSDARG00000096906	27.8
tnni2a.1	*troponin I type 2a (skeletal, fast), tandem duplicate 1*	ENSDARG00000045592	16.1
zgc:136930	*thread keratin gamma*	ENSDARG00000055192	13.6
myhb	*myosin, heavy chain b*	ENSDARG00000001993	8.8
tcnbb	*transcobalamin beta b*	ENSDARG00000091996	7.4
hspb11	*heat shock protein, alpha-crystallin-related, b11*	ENSDARG00000002204	7.4
mrap	*melanocortin 2 receptor accessory protein*	ENSDARG00000091992	6.7
scpp1	*secretory calcium-binding phosphoprotein 1*	ENSDARG00000090416	6.7
slco2a1	*solute carrier organic anion transporter family, member 2A1*	ENSDARG00000061896	5.6
CABZ01072043.1		ENSDARG00000102907	5.6
tmem196a	*transmembrane protein 196a*	ENSDARG00000013935	5.4
hpcal1	*hippocalcin-like 1*	ENSDARG00000022763	5.1
asic2	*acid-sensing (proton-gated) ion channel 2*	ENSDARG00000006849	5.0
krt17	*keratin 17*	ENSDARG00000094041	4.3
cthrc1a	*collagen triple helix repeat containing 1a*	ENSDARG00000087198	4.1
col12a1b	*collagen, type XII, alpha 1b*	ENSDARG00000019601	4.1
sh3glb2b	*SH3-domain GRB2-like endophilin B2b*	ENSDARG00000035470	4.0
mamdc2b	*MAM domain containing 2b*	ENSDARG00000073695	3.9
si:ch211-105c13.3		ENSDARG00000089441	3.8
krt15	*keratin 15*	ENSDARG00000036840	3.8
tspan34	*tetraspanin 34*	ENSDARG00000103951	3.7
apnl	*actinoporin-like protein*	ENSDARG00000090900	3.4
myoz3a	*myozenin 3a*	ENSDARG00000067701	3.3
FO834829.3		ENSDARG00000102718	3.3
im:7150988		ENSDARG00000098058	3.3
BX664721.3	*endonuclease domain-containing 1 protein-like*	ENSDARG00000073995	3.2
si:ch211-150d5.3	*von Willebrand factor A domain-containing protein 7-like*	ENSDARG00000093384	3.0
Unknown role			
FO704758.1		ENSDARG00000098478	6.3
CABZ01056516.1		ENSDARG00000102467	5.0
si:dkey-248g15.3		ENSDARG00000097959	4.2
si:ch211-113d11.8		ENSDARG00000105494	3.4

synthase 2 (*alas2*), *choride channel K* (*clcnk*) and *nephrosis 1, congenital, Finnish type (nephrin)* (*nphs1*) (Supplementary Fig. 1) as well as genes typically used as markers for different types of immune cells (Supplementary Fig. 2), indicating that the samples represent the kidney and different types of blood cells.

3.2. Innate immune response is dominant at 14 days post *M. marinum* infection

The largest group of the protein coding genes induced upon infection consisted of genes with a documented or predicted immunological role. To further examine the induced immune response, these genes were divided into subcategories comprising acute phase response and

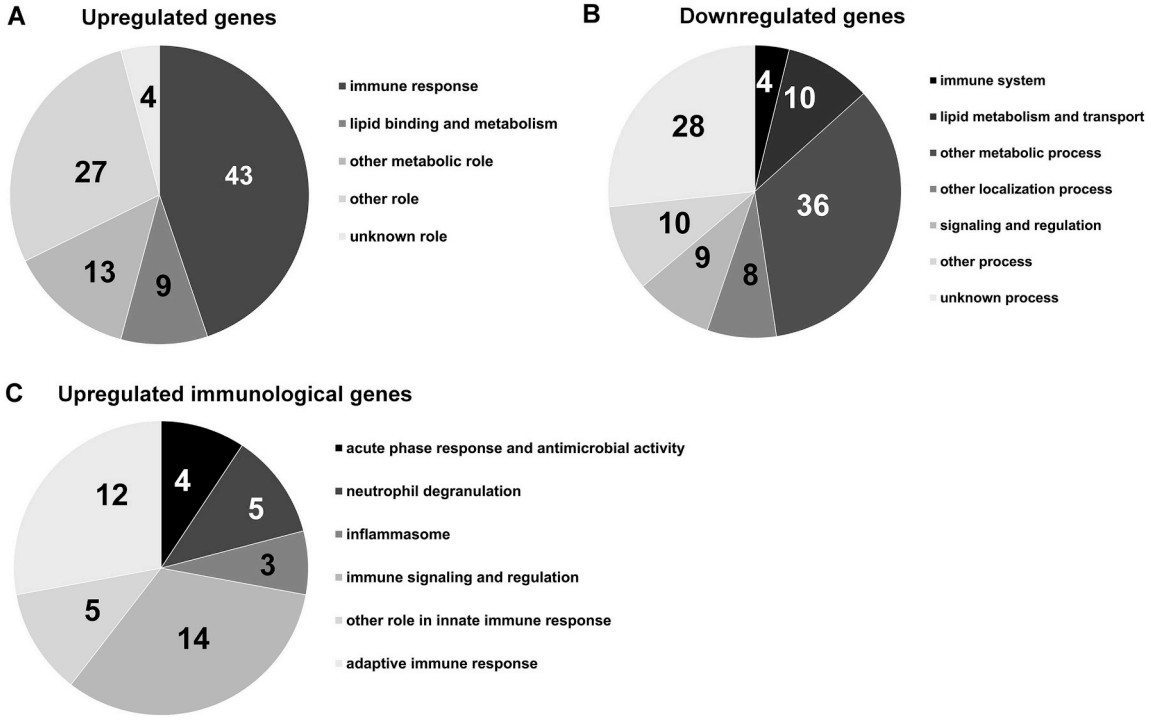

Fig. 1. Categorization of mycobacterium-responsive genes. Differentially expressed genes at 14 days post a low-dose (5–9 CFU, colony forming units) *M. marinum* infection were divided into categories based on their **roles** and/or the **biological processes** they participate in. **(A, C)** The upregulated genes were classified based on their roles as detailed in Materials and methods. **(B)** Downregulated genes were classified according to the gene ontology process data as detailed in Materials and methods. **(C)** Upregulated immunological genes were classified as in panel **(A)**.

antimicrobial activity (4 genes), neutrophil degranulation (5 genes), inflammasome (3 genes), immune signaling and regulation (14 genes), other role in innate immune response (5 genes) and adaptive immune response (12 genes) (Fig. 1C, Table 2). Most of these subcategories were related to innate immunity, highlighting that these responses still have an important role in the immune defense at 14 days post *M. marinum* infection and in the initiation of the adaptive immune response.

The innate immunity processes associated with defense response to bacteria were represented in our data. Acute phase protein coding gene *serum amyloid A* (*saa*) was induced together with three genes encoding proteins with antimicrobial activity: *hepcidin antimicrobial peptide* (*hamp*), *liver-expressed antimicrobial peptide 2* (*leap2*) and predicted *lysozyme g-like 2* (*lygl2*). Out of the other innate defense mechanisms, neutrophil degranulation was evidenced by the induction of five protein-coding genes.

An inflammatory response against pathogenic *M. tuberculosis* includes inflammasome activation (Wawrocki and Druszczynska, 2017). Three of the induced genes were associated with inflammasomes. These were: *si:ch211-236p5.3* encoding predicted NACHT, LRR and PYD domains-containing protein 3-like protein; *si:ch211-233m11.1* coding for a putative NOD-like receptor, NACHT, LRR and PYD domains-containing protein 12 (Hu et al., 2017; Vladimer et al., 2012); and *v-fos FBJ murine osteosarcoma viral oncogene homolog Ab* (*fosab*). The latter codes for an ortholog of a transcription factor that is a part of activator protein 1 (AP-1) protein complex (Chinenov and Kerppola, 2001; van Dam and Castellazzi, 2001) which participates in inflammasome activation (Malik and Kanneganti, 2017). Also related to inflammasome activation, another upregulated gene, *egr3*, codes for Early growth response 3 transcription factor that positively regulates *interleukin 1, beta,* (*il1b*) expression (Kenyon et al., 2017).

Some genes upregulated in our data, or their orthologs, are expressed in various innate immune cell types. These included genes expressed in macrophages (*forkhead box Q1a*, *foxq1a* (Earley et al., 2018) and one putative *microfibril-associated glycoprotein 4-like, MFAP4* variant (Walton et al., 2015)); dendritic cells (*purinergic receptor P2Y G-protein coupled, 11*, *p2ry11* (Berchtold et al., 1999) and putative *cytochrome P450, family 21, subfamily A, polypeptide 2*, *cyp21a2* (Poliani et al., 2010)); and mast cells (putative *mast cell protease 1A, si:dkey-21e2.15*). Related to cell migration, genes and putative genes encoding chemokines and chemokine-like proteins formed the largest subset of the genes with a postulated or documented role in immune signaling and regulation. On top of this, the human ortholog of upregulated signaling protein *relaxin family peptide receptor 1* (*rfxp1*), *LGR7*, has a reported role in leukocyte migration (Figueiredo et al., 2006).

In addition to the genes involved in innate immune response, twelve genes coding for proteins with a suggested or documented role in adaptive immunity were upregulated upon *M. marinum* infection. Four of these: predicted *CU896602.3*, *si:dkey-234i14.12*, *zgc:153659* and *immunoglobulin light 3 variable 1* (*igl3v1*), code for proteins having a postulated role in immunoglobulin production. Five potential genes (or their human orthologs) have suggested roles in T and B lymphocyte signaling and regulation: *early growth response 1* (*egr1*) (Gomez-Martin et al., 2010), *immunoglobulin heavy variable 4-1* (*ighv4-1*), *immunoglobulin heavy variable 5-5* (*ighv5-5*), *immunoglobulin heavy variable 13-2* (*ighv13-2*) and *RAC1* coding for a putative ras-related C3 botulinum toxin substrate 1-like protein.

3.3. *M. marinum* infection changes expression of metabolism associated genes

In addition to the immune system-related genes presented in Table 2, nine genes involved in lipid binding or lipid metabolism and 13 genes classed as 'other metabolic role' were induced (Table 3). Significantly, the most induced gene, *fatty acid binding protein 6* (*fabp6*) has a postulated role in lipid binding and metabolism. The upregulation of lipid metabolism associated genes due to *M. marinum* infection is supported by the literature, as the ability of *M. tuberculosis* to manipulate host metabolism and to use the host lipids for its pathogenic activities

and survival has been widely studied in a number of different research models (Barisch and Soldati, 2017; Korb et al., 2016; Stutz et al., 2018).

Involvement of lipid metabolism in the response to *M. marinum* is also apparent when analyzing the set of downregulated genes. This group included nine genes under the gene ontology term 'lipid metabolism', including 5 genes encoding apolipoproteins or predicted apolipoproteins and one, *apolipoprotein Bb, tandem duplicate 2* (*apobb.1*), especially associated with lipid transport. Among the most enriched processes of the downregulated genes were lipid localization, cellular lipid catabolic process and lipid transport (Supplementary Table 2). The downregulation of lipid catabolism and transport could be associated with the tendency of mycobacteria to manipulate the host to store the lipids inside the cell for the bacteria to use for its own purposes (Peyron et al., 2008; Russell et al., 2009; Stutz et al., 2018).

3.4. A forward genetic screen identifies a fish line with increased susceptibility to M. marinum infection

In order to identify genes that are important for defense against *M. marinum* infection in zebrafish, we conducted a forward genetic screen using gene-breaking transposon-based mutagenesis. 127 zebrafish lines, carrying a set of random mutations in F3–F5 inbred generations, were screened for altered susceptibility to *M. marinum* infection. To do this, we infected 3–15 month-old zebrafish from each mutant line with a low-dose (1–72 CFU) of *M. marinum* and followed the survival of the fish for 12–14 weeks. *rag1*$^{hu1999/hu1999}$ mutant fish, which have no functional T and B cells (Wienholds et al., 2002) were used as a positive control. During the screen, we identified 10 lines with impaired survival and one line with improved survival against *M. marinum* infection.

The most susceptible line, designated mutant463, showed drastically impaired survival (8% at the 14 weeks post infection, wpi endpoint) compared to the WT zebrafish (74%, $P < 0.0001$) (Fig. 2A). For reference, Fig. 2A also shows the survival rates of two mutant lines as compared to WT; mutant189 (endpoint survival 83%) and mutant229 (endpoint survival 68%). Supplementary Fig. 3A shows the survival of mutant463 fish prior to RNA extraction for the RNA sequencing experiment (survival at the 4 wpi endpoint of 38% in the mutant463 group vs. 100% in the WT group, $P = 0.002$). This survival was also similar to the mutant463 zebrafish survival kinetics observed during the screen. Buffer injection (PBS) did not cause any mortality among the mutant463 fish (Supplementary Fig. 3A). Infection with 220–328 CFU of *S. pneumoniae* (T4 serotype) resulted in a minor impairment to mutant463 embryo survival when compared to WT embryo survival (Supplementary Fig. 3B).

In our previous studies, high mortality after *M. marinum* infection associated with high bacterial burden (Harjula et al., 2018a; Myllymäki et al., 2018; Parikka et al., 2012). To clarify whether a defect in tolerance or resistance underlies the decreased survival of the mutant463 fish, we studied the *M. marinum* burden in the mutants and WT control zebrafish at 7 and 14 dpi (Fig. 2B). At 7 dpi, there was no difference in the bacterial count between mutant463 and WT fish (estimated copy number median 206 vs. 440 CFU, respectively), whereas at 14 dpi there was significantly higher bacterial burden in mutant463 fish compared to WT fish (copy number median 23,076 vs. 701 CFU, $P = 0.042$, respectively).

To further study whether the decreased survival and the increased mycobacterial burden in mutant463 fish is attributed to defects in the innate immune response, we performed a survival assay on WT and mutant463 zebrafish embryos, which rely solely on the innate immune system for protection against infection. We infected 32–198 CFU into the caudal vein of the embryos at 1 dpf and followed their survival for 7 days (Supplementary Fig. 3C). In contrast to the situation in adult fish, mutant463 embryos showed similar survival kinetics to WT embryos. This result suggests that the increased susceptibility of the mutant463 zebrafish line against a mycobacterial infection is due to defective adaptive rather than innate immunity.

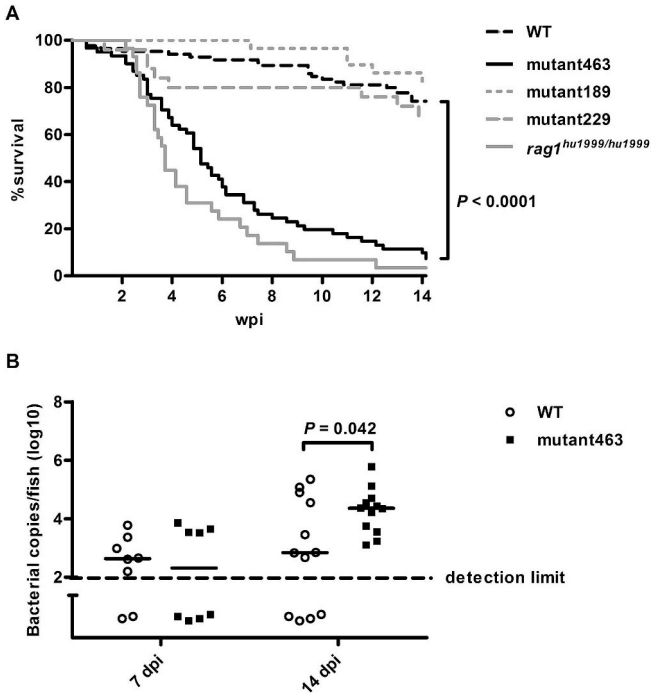

Fig. 2. Survival and bacterial burden of the mutant463 zebrafish after *M. marinum* infection. Adult mutant463 zebrafish have impaired survival and elevated bacterial burden after a low-dose *M. marinum* infection. **(A)** The survival of WT (wild type) ($n = 84$), *rag1*$^{hu1999/hu1999}$ ($n = 29$, a positive control) and three mutant zebrafish lines, mutant189 ($n = 29$), mutant229 ($n = 25$) and mutant463 ($n = 61$), was monitored for 14 weeks post a low-dose (2–35 CFU, colony forming units) *M. marinum* infection. The graphs represent the data from one (mutant189 and mutant229), two (*rag1*$^{hu1999/hu1999}$) or three (WT, mutant463) independent experiments. The survival data is presented as a Kaplan-Meier survival curve. wpi, weeks post infection **(B)** The *M. marinum* burden in the abdominal organ blocks (excluding kidney) of the low-dose (5–9) infected WT ($n = 8$–12) and mutant463 ($n = 8$–12) adult zebrafish was measured with qPCR at 7 and 14 dpi (days post infection). The bacterial count is presented as a scatter dot plot and as the median of total bacterial copies (log10). A log-rank (Mantel-Cox) test for panel (A) and a one-tailed Mann-Whitney test for panel (B) was used to perform the statistical comparison of differences.

3.5. The hypersusceptible mutant463 zebrafish have decreased expression of si:ch211-236p5.3 and unc119b involved in the innate and adaptive immune response

To further elucidate the reasons behind increased susceptibility of the mutant463 fish to mycobacterial infection, we analyzed the transcriptome of mutant463 zebrafish at 14 days post *M. marinum* infection and compared it to WT controls. Out of the 96 upregulated protein coding genes in WT fish, 27 had reduced expression in mutant463 compared to WT fish (Table 4). In addition, out of the 21 non-coding upregulated RNAs, 8 had reduced expression in the mutant463 zebrafish (Supplementary Table 6). None of the typical immune cell marker genes were clearly (≥ 3-fold) downregulated in mutant463 zebrafish compared to WT (Supplementary Fig. 2). However, there were other protein coding genes with a potential immunological role and reduced expression in mutants. For selected genes, the RNA sequencing result was confirmed by qPCR at 7, 14 and 28 dpi (Fig. 3).

Among the genes with reduced expression in mutant463 fish were four genes with metabolic roles. One of these, *fabp6*, showed a 4.5-fold reduction at 14 dpi compared to WT fish in RNA sequencing and a 6.1-fold reduction at 28 dpi in qPCR ($P < 0.016$) (Fig. 3A). In addition to its roles in lipid binding and metabolism, Fabp6 is a bile acid binding protein (Capaldi et al., 2009). Bile acid mediates inflammation through different mechanisms in the liver (Li et al., 2017) and attenuates

Table 4
The protein coding genes downregulated in mycobacterial infection in mutant463 zebrafish kidney compared to WT (wild type) at 14 dpi (days post infection). The table shows the fold change in expression of the genes upregulated in mycobacterial infection between mutant463 and WT zebrafish ($n = 4$ in both groups). Fold change represents the fold change between the group medians. The table includes only the genes with at least two samples with ≥20 normalized reads in the WT group and whose expression was reduced at least 3.0-fold (calculated both with the DEseq-tool and from the medians). The fold changes are presented as the quotient of medians when the divisor is zero.

Gene symbol	Gene name/description	Ensembl gene ID	Fold change (median)
Immune response			
si:ch211-236p5.3	*NACHT, LRR and PYD domains-containing protein 3-like*	ENSDARG00000086418	−20:0
unc119b	*unc-119 homolog b (C. elegans)*	ENSDARG00000044362	−23.9
plaub	*plasminogen activator, urokinase b*	ENSDARG00000039145	−12.5
chad	*chondroadherin*	ENSDARG00000045071	−7.5
leap2	*liver-expressed antimicrobial peptide 2*	ENSDARG00000104654	−4.5
si:dkey-21e2.15	*mast cell protease 1A*	ENSDARG00000092788	−4.2
ighv5-5	*immunoglobulin heavy variable 5-5*	ENSDARG00000096342	−3.0
Metabolism			
TPO	*thyroid peroxidase*	ENSDARG00000033280	−43
CR559945.2	*probable N-acetyltransferase CML5*	ENSDARG00000106491	−13
hoxb5b	*homeobox B5b*	ENSDARG00000054030	−4.6
fabp6	*fatty acid binding protein 6, ileal (gastrotropin)*	ENSDARG00000044566	−4.5
Other role			
si:ch211-150d5.3	*von Willebrand factor A domain-containing protein 7-like*	ENSDARG00000093384	−24
myhb	*myosin, heavy chain b*	ENSDARG00000001993	−22
tmem196a	*transmembrane protein 196a*	ENSDARG00000013935	−21.6
mamdc2b	*MAM domain containing 2b*	ENSDARG00000073695	−20.8
tspan34	*tetraspanin 34*	ENSDARG00000103951	−16
tnni2a.1	*troponin I type 2a (skeletal, fast), tandem duplicate 1*	ENSDARG00000045592	−14.8
myoz3a	*myozenin 3a*	ENSDARG00000067701	−13.1
scpp1	*secretory calcium-binding phosphoprotein 1*	ENSDARG00000090416	−8.0
slco2a1	*solute carrier organic anion transporter family, member 2A1*	ENSDARG00000061896	−4.5
tcnbb	*transcobalamin beta b*	ENSDARG00000091996	−4.4
lum	*lumican*	ENSDARG00000045580	−4.3
BX664721.3	*endonuclease domain-containing 1 protein-like*	ENSDARG00000073995	−4.1
krt15	*keratin 15*	ENSDARG00000036840	−3.5
FO834829.3		ENSDARG00000102718	−3
Unknown role			
FO704758.1		ENSDARG00000098478	−33.3
CABZ01056516.1		ENSDARG00000102467	−6.4

inflammation through its receptors in the kidney (Herman-Edelstein et al., 2018). *fabp6* as well as another lipid metabolism associated gene *stearoyl-CoA desaturase (delta-9-desaturase)* (*scd*) were significantly induced upon *M. marinum* infection also when measured by qPCR. *fabp6* was induced 131.5-fold in RNA sequencing at 14 dpi and in qPCR; 14.9-fold at 7 dpi ($P = 0.007$) and 25.1-fold at 14 dpi ($P = 0.015$) (Fig. 3A). *scd* was induced 6.3-fold in RNA sequencing at 14 dpi and 2.1-fold ($P = 0.007$) in qPCR at 14 dpi (Fig. 3B).

Out of the 27 protein coding genes with reduced expression in mutant463 fish, 7 have a documented or predicted role in innate immunity (Table 4). For example, the expression of predicted *NACHT, LRR and PYD domains-containing protein 3-like* (*si:ch211-236p5.3*) was almost completely absent in mutant463 zebrafish upon *M. marinum* infection, whereas it was induced by infection in WT fish (13.3-fold induction in RNA sequencing). The diminished expression of this gene in mutant463 compared to WT fish was also seen in qPCR analysis with larger sample sizes (Fig. 3C) at 14 dpi (1848.4-fold reduction, $P < 0.0001$) and at 28 dpi (493.7-fold reduction, $P = 0.0002$). This gene and its paralogs have been predicted to code for a component of the inflammasome and to have a role in intracellular microbial recognition and immune activation.

Two of the genes with reduced expression have a role in adaptive immunity (Table 4). While *ighv5-5* (3.0-fold reduction) has a predicted role in the B cell receptor signaling pathway and in the positive regulation of B cell activation, *unc-119 homolog b (C. elegans)* (*unc119b*) is a zebrafish ortholog for the human gene coding for Unc-119 lipid binding chaperone (*UNC119*), which has been shown to be essential for T cell activation (Gorska et al., 2004; Stephen et al., 2018). *unc119b* showed 23.9-fold reduction in RNA sequencing and in qPCR showed the following; 11.4-fold reduction at 7 dpi ($P = 0.019$), 8.4-fold reduction at 14 dpi ($P = 0.003$) and 76.0-fold reduction at 28 dpi ($P = 0.0002$) (Fig. 3D).

4. Discussion

The transcriptional responses to mycobacterial infection are an outcome of the dynamic interplay between the host and the pathogen. Thus, it is implicit that the gene expression profile over the duration of *M. marinum* infection in zebrafish depends on the type of cells analyzed as well as the time-point of the analysis, the host and bacterial strains and the bacterial dosage, particularly when the analysis is carried out at the whole organismal level (Benard et al., 2016; Kenyon et al., 2017; Ojanen et al., 2019; Rougeot et al., 2014; van der Sar et al., 2009). Here, we utilized a zebrafish *M. marinum* infection model to study response to infection at the transcriptional level, using kidney samples to obtain a focused view of transcriptional changes in hematopoietic cells. Since head kidney is the main hematopoietic organ of zebrafish, it contains immune precursor cells. In our previous studies we have reported the presence of granulomas (Oksanen et al., 2013; Parikka et al., 2012) in adult zebrafish kidney. Therefore our data can give information about the interaction between the mature host immune cells and bacteria in addition to the production of immune cells during infection. As expected, the expression of many genes involved in the innate immune response were induced, such as genes involved in bacterial elimination, immune cell migration and neutrophil degranulation.

Activation of an inflammasome is a typical response to intracellular

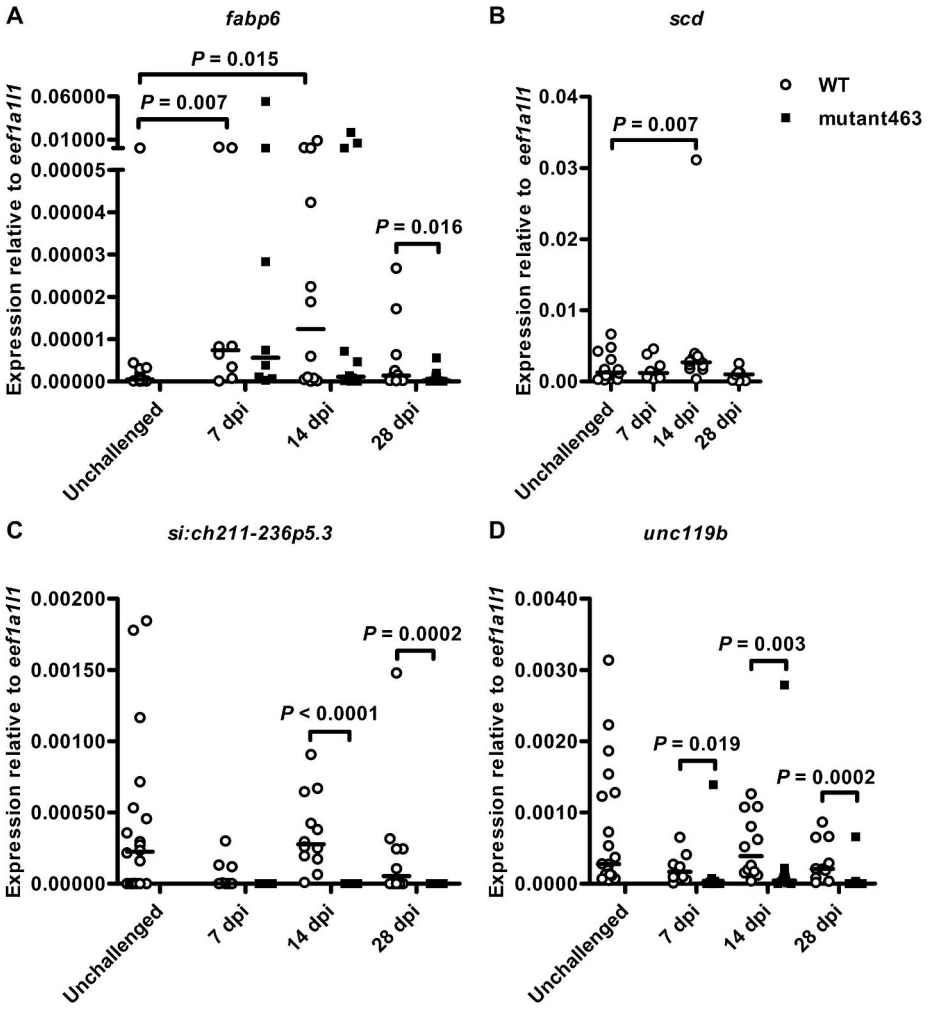

Fig. 3. The expression of selected genes following *M. marinum* infection. Hypersusceptible mutant463 zebrafish have decreased expression levels of the postulated inflammasome-related gene *si:ch211-236p5.3* and potentially T cell -related *unc119b* at various time-points during *M. marinum* (5–9 CFU, colony forming units) infection. **(A-D)** The relative expression levels of genes upregulated during mycobacterial infection in WT (wild type) (*scd*) and downregulated in mutant463 compared to WT (*fabp6*, si:*ch211-236p5.3* and *unc119b*) according to the transcriptome analysis were measured with qPCR from the kidney samples of unchallenged WT (n = 20) as well as WT (n = 8–12) and mutant463 (n = 8–14) zebrafish at 7, 14 and 28 dpi (days post infection). Data are presented as a scatter dot plot and median. Note the different scales of y-axes and the divided y-axis in panel (A). The expression of *eef1a1l1* was used to normalize the measured gene expressions. Data was collected from one experiment. A one-tailed Mann-Whitney test was used to perform the statistical comparison of differences. dpi, days post infection.

microbes (Mitchell and Isberg, 2017). Kenyon et al. (2017) used transcriptome analysis in neutrophils from zebrafish larvae at 3 dpi, to show differential expression of genes encoding inflammasome components (Kenyon et al., 2017). Our new data in adult zebrafish also revealed the upregulation of several genes associated with inflammasome function. Of note, one of the genes with substantially reduced expression in the hypersusceptible mutant463 fish was an inflammasome-associated gene, predicted *NACHT, LRR and PYD domains-containing protein 3-like* (*si:ch211-236p5.3*). There was no measurable expression of *si:ch211-236p5.3* detected by RNA sequencing post *M. marinum* infection and *si:ch211-236p5.3* expression was detected only in one fish out of 34 by qPCR. Conclusive data about the significance of this gene as well as other inflammasome components requires the phenotypic analysis of mutant fish lines. As such, the importance of inflammasome activation, as a defense mechanism against mycobacteria in zebrafish, remains to be determined.

Based on the known role of adaptive immunity in the defense against tuberculosis (O'Garra et al., 2013), our analysis, like previous corresponding studies (Meijer et al., 2005; van der Sar et al., 2009), also showed induction of genes coding for proteins that potentially participate in adaptive immune response, particularly in T cell function. Kidney is not a secondary lymphoid organ of zebrafish and therefore antigen presentation and lymphocyte activation do not typically occur in kidney (Renshaw and Trede, 2012). However, several genes with postulated roles in these processes were upregulated in our data, possibly as a result of granuloma formation in kidney and related presence of antigen presenting cells and lymphocytes. This is supported, for example, by a notion that genes related to neutrophil degranulation were among the upregulated genes. Alternatively the upregulation is related to the other possible functions of these genes. Based on the literature, two predicted genes upregulated in our data and having possible roles related to adaptive immunity are *transmembrane protein 176l.3b* (*tmem176l.3b*), and a member of the Claudin 1 -protein family, *BX649608.2*. The rat gene *Tmem176b* has been shown to be upregulated in inactive and immature antigen presenting cells in rats with allografts (Louvet et al., 2005) and conserved genes encoding the proteins of this family have also been found in zebrafish (Zuccolo et al., 2010). Therefore, *tmem176l.3b* could have an adaptive immunity related function also in zebrafish. Claudin 1 expression has been reported in human T and B lymphocytes and also in monocytes (Mandel et al., 2012), suggesting a potential immunological role for *BX649608.2*. Our analysis also revealed the upregulation of genes participating in B cell activation and immunoglobulin production. In line with these results, and related to the function of adaptive immune cells, Th2 type response has been suggested to have an important role in resistance against *M. marinum* in adult zebrafish (Hammarén et al., 2014).

One of the adaptive immune genes induced in WT zebrafish but with impaired expression in the hypersusceptible mutant463 line was *unc119b*. The human ortholog *UNC119* has been linked to T cell lymphopenia (Gorska and Alam, 2012), having been found to activate Lymphocyte-specific protein tyrosine kinase (LCK) and Proto-oncogene tyrosine-protein kinase Fyn (FYN) (Gorska et al., 2004). Thus, UNC119 is involved in T cell receptor (TCR) signaling and is essential for immune synapse formation (Gorska et al., 2004, 2009). On top of this, Stephen et al. (2018) recently showed that UNC119 binds LCK and further described the mechanisms by which UNC119 participates in the

immune synapse and in the initiation of T cell signaling by affecting the membrane localization of LCK (Stephen et al., 2018). They also proposed a new role for ciliary machinery in maintaining activated LCK at the immune synapse (Stephen et al., 2018). Since the $CD4^+$ T cells are indispensable in the immune defense against tuberculosis (Jasenosky et al., 2015), the increased susceptibility of mutant463 fish to *M. marinum* infection could be due to Unc119b deficiency. However, there are no clear differences in the expression of the genes coding for the most common T cell markers, *cd4-1*, *cd8a*, *lck*, *rag1*, *rag2* but instead, there is a 2.3-fold reduction in the eosinophil marker *gata2a*. In addition to T cell activation, UNC119 also plays a role in eosinophil survival (Cen et al., 2003). Since eosinophils have an ability to present antigens to $CD4^+$ Th cells in response to *Mycobacterium tuberculosis* purified protein derivate (Farhan et al., 2016), the defects in mycobacterial response in the mutant463 fish could derive from the complex effects of *unc119b* repression both in T cell signaling and eosinophil survival.

As mentioned above, the ability of *M. tuberculosis* to benefit from host lipids has been widely studied (Wilburn et al., 2018). Defects in certain host metabolic genes have been shown to affect the survival of the bacteria and the disease outcome (Kumar et al., 2010). Several genes involved in lipid metabolism, binding and transport were differentially expressed in our transcriptome data describing *M. marinum* infection in zebrafish. This has also been reported previously by others (Benard et al., 2016; Kenyon et al., 2017; Meijer et al., 2005; Ojanen et al., 2019; Rougeot et al., 2019; van der Sar et al., 2009). Two upregulated genes in our data code for lipid binding proteins *retinol binding protein 7b, cellular* (*rbp7b*) and *cellular retinoic acid binding protein 1b* (*crabp1b*). The mouse ortholog of *rbp7*, has been linked to abnormal levels of vitamin A (Piantedosi et al., 2005) and *crabp1b* has postulated roles in retinoic acid biosynthesis, signaling, binding and catabolism. Vitamin A and retinoic acid in turn have antimicrobial activity against *M. tuberculosis* (Wheelwright et al., 2014) and enhance autophagy in macrophages during intracellular bacterial infection (Coleman et al., 2018). An *in vitro* study in human macrophages infected with *M. tuberculosis* showed that a set of host genes involved in autophagy affect the survival of the bacteria inside macrophages (Kumar et al., 2010).

Some other genes which, according to our data, are upregulated during *M. marinum* infection and encode proteins associated to lipid metabolism, have reported or possible immunological functions. *dipeptidase 1* (*dpep1*), for example, has a potential role in leukotriene synthesis. Leukotriene B_4 is a known chemoattractant (Samuelsson, 1983) and an excess of leukotriene B_4 has been shown to lead to hyperinflammation and susceptibility to tuberculosis in zebrafish larvae (Tobin et al., 2012). On the other hand, from a genetic screen by Ramakrishnan's group, a deficiency in the leukotriene B_4 synthesis pathway in zebrafish larvae has been reported to cause an anti-inflammatory state, resulting in increased mycobacterial susceptibility (Tobin et al., 2010). Moreover, the human ortholog of another upregulated gene in our data, *Arachidonate 5-lipoxygenase b, tandem duplicate 2* (*alox5b.2*) encodes a protein which participates in leukotriene B_4 synthesis (Rådmark et al., 2015). Liu et al. (2013) showed that downregulation of *alox5b.2* leads to a reduced migration of leukocytes to a tail fin amputation site (Liu et al., 2013). Related to cell migration, Ramakrishnan's group has also recognized a role in macrophage migration for *cathepsin L.1* (*ctsl.1*) in zebrafish larvae during *M. marinum* infection (Berg et al., 2016).

Head kidney is also a part of endocrine system in teleosts (Geven and Klaren, 2017) and a key thyroid signaling organ (Quesada-García et al., 2014). Accordingly, *thyroid peroxidase* (*TPO*) was among most highly induced genes in our study (Table 3). In addition, *relaxin family peptide receptor 1* (*rxfp1*) and *melanocortin 2 receptor accessory protein* (*mrap)* with postulated roles in hormone-mediated signaling and in peptide hormone binding, respectively, were among the upregulated genes (Tables 2 and 3). Of note, *TPO* is downregulated in mutant463 compared to WT (Table 4). Out of the downregulated genes, *glycoprotein hormone beta 5* (*gphb5*) has a postulated role in the regulation of thyroid hormone mediated signaling pathway and *CABZ01111515.1* in thyroid hormone transport (Supplementary Table 3).

Our analysis also revealed differential expression of muscle-related genes in the kidney. The following muscle-related genes: *myosin, heavy chain b* (*myhb*); *troponin I type 2a (skeletal, fast); tandem duplicate 1* (*tnni2a.1*) and *myozenin 3a* (*myoz3a*); whilst upregulated in WT fish infected with *M. marinum*, were downregulated compared to WT in the hypersusceptible mutant463 at 14 dpi. The downregulation of muscle-related genes in selected neutrophil populations, during *M. marinum* infection in zebrafish, has been shown in a transcriptome analysis by Kenyon et al. (2017). Their results are thus suggestive of other immunological roles for muscle-related genes. The differential expression of genes traditionally associated with muscle function during infection, has also been shown in several other transcriptome level analyses in zebrafish (Meijer et al., 2005; Ojanen et al., 2019; van der Sar et al., 2009) as well as in a study in *Drosophila melanogaster* (Apidianakis et al., 2005). Moreover, expression of such genes has been shown in the macrophages, neutrophils and lymphocytes of zebrafish larvae (Rougeot et al., 2019). It has also been shown that, after exercise, the same functional gene networks are found in both human skeletal muscle and blood neutrophils (Broadbent et al., 2017).

5. Conclusions

In this study, we report a whole-genome level transcriptome analysis using adult zebrafish kidney cells at 14 days post a *M. marinum* infection. Kidney is the main hematopoietic organ of zebrafish and thus informative for studying immune response. Our results show that, at this time-point, the innate immune response is dominant. Furthermore, through a forward genetic screen, we identified a line with impaired resistance against *M. marinum*. The importance of immune responsive genes for resistance against mycobacterial disease can be studied using reverse genetics utilizing for example Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) -based mutagenesis. In this way, it is possible to solve which genes and pathways are most critical to harness for therapeutic strategies against mycobacterial diseases.

Declaration of competing interest

None.

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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.2019.103523.

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