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ORIGINAL ARTICLE

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Proprotein convertase subtilisin/kexin type 9 regulates the production of acute-phase reactants from the liver

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Abstract

Background & Aims: Proprotein convertase subtilisin/kexin type 9 (PCSK9) controls blood cholesterol levels by fostering the LDL receptor (LDLR) degradation in hepatocytes. Additionally, PCSK9 has been suggested to participate in immunoregulation by modulating cytokine production. We studied the immunological role of PCSK9 in *Streptococcus pneumoniae* bacteraemia in vivo and in a human hepatocyte cell line.

Methods: CRISPR/Cas9 mutagenesis was utilized to create *pcsk9* knock-out (KO) zebrafish, which were infected with *S pneumoniae* to assess the role of PCSK9 for the survival of the fish and in the transcriptomic response of the liver. The direct effects of PCSK9 on the expression of acute-phase reaction (APR) genes were studied in HepG2 cells.

Results: The *pcsk9* KO zebrafish lines (*pcsk9*$^{tpu-13}$ and *pcsk9*$^{tpu-2,+15}$) did not show developmental defects or gross phenotypical differences. In the *S pneumoniae* infected zebrafish, the mortality of *pcsk9* KOs was similar to the controls. A liver-specific gene expression analysis revealed that a pneumococcal challenge upregulated *pcsk9*, and that the *pcsk9* deletion reduced the expression of APR genes, including *hepcidin antimicrobial peptide* (*hamp*) and *complement component 7b* (*c7b*). Accordingly, silencing *PCSK9* in vitro in HepG2 cells using small interfering RNAs (siRNAs) decreased *HAMP* expression.

Abbreviations: APR, acute-phase reaction; C, complement component; CFU, colony forming unit; dpi, days post infection; gDNA, genomic DNA; GO, gene ontology; gRNA, guide RNA; HAMP, hepcidin antimicrobial peptide; KO, knock-out; LDLR, low-density lipoprotein receptor; PBS, phosphate-buffered saline; PCSK9, proprotein convertase subtilisin/kexin type 9; qPCR, quantitative PCR; siRNA, small interfering RNA; SOCS3, suppressor of cytokine signaling 3; TNF, tumour necrosis factor; WT, wild type.

Anni K. Saralahti and Meeri Pekkarinen contributed equally for the second authorship and Markus J.T. Ojanen and Marko Pesu for the last authorship.

Conclusions: We demonstrate that PCSK9 is not critical for zebrafish survival in a systemic pneumococcal infection. However, *PCSK9* deficiency was associated with the lower expression of APR genes in zebrafish and altered the expression of innate immunity genes in a human hepatocyte cell line. Overall, our data suggest an evolutionarily conserved function for PCSK9 in APR in the liver.

KEYWORDS

innate immunity, *Pneumococcus*, sepsis, zebrafish

1 | INTRODUCTION

Proprotein convertase subtilisin/kexins (PCSKs) are serine endoproteases that enzymatically process and convert biologically inactive proproteins into functional end-products. PCSKs are important not only in maintaining homeostasis in the body, but also in a number of pathological conditions, such as malignancies and inflammatory disorders.[1,2] In contrast to the other PCSK enzymes, PCSK9 has a fundamental non-enzymatic role in cholesterol homeostasis by targeting the LDL receptor (LDLR) for lysosomal degradation.[3,4] Consequently, loss-of-function mutations of PCSK9 are associated with decreased plasma LDL-cholesterol levels and protection against coronary heart disease (CHD),[5] whereas gain-of-function PCSK9 variants have been reported to cause hypercholesterolemia.[6] Collectively, mechanistic and genetic insights into the PCSK9-mediated regulation of hepatic clearance of blood LDL-cholesterol have paved the way for the existing anti-PCSK9 antibodies and small interfering RNAs (siRNAs) as therapeutics to prevent CHD.[7–9]

In addition to its undisputable significance in lipid metabolism, PCSK9 has recently been suggested to affect the pathogenesis of dengue virus infection[10] as well as to regulate the production of inflammatory cytokines. In fact, although tomographic analysis in patients receiving PCSK9 inhibitors has suggested that the arterial inflammation is not affected by the PCSK9 levels,[11] experimental evidence from mice argues that PCSK9 can increase the magnitude of the pro-inflammatory response in macrophages, and subsequently exacerbate foam cell formation and atherosclerosis.[12,13] Moreover, the PCSK9-controlled production of inflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin 6 (IL6), leads to a dysregulated systemic inflammatory response in septic infections.[14,15] Importantly, since lipoprotein particles, particularly VLDL, LDL, and HDL, are able to bind and neutralize pathogen-associated lipids such as lipopolysaccharide (LPS) and lipoteichoic acid from the circulation,[14,16] the inhibition of PCSK9 has potentially a dual beneficial effect in treating septic patients. Accordingly, improved survival has been reported in septic patients with loss-of-function mutations in *PCSK9* as well as in situations where the levels of PCSK9 in the plasma are lowered.[14,15,17] In contrast, recent clinical data have suggested that neither loss- nor gain-of-function variants of PCSK9 are associated with morbidity,[18] and that low PCSK9 levels in bacteraemia patients are associated with increased mortality.[19–21] All in all, the contradictory clinical data necessitates additional research into the role of PCSK9 in systemic bacterial infections.

Lay summary

We deleted the proprotein convertase *pcsk9* gene in zebrafish and demonstrated that the lack of *pcsk9* does not cause developmental defects or impact the survival from pneumococcal infection. However, gene expression studies indicated that deleting PCSK9 is associated with lower production of the acute-phase response genes from the liver. Our findings unravel a novel immunoregulatory function for the proprotein convertase PCSK9 in the liver.

Key points

- Deleting pcsk9 in zebrafish using CRISPR/Cas9 mutagenesis does not cause developmental defects or morphological abnormalities.
- Pcsk9 is dispensable for zebrafish survival in a systemic S. pneumoniae infection.
- Pcsk9 KO is associated with reduced expression of acute-phase reaction genes in pneumococcus infected zebrafish.
- PCSK9 siRNA alters the expression of innate immunity genes in a human hepatocellular carcinoma cell line.

We have previously shown that a *Streptococcus pneumoniae* infection can model the pathogenesis of streptococcal bacteraemia in both zebrafish (*Danio rerio*) larvae and in adult fish.[22,23] Although the larvae can be specifically used to study the innate immunity against bacteria, the adult zebrafish model enables systemic and organ-specific studies of the immune response as a whole.[24,25] Furthermore, the zebrafish CRISPR/Cas9 mutagenesis system enables targeted modification of the fish genome to create gene knockout (KO) animals.[26] In this study, we used CRISPR/Cas9 to create two *pcsk9* KO zebrafish lines ($pcsk9^{tpu\text{-}13}$ and $pcsk9^{tpu\text{-}2,+15}$) and determined whether *pcsk9* is critical for zebrafish survival in a systemic *S pneumoniae* infection. Additionally, we used RNA profiling in the

in vivo zebrafish pneumococcal model and in human HepG2 cells to evaluate the immunoregulatory function of PCSK9 at the molecular level in the liver.

2 | EXPERIMENTAL PROCEDURES

2.1 | Zebrafish use and ethics statement

The zebrafish maintenance and the experiments followed the Reporting of In Vivo Experiments (ARRIVE) and EU Directive (2010/63/EU) guidelines and the Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013). The experiments were approved by the Animal Experiment Board of Finland (permits: ESAVI/10079/04.10.06/2015 and ESAVI/2235/04.10.07/2015). F2-generation *pcsk9*$^{tpu-13}$ and *pcsk9*$^{tpu-2,+15}$ mutants were used in the larval infections. In the adult zebrafish experiments, 4- to 7-month-old wild type (WT) AB, F2-generation *pcsk9*$^{tpu-13}$ and F3-generation *pcsk9*$^{tpu-2,+15}$ zebrafish were used. Zebrafish larvae were maintained in E3-medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.0003 g/l methylene blue) at 28.5°C until 7 days postfertilization (dpf). Unchallenged adult fish were maintained in a conventional flow through system (Aquatic Habitats, Florida, USA) with an automated light/dark cycle (14 h/10 h) and fed once a day with Gemma Micro 500 (Skretting, Stavanger, Norway). *S pneumoniae* infected adults were kept in a separate flow through system (Aqua Schwarz GMbH, Göttingen, Germany) or conductivity- (800 μs) and pH- (7.6) adjusted water was manually changed twice a day and the tanks kept at 28.5°C for the duration of the experiment. An identical light/dark cycle compared with the unchallenged fish was used and the fish were fed once a day with Gemma Micro 500 (Skretting). Adult zebrafish infected with pneumococcus were monitored at least two times a day to follow the humane endpoint criteria as defined in the permit for animal experiments.

2.2 | Zebrafish genotyping

Both *pcsk9*$^{tpu-13}$ and *pcsk9*$^{tpu-2,+15}$ mutant zebrafish lines were genotyped using Sanger sequencing and the following primers; F: 5´-AGTAAAGTTGCCCCATGTGG-3´ and R: 5´-TAAGTGCAAAGAGTGTGATTTGG-3´. CRISPR/Cas9 mutagenesis efficiency was estimated with the following formula: % mutagenesis = $100 \times [1 - (1 - \text{fraction of cleavage})^{1/2}]$ [27] and the effects of the indel mutations on the protein sequence using the Translate tool (Expasy; SIB, Swiss Institute of Bioinformatics, https://web.expasy.org/translate/).[28]

2.3 | CRISPR/Cas9 mutagenesis

Nonsense *pcsk9* mutation carrying zebrafish lines (*pcsk9*$^{tpu-13}$ and *pcsk9*$^{tpu-2,+15}$) were created as previously described with slight adjustments.[26,29] Susceptible guide RNA (gRNA) target sites were identified using CHOPCHOP v2 (https://chopchop.cbu.uib.no/),[30] and 170 ng of *pcsk9* exon 3 gRNA together with 300 pg of in-house produced Cas9 protein (Protein Service core facility, Tampere University) (Supplementary Methods) was used for mutagenesis. The Cas9 expression plasmid 1xNLS-pMJ915v2 was a gift from Jennifer Doudna (Addgene plasmid #88915; http://n2t.net/addgene:88915).[31]

2.4 | Experimental *S pneumoniae* infections

The pneumococcal (*S pneumoniae* serotype 4, T4, sequence type 205) culture and infections followed previously established protocols.[22,23] In brief, 2-day-old zebrafish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, Missouri, USA) and 2 nl of bacteria were suspended in 0.2 M potassium chloride (KCl) with 7 mg/ml of tetramethylrhodamine dextran (Thermo Fisher Scientific, Massachusetts, USA) and microinjected into the blood circulation valley. The survival of the larvae was monitored twice a day during the first 50 hours post infection (hpi) and subsequently once a day until 5 days post infection (dpi). Adult zebrafish were similarly anesthetized, and then injected with 5 μl of *S pneumoniae* in a suspension of 10 mM phosphate-buffered saline (PBS) and 0.3 mg/ml phenol red (Sigma-Aldrich) into the abdominal cavity using a 30-gauge Omnican 100 insulin needle (Braun, Melsungen, Germany). Colony forming units (CFU) of viable *S pneumoniae* were counted after culturing inoculates of bacteria on 5% lamb blood agar plates overnight at 37°C with 5% CO_2. To create randomized and blinded experimental set up for the survival experiments, both fish lines were infected prior genotyping as larvae, whereas the *pcsk9*$^{tpu-2,+15}$ fish line was infected prior genotyping in the adult fish experiments. Thirteen samples were excluded from the larvae survival analysis and four samples from the adult zebrafish experiments because of failed genotyping. Acclimatization periods were not used prior infections.

2.5 | RNA isolation and quantitative PCR

Adult zebrafish tissues or HepG2 cells were homogenized and the removal of genomic DNA (gDNA) and RNA isolation were performed according to the manufacturer's instructions using the RNeasy Mini Kit or RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Reverse transcriptions were done using the iScript cDNA synthesis kit (Bio-Rad Laboratories, California, USA), and the relative gene expression of the target genes were determined with quantitative PCR (qPCR) using the PowerUp SYBR master mix (Thermo Fisher Scientific) and either the CFX96 (Bio-Rad Laboratories) or the QuantStudio 12K Flex (Applied Biosystems, California, USA) detection systems. CFX Manager software (v.3.1; Bio-Rad Laboratories) and QuantStudio 12K Flex Software (v.1.2.2; Applied Biosystems) was used for data analysis. The target gene expression was normalized to either the *eukaryotic translation elongation factor 1 alpha 1, like 1* (*eef1a1l1* or *ef1a*, zebrafish

data) expression [32] or to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*, HepG2 data) using the 2^(-ΔCt) method. PCR contamination as well as the specificity of the qPCR products were monitored using non-template (H_2O) as well as no-reverse transcriptase controls and performing a melt curve analysis and 1.5% agarose TAE gel electrophoresis. qPCR primer sequences are depicted in Table S1.

2.6 | RNA sequencing

The RNA quality was controlled with the Fragment Analyzer system (Advanced Analytical, Inc, Ankeny, USA) and the Standard Sensitivity RNA Analysis Kit (15 nt) (Advanced Analytical). RNA sequencing was done by Novogene Co. (Hong Kong, Special Administrative Region of the People's Republic of China) and the data were provided in a FASTQ format. Detailed description of the differential gene expression analysis is found in the Supplementary Methods.

2.7 | Cell culture and transfections

The human HCC cell line HepG2 was cultured in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 6 mM of both penicillin and streptomycin at 37°C and 5% CO_2. ON-TARGETplus Non-targeting Control Pool (Dharmacon, Colorado, USA) or ON-TARGETplus Human PCSK9 small interfering RNA (siRNA)—SMART pool (GE Dharmacon) was transfected at a final concentration of 6 nM using Transfection Reagent 4 (GE Dharmacon).

2.8 | Statistical analysis

Sample sizes are based on our previous calculations and experimental observations.[23,33] Statistical analyses were performed with the Prism program (v. 5.02, GraphPad Software, Inc, California, USA). In zebrafish survival experiments a log-rank (Mantel-Cox) test was used to compare differences between the experimental groups, whereas a nonparametric two-tailed Mann-Whitney was used to analyse the zebrafish qPCR data. A 2-tailed *t* test was used in the analysis of HepG2 qPCR results. Statistics of the RNA sequencing have been described in the Supplementary Methods. *P*-values (or the adjusted *P*-value in RNA sequencing data) less than .05 was considered statistically significant.

3 | RESULTS

3.1 | Nonsense *pcsk9*$^{tpu-13/tpu-13}$ and *pcsk9*$^{tpu-2,+15/tpu-2,+15}$ mutant zebrafish have diminished *pcsk9* expression and are morphologically comparable with WT fish

The engineered type II CRISPR/Cas system known as CRISPR/Cas9 is an efficient tool for reverse genetics,[27,34] and it has been widely used in both cell and animal models of human diseases. Here, we used CRISPR/Cas9 mutagenesis to disrupt the open reading frame of zebrafish *pcsk9* (ENSDARG00000074185), and subsequently to create *pcsk9* KO zebrafish lines. By using a functional gRNA that targeted the third exon of *pcsk9* gene, we could induce parental (F0-generation) insertion/deletion (indel) mutations at the target site with an average efficiency of 40% (Figure 1A and 1B). Sequencing the mutated loci in the outcrossed F1-progeny [F0-generation × WT (Tüpfel long fin) zebrafish] revealed 2 germ-line transmitted nonsense *pcsk9* mutations; one with a loss of 13 bp (AACCTGCAGCGGG) and another with a loss of 2 bp (TG) and gain of 15 bp (AGCATCCATGCGAAC) leading to disrupted reading frames after 147 and 148 amino acids (aa) and to predicted premature stop-codons at the beginning of the core-protein regions at 173 aa and 159 aa respectively (Figure 1C). The *pcsk9* KO lines were concomitantly named *pcsk9*$^{tpu-13}$ (loss of 13 bp mutation) and *pcsk9*$^{tpu-2,+15}$ (loss of 2 bp and gain of 15 bp mutation).

Our previous work has demonstrated that CRISPR/Cas9-mediated indel mutations not only affect the protein translation, causing inability to produce intact protein, but can also lead to changes in the transcription of the target gene.[26] In line with this, qPCR quantification of *pcsk9* expression in adult steady-state zebrafish revealed significantly lower *pcsk9* mRNA levels in homozygous *pcsk9*$^{tpu-13/tpu-13}$ and *pcsk9*$^{tpu-2,+15/tpu-2,+15}$ mutants (residual expression medians of 15.9% and 8.0%; $P = .008$ in both comparisons) compared with their WT siblings (Figure 1D). Importantly, although earlier knock-down studies using morpholinos in zebrafish have indicated a crucial role for *pcsk9* in neural development,[35] *pcsk9* KO zebrafish did not have any apparent developmental abnormalities during the first 7 dpf (Figure S1). Furthermore, as adults, *pcsk9*$^{tpu-13/tpu-13}$ and *pcsk9*$^{tpu-2,+15/tpu-2,+15}$ mutants were morphologically similar to the WT fish (Figure 1E).

3.2 | *pcsk9* deficiency is dispensable for the survival of zebrafish upon a systemic pneumococcal infection

Both experimental and clinical data about the harmful/beneficial effects of PCSK9 in sepsis are contradictory.[14,17-21,36] To study the significance of the *pcsk9* gene in a systemic *S pneumoniae* infection of adult zebrafish,[23] we inoculated bacteria (4 700 000 CFU; SD 990 000 CFU) into the abdominal cavity of fish from both *pcsk9* mutation carrying lines; *pcsk9*$^{tpu-13}$ and *pcsk9*$^{tpu-2,+15}$. After the 7-day follow-up, we observed average mortalities of 31.8% and 15.6% in the *pcsk9*$^{tpu-13}$ and *pcsk9*$^{tpu-2,+15}$ zebrafish lines respectively (Figure 2A and 2B). However, we did not observe any statistically significant difference in the survival of homozygous *pcsk9*$^{tpu-13/tpu-13}$ mutants (76.2% survival) compared with the corresponding heterozygous *pcsk9*$^{tpu-13/+}$ (70.8% survival; $P = .31$) and WT siblings (57.7%; $P = .16$) (Figure 2A), nor when comparing *pcsk9*$^{tpu-2,+15/tpu-2,+15}$ mutants (80.0% survival) with their sibling controls (90.7% of *pcsk9*$^{tpu-2,+15/+}$; $P = .30$ and 82.6% of WT fish; $P = .79$) (Figure 2B).

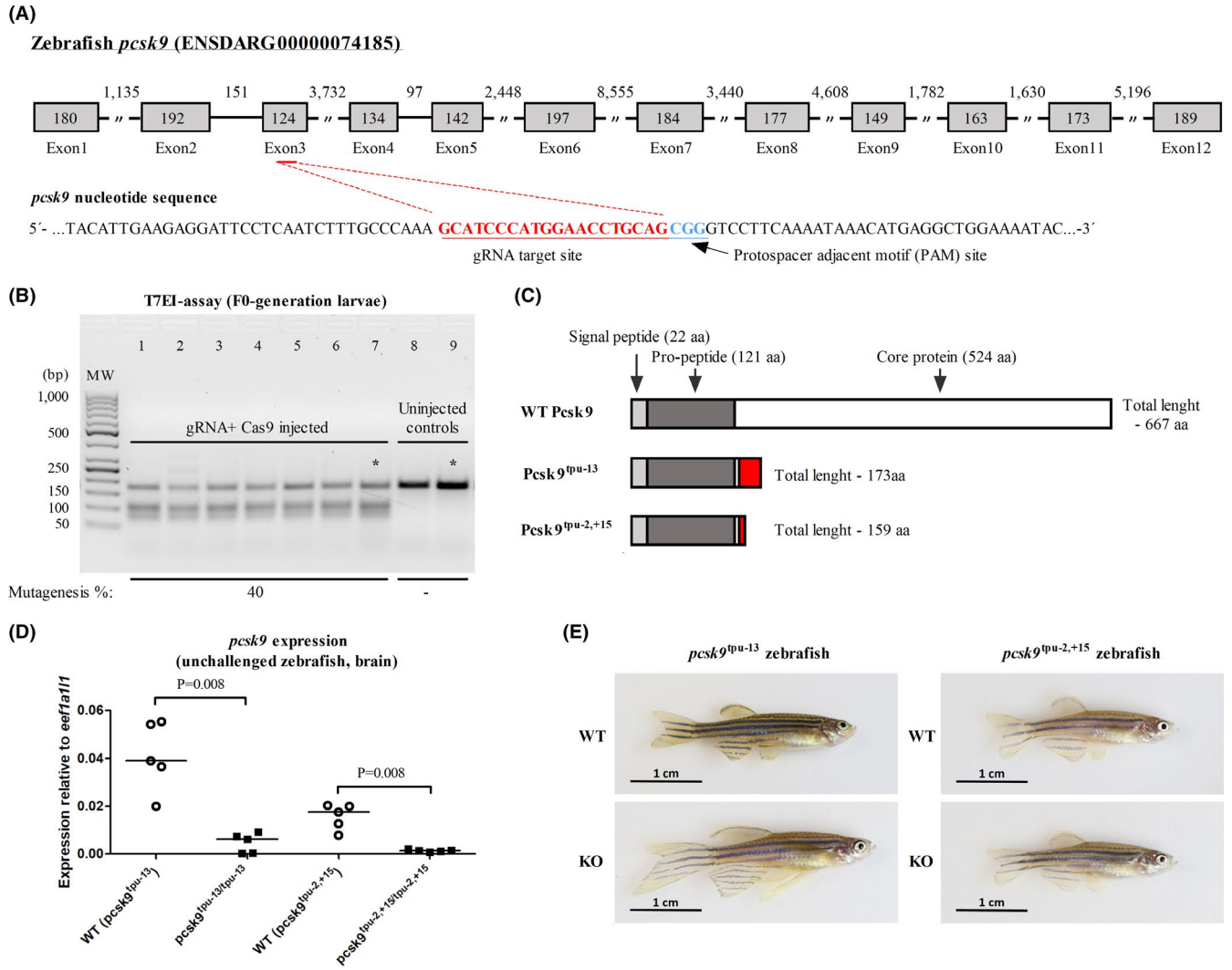

FIGURE 1 Homozygous *pcsk9*$^{tpu-13/tpu-13}$ and *pcsk9*$^{tpu-2,+15/tpu-2,+15}$ mutant zebrafish show reduced *pcsk9* expression but normal morphology. A, A schematic representation of zebrafish *pcsk9* (ENSDARG00000074185), and the gRNA target site for CRISPR/Cas9 mutagenesis in the third exon. B, The in vivo mutagenesis efficiency was estimated in the gRNA and Cas9 protein injected F0-generation embryos and in the uninjected controls using a T7 endonuclease I (T7EI) assay and 2.5% agarose TAE gel electrophoresis. The uninjected controls: a 169-bp WT PCR product (lanes 8 and 9); the gRNA and Cas9-injected mutant embryos: three bands of 169 bp (WT), ~100 bp and ~70 bp (lanes 1-7). The mutagenesis efficiency was calculated as described previously.[27] C, A schematic representation of the indel mutations (−13 bp deletion, *pcsk9*$^{tpu-13}$ and −2 bp deletion, +15 bp insertion, *pcsk9*$^{tpu-2,+15}$), leading to truncated protein products of 173 and 159 amino acids (aa) respectively. Red boxes indicate altered amino acids caused by the frameshift. D, The expression of *pcsk9* was determined in the brain of *pcsk9*$^{tpu-13/tpu-13}$ (n = 5; 2 females, 3 males) and *pcsk9*$^{tpu-2,+15/tpu-2,+15}$ zebrafish (n = 5; 2 females, 3 males) and in the WT siblings (n = 5 in both control groups; 2 females, 3 males) with qPCR. Gene expression levels were normalized to *eef1a1l1* and a 2-tailed Mann-Whitney test was used for statistics. E, Anesthetized WT and homozygous *pcsk9* mutant zebrafish were imaged using a Canon EOS 7D Mark II camera with an exposure time of 17 ms. Fish were kept submerged in water during image acquisition. Images in (B) and (E) were cropped to exclude empty background from the figure

The host defense against pneumococcus is critically dependent on the innate immune response.[22] Consequently, we next used zebrafish larvae to specifically address if PCSK9 is important for the protective innate immunity in a pneumococcal infection. To this end, we microinjected *S pneumoniae* (240 CFU; SD 76 CFU) into the blood circulation valley of the F2-generation progeny of heterozygous *pcsk9*$^{tpu-13/+}$ and *pcsk9*$^{tpu-2,+15/+}$ zebrafish at 2 dpf and followed their survival for 5 days. At the end of the experiment, an average of 25.7% (*pcsk9*$^{tpu-13}$ background) and 77.7% (*pcsk9*$^{tpu-2,+15}$ background) survival was observed (Figure 2C and 2D). More specifically, 20.8% of the *pcsk9*$^{tpu-13/tpu-13}$, 39.6% of the *pcsk9*$^{tpu-13/+}$ and 16.7% of the WT (*pcsk9*$^{tpu-13}$ background) fish (Figure 2C) had survived, whereas survival fractions of 58.8% in the *pcsk9*$^{tpu-2,+15/tpu-2,+15}$, 87.5% in the *pcsk9*$^{tpu-2,+15/+}$ and 86.7% in the WT (*pcsk9*$^{tpu-2,+15}$) fish were seen (Figure 2D). Similarly to our experiments in adult zebrafish, no statistically significant differences in the survival rates of the *pcsk9* KO and WT larvae were observed ($P = .57$ and $P = .078$ in *pcsk9*$^{tpu-13}$ and *pcsk9*$^{tpu-2,+15}$ lines respectively), although the difference between *pcsk9* KO and heterozygous *pcsk9*$^{tpu-2,+15/+}$ larvae was significant ($P = .038$). Overall, we conclude that *pcsk9* is

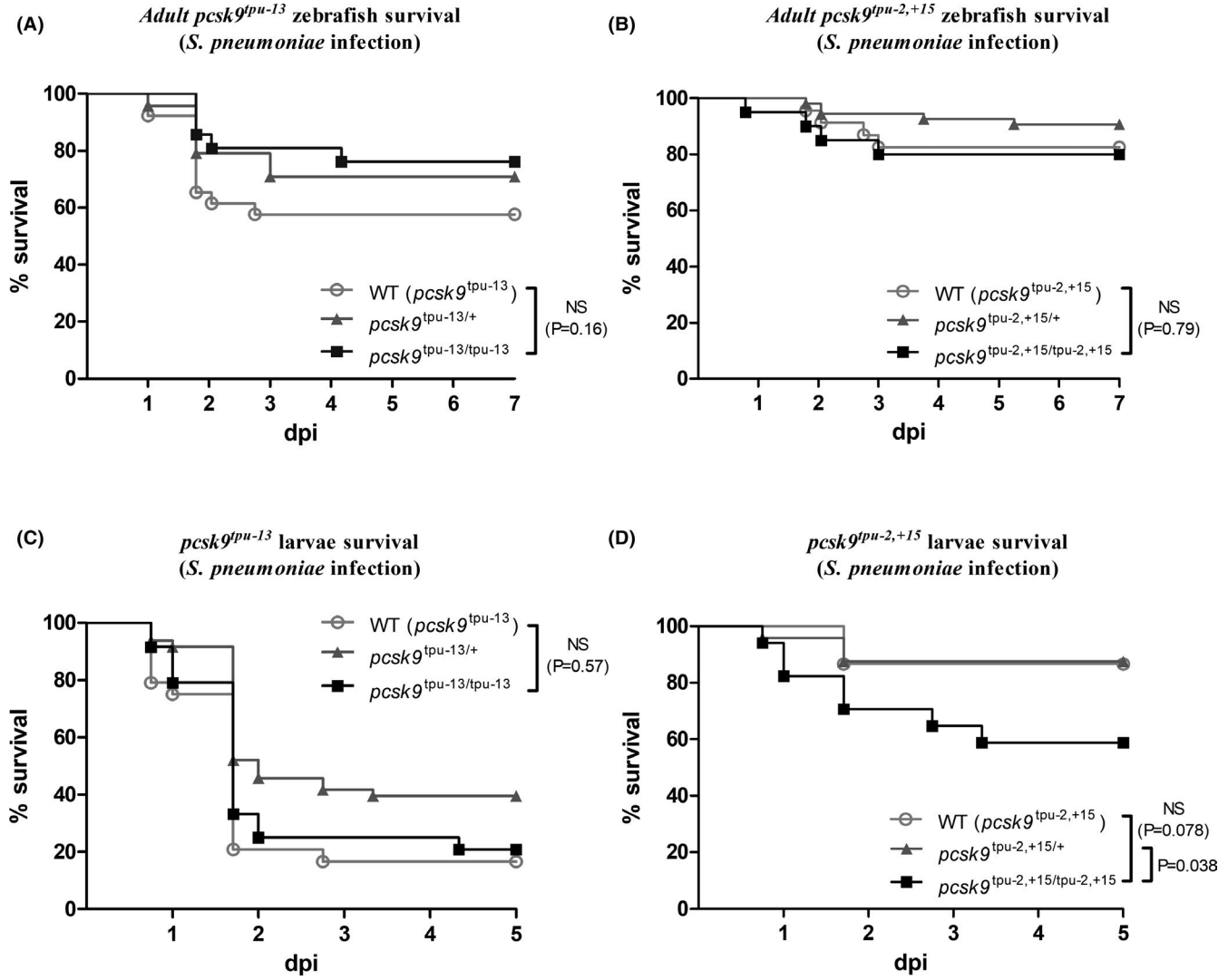

FIGURE 2 Nonsense mutations in *pcsk9* do not affect zebrafish survival upon an *S pneumoniae* infection. *S pneumoniae* (4 700 000 CFU; SD 990 000 CFU) was injected into the abdominal cavity of (A) $pcsk9^{tpu\text{-}13/tpu\text{-}13}$ (n = 21; 8 females, 13 males; survived: n = 16; 8 females, 8 males), $pcsk9^{tpu\text{-}13/+}$ (n = 24; 7 females, 17 males; survived: n = 17; 6 females, 11 males) and WT ($pcsk9^{tpu\text{-}13}$) (n = 26; 5 females, 21 males; survived: n = 15; 4 females, 11 males) as well as (B) $pcsk9^{tpu\text{-}2,+15/tpu\text{-}2,+15}$ (n = 20; 4 females, 16 males; survived: n = 16; 3 females, 13 males), $pcsk9^{tpu\text{-}2,+15/+}$ (n = 54; 10 females, 44 males; survived: n = 49; 9 females, 40 males) and WT ($pcsk9^{tpu\text{-}2,+15}$) (n = 23; 12 females, 11 males; survived: n = 19; 10 females, 9 males) adult zebrafish, and their survival was followed for 7 days. *Pneumococcus* infected (240 CFU; SD 76 CFU) zebrafish larvae from both the (C) $pcsk9^{tpu\text{-}13}$ and (D) $pcsk9^{tpu\text{-}2,+15}$ background were followed until 5 dpi and their survival was recorded. Group sizes; $pcsk9^{tpu\text{-}13/tpu\text{-}13}$ (n = 24; survived: n = 5), $pcsk9^{tpu\text{-}13/+}$ (n = 48; survived n = 19), WT ($pcsk9^{tpu\text{-}13}$) (n = 24; survived n = 4) and $pcsk9^{tpu\text{-}2,+15/tpu\text{-}2,+15}$ (n = 17; survived n = 10), $pcsk9^{tpu\text{-}2,+15/+}$ (n = 24; survived n = 21), WT ($pcsk9^{tpu\text{-}2,+15}$) (n = 15; survived n = 13). The data were collected from single experiments. A log-rank (Mantel-Cox) test was used for statistics. dpi, days post infection

not critical for the adult or larvae zebrafish survival on a *S pneumoniae* infection.

3.3 | Expression of *pcsk9* is upregulated on a *S pneumoniae* infection, and it is required for a normal acute-phase response

Previously, it has been shown that hepatic PCSK9 expression is increased in LPS-induced inflammation,[37] and that plasma PCSK9 levels are upregulated in a liver-dependent manner in bacteraemia patients.[21] To directly test whether hepatic PCSK9 is upregulated in a pneumococcal infection, we injected adult WT zebrafish (AB line) with PBS or *S pneumoniae* (635 000 CFU; SD 276 000 CFU) into the abdominal cavity and quantified the expression of *pcsk9* in the liver at 7 dpi. Our qPCR analysis revealed a 4.5-fold increase ($P = .008$) in the relative levels of *pcsk9* mRNA in the pneumococcus infected zebrafish (Figure 3A), suggesting that the inflammation-mediated inducibility of hepatic PCSK9 is evolutionarily conserved.

To gain a genome-wide perspective on the PCSK9-dependent liver-specific transcriptional response in a *S pneumoniae* infection, we performed RNA sequencing on the livers of unchallenged, PBS injected and *S pneumoniae* infected (1 dpi, 3 370 000 CFU; SD 840 000 CFU) adult $pcsk9^{tpu\text{-}13/tpu\text{-}13}$ zebrafish and their WT siblings.

The principal component analysis revealed that the experimental group was the largest variant affecting the sample clustering (Figure S2). In addition, the *pcsk9*$^{tpu-13/tpu-13}$ mutants and the WT (*pcsk9*$^{t-pu-13}$) controls grouped separately among the unchallenged and the pneumococcus infected fish, indicating *pcsk9*-mediated effects also on gene expression in the liver. In fact, a comparison of transcript levels in *pcsk9*$^{tpu-13/tpu-13}$ and WT fish using DESeq2, revealed a total of 828 differentially expressed genes, of which 609 (adjusted *P*-value < .05) were differentially expressed at steady-state, 81 on a PBS injection and 241 in a *S pneumoniae* infection (Figure 3B, Tables S2-S4). Although 87 genes were expressed differentially in at least two different experimental groups, most of the identified transcripts were specific for a particular treatment; 526 transcripts in unchallenged zebrafish, 25 in the PBS-injected group and 190 in infected fish.

To decipher the immunoregulatory functions of PCSK9 at a molecular level in vivo on infection, we next focused on the 112 up- and 77 down-regulated protein coding genes in the *S pneumoniae* challenged *pcsk9*$^{tpu-13/tpu-13}$ mutants compared with the WT controls. To this end, gene ontology (GO) enrichment analysis of the induced transcripts in the *pcsk9* KO zebrafish, revealed a total of 15 enriched processes that were related to hormonal responses, such

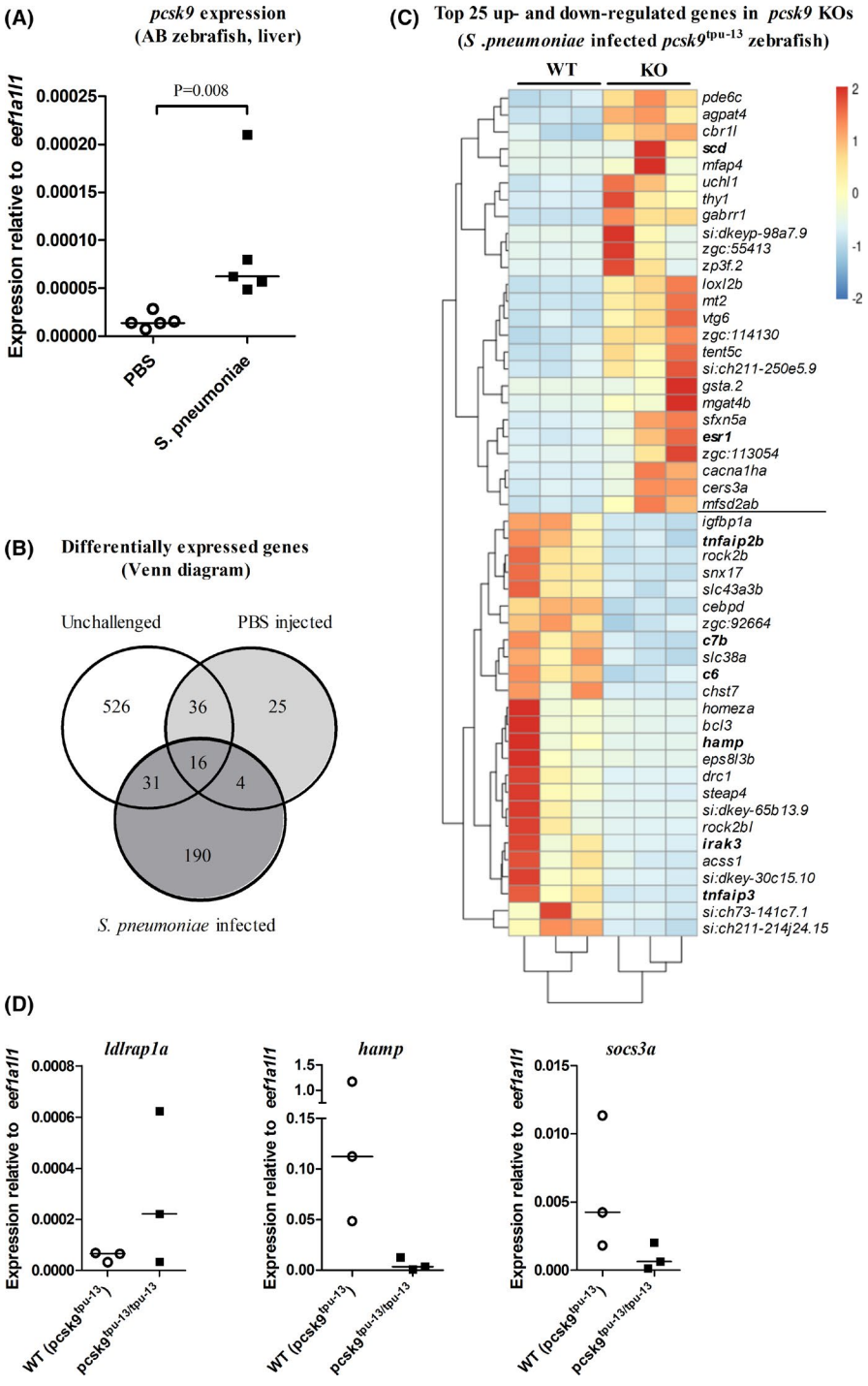

FIGURE 3 *pcsk9* is up-regulated on a pneumococcal infection and associated with the expression of acute-phase reactants. A, The relative expression of *pcsk9* was determined in the liver of PBS injected (n = 5; all males) and *S pneumoniae* infected (635 000 CFU; SD 276 000 CFU, n = 5; all males) WT AB zebrafish at 7 dpi using qPCR. Gene expression levels were normalized to *eef1a1l1* expression and target genes were run once as technical duplicates. A 2-tailed Mann-Whitney test was used for statistics. B-C, RNA was isolated from the liver of unchallenged (n = 2 in both groups; 2 females/group), PBS injected (n = 3 in both groups; 3 females/group) and *S pneumoniae* infected (3 370 000 CFU; SD 840 000 CFU, n = 3 in both groups; 3 females/group) adult *pcsk9*$^{tpu-13/tpu-13}$ and WT (*pcsk9*$^{tpu-13}$) zebrafish at 1 dpi and the transcriptome was analysed using RNA sequencing. B, Venn diagram for the differentially expressed genes within treatment groups. C, The top 25 up- and down-regulated genes in *S pneumoniae* infected *pcsk9*$^{tpu-13/tpu-13}$ zebrafish in comparison with the WT controls are depicted using a heat-map. Statistics were done using DESeq2 and adjusted using the Benjamini-Hochberg (BH) method. D, The relative expression of selected genes up- or down-regulated in the RNA sequencing data (*ldlrap1a*, *hamp* and *socs3a*) was quantified using qPCR. Gene expression levels were normalized to *eef1a1l1* expression and target genes were run once as technical duplicates

as the cellular response to an estrogen stimulus (GO:0071391), but also to a lipid metabolism, e.g., the response to lipids (GO:0033993) and lipid transport (GO:0006869) (Table 1). Among the up-regulated genes in *pcsk9* KO fish, we found *stearoyl-CoA desaturase* (*scd*, log 2-fold change 5.1, $P < .001$), *estrogen receptor 1* (*esr1*, log 2-fold change 3.5, $P < .001$) as well as several *vitellogenin* (eg *vtg3*, log 2-fold change 2.3, $P = .010$) genes (Figure 3C, Table S5). It is additionally important to note that the *PCSK9* promoter region contains estrogen response elements (EREs),[38] which can in principle explain the effects on *esr1* expression in the $pcsk9^{tpu\text{-}13/tpu\text{-}13}$ mutants. Interestingly, among the genes induced in $pcsk9^{tpu\text{-}13/tpu\text{-}13}$ zebrafish we also observed a 2.5-fold induction (log 2 change, $P = .049$) in the low-density *lipoprotein receptor adaptor protein 1a* (*ldlrap1a*) gene, encoding a homologue of the human mediator of LDLR endocytosis.[39]

Importantly, the GO-analysis of the down-regulated genes showed that from a total of six processes two were directly related to the immune system; the immune system process (GO:0002376) and the humoral immune response (GO:0006959) (Table 2). In fact, among the 78 genes whose expression levels were reduced in the $pcsk9^{tpu\text{-}13/tpu\text{-}13}$ fish, we found at least 18 genes (23%) with immunological functions, including *hepcidin antimicrobial peptide* (*hamp*, log 2-fold change −5.8, $P < .001$), *complement component 7b* (*c7b*, log 2-fold change −3.53, $P < .001$), *interleukin-1 receptor-associated kinase 3* (*irak3*, log 2-fold change −3.29, $P < .001$), *tumour necrosis factor, alpha-induced protein 3* (*tnfaip3*, log 2 fold change −3.02, $P < .001$) and *suppressor of cytokine signalling 3a* (*socs3a*, log 2 fold change −2.97, $P = .008$) (Figure 3C, Table S5). We replicated our RNA sequencing analysis for *ldlrap1a* (3.4-fold increase in median), *hamp* (residual expression median of 3.1%), and *socs3a* (residual expression median of 15.0%) using qPCR (Figure 3D). Overall, our findings indicate that upon a *S pneumoniae* infection *pcsk9* expression becomes up-regulated in vivo in the zebrafish liver, and that the lack of *pcsk9* favours the expression of genes associated with lipid and estrogen metabolism, whereas several liver-expressed acute-phase reaction (APR) genes are down-regulated.

3.4 | PCSK9 regulates the expression of innate immunity genes in human HepG2 cells

Suggesting a direct regulatory function for PCSK9 in the immune response in the absence of a microbial insult, in vitro administration of recombinant PCSK9 into a mouse and human macrophage culture was shown to induce the expression of genes coding for pro-inflammatory cytokines such as TNF, IL6 and IL1B.[13] To address whether PCSK9 controls the expression of the above identified innate immunity genes also in human hepatocytes, we knocked-down *PCSK9* expression in HepG2 cells (residual expression median of 41.8% compared with controls) (Figure 4, Figure S3A), and quantified the expression of *HAMP*, *C7*, *SOCS3*, *TNF*, *TNFAIP3*, *C6* and *LDLRAP1* using qPCR (Figure 4). In these experiments the mRNA levels of *HAMP* (25% decrease in median, $P < .001$), *C7* (1.41-fold increase in median, $P = .008$), *SOCS3* (1.29-fold increase in median 1.29, $P = .019$) and *TNF* (2.52-fold increase in median, $P = .017$) were significantly affected by the reduced PCSK9 expression. In addition, western blot analysis confirmed the down-regulation of HAMP also at the protein level (Figure S3B), underscoring the direct immunoregulatory role of PCSK9 also in a human hepatocyte cell line.

4 | DISCUSSION

PCSK9 antibodies efficiently lower cholesterol and provide protection against CHD, but whether inhibiting PCSK9 could also be leveraged to prevent an exacerbated immune response in septic patients remains under investigation. Here, we used the CRISPR/Cas9 system to create two zebrafish lines with nonsense *pcsk9* mutations ($pcsk9^{tpu\text{-}13}$ and $pcsk9^{tpu\text{-}2,+15}$) and demonstrated that the mutant zebrafish have a normal morphology and development. Infecting *pcsk9* mutant fish with *S pneumoniae* did not reveal any differences in the mortality of either the larvae or the adult fish compared with controls. Nevertheless, the expression of *pcsk9* was induced on a pneumococcal challenge, and the genome-wide expression analysis of zebrafish liver revealed the down-regulation of several genes associated with the innate immunity, suggesting a role in the innate host response against a pneumococcus infection. Accordingly, silencing *PCSK9* in a human hepatocarcinoma cell line (HepG2) led to decreased *HAMP* expression as well as to alterations in the expression of other innate immune genes such as *TNF*, *C7* and *SOCS3*.

In mammals, PCSK9 is expressed not only in the liver but also in, e.g., the pancreas and brain.[40,41] Our previous analysis of genes of the *pcsk* family in adult zebrafish tissues revealed high relative *pcsk9* expression in the fish brain,[42] which is in accordance with an earlier report, where *pcsk9* was inhibited with morpholinos and a critical role for PCSK9 in neural tissues and early fish development were described.[35] By creating targeted nonsense mutations, we have demonstrated that the CRISPR/Cas9 mutagenesis method can be efficiently used to knock out genes of interest in zebrafish.[26,29] Accordingly, we created 2 *pcsk9* KO zebrafish lines; $pcsk9^{tpu\text{-}13}$ and $pcsk9^{tpu\text{-}2,+15}$, with disrupted open reading frames (residual expression medians of the mutated mRNAs in the brain; 15.9% and 8.0%, respectively). However, in contrast with previously published data demonstrating the absence of tectum and midbrain-hindbrain boundary in the *pcsk9* morphants at 24 hours post fertilization,[35] homozygous $pcsk9^{tpu\text{-}13/tpu\text{-}13}$ and $pcsk9^{tpu\text{-}2,+15/tpu\text{-}2,+15}$ mutants did not show morphological abnormalities during embryogenesis prior to 7 dpf. Moreover, adult fish were obtained in predicted Mendelian ratios, a result that is in line with the normal development of *Pcsk9* KO mouse and humans with null *PCSK9* alleles.[43] Clarity to whether the differing developmental results in zebrafish are indicative of morpholino off-target effects caused by p53-mediated neural cell death [44] or genetic compensation observed in experimental KO animals [45] could add to our understanding of the extra-hepatic functions of PCSK9.

TABLE 1 Enriched processes from a gene ontology (GO) analysis of up-regulated genes in *S pneumoniae* infected zebrafish

GO term	Description	*P* value
GO:0071391	Cellular response to estrogen stimulus	6.03 E−12
GO:0043627	Response to estrogen	1.63 E−11
GO:0032355	Response to estradiol	1.67 E−10
GO:0033993	Response to lipid	8.23 E−08
GO:0014070	Response to organic cyclic compound	2.00 E−06
GO:1901700	Response to oxygen-containing compound	5.72 E−06
GO:0009719	Response to endogenous stimulus	7.62 E−06
GO:0009725	Response to hormone	2.31 E−05
GO:0070887	Cellular response to chemical stimulus	2.49 E−05
GO:0042221	Response to chemical	3.12 E−05
GO:0010033	Response to organic substance	4.49 E−05
GO:0006869	Lipid transport	1.70 E−04
GO:0009082	Branched-chain amino acid biosynthetic process	2.91 E−04
GO:0046394	Carboxylic acid biosynthetic process	5.21 E−04
GO:0016053	Organic acid biosynthetic process	5.40 E−04

Note: A genome-wide transcriptome analysis in zebrafish liver was performed using RNA sequencing and a GO enrichment analysis was used to study the up-regulated transcripts in the *pcsk9*$^{tpu\text{-}13/tpu\text{-}13}$ mutants challenged with *S pneumoniae* compared with the WT controls. A target list of differentially expressed genes (adj. $P < .05$) was compared with a background list (adj. $P \geq .05$).

Abbreviation: GO term, gene ontology term.

TABLE 2 Enriched processes from a gene ontology (GO) analysis of down-regulated genes in *S pneumoniae* infected zebrafish

GO term	Description	*P* value
GO:0070589	Cellular component macromolecule biosynthetic process	5.89 E−05
GO:0044038	Cell wall macromolecule biosynthetic process	5.89 E−05
GO:0044036	Cell wall macromolecule metabolic process	1.18 E−04
GO:0002376	Immune system process	1.34 E−04
GO:0051241	Negative regulation of multicellular organismal process	4.20 E−04
GO:0006959	Humoral immune response	6.42 E−04

Note: A genome-wide transcriptome analysis in zebrafish liver was performed using RNA sequencing and a GO enrichment analysis was used to study the down-regulated transcripts in the *pcsk9*$^{tpu\text{-}13/tpu\text{-}13}$ mutants challenged with *S pneumoniae* compared with the WT controls. A target list of differentially expressed genes (adj. $P < .05$) was compared with a background list (adj. $P \geq .05$).

Abbreviation: GO term, gene ontology term.

Sepsis is a clinical syndrome caused by the interplay of micro-organisms and an exacerbated immune reaction leading to multiple organ failure.[46] Although mammalian sepsis models remain highly important for pre-clinical drug development, they are laborious, expensive and raise ethical concerns. Zebrafish is a non-mammalian alternative for studying host-pathogen interactions in vivo. As it takes several weeks for lymphocytes and the adaptive immune system to develop in zebrafish, the fish larvae can be used to specifically study the innate immunity.[24] Importantly, adult zebrafish have a highly similar immune system compared with humans with both innate immune cells, lymphocytes [24] as well as humoral components like complement components.[47] To assess how the KO of *pcsk9* affects zebrafish mortality on a pneumococcal challenge, we infected both zebrafish larvae and adult fish with *S pneumoniae* and followed their survival until 5 or 7 dpi respectively. Similarly to studies using LPS-induced endotoxemia in *Pcsk9* KO mice,[36] the mortality of pneumococcus infected homozygous *pcsk9*$^{tpu\text{-}13/tpu\text{-}13}$ and *pcsk9*$^{tpu\text{-}2,+15/tpu\text{-}2,+15}$ mutants was comparable with the WT controls in both the larvae and adult zebrafish. Overall, we conclude that PCSK9 is not essential for zebrafish survival in a systemic *S pneumoniae* infection.

More than 70% of the human genes have zebrafish orthologues,[48] and consequently gene KO zebrafish accompanied with genome-wide transcriptome analysis can be used to model both systemic and tissue-specific functions of human genes.[25,26] Here, our genome-wide transcriptomic analysis of pneumococcus infected *pcsk9*$^{tpu\text{-}13/tpu\text{-}13}$ mutants and WT fish revealed a substantial proportion (18/78 genes)

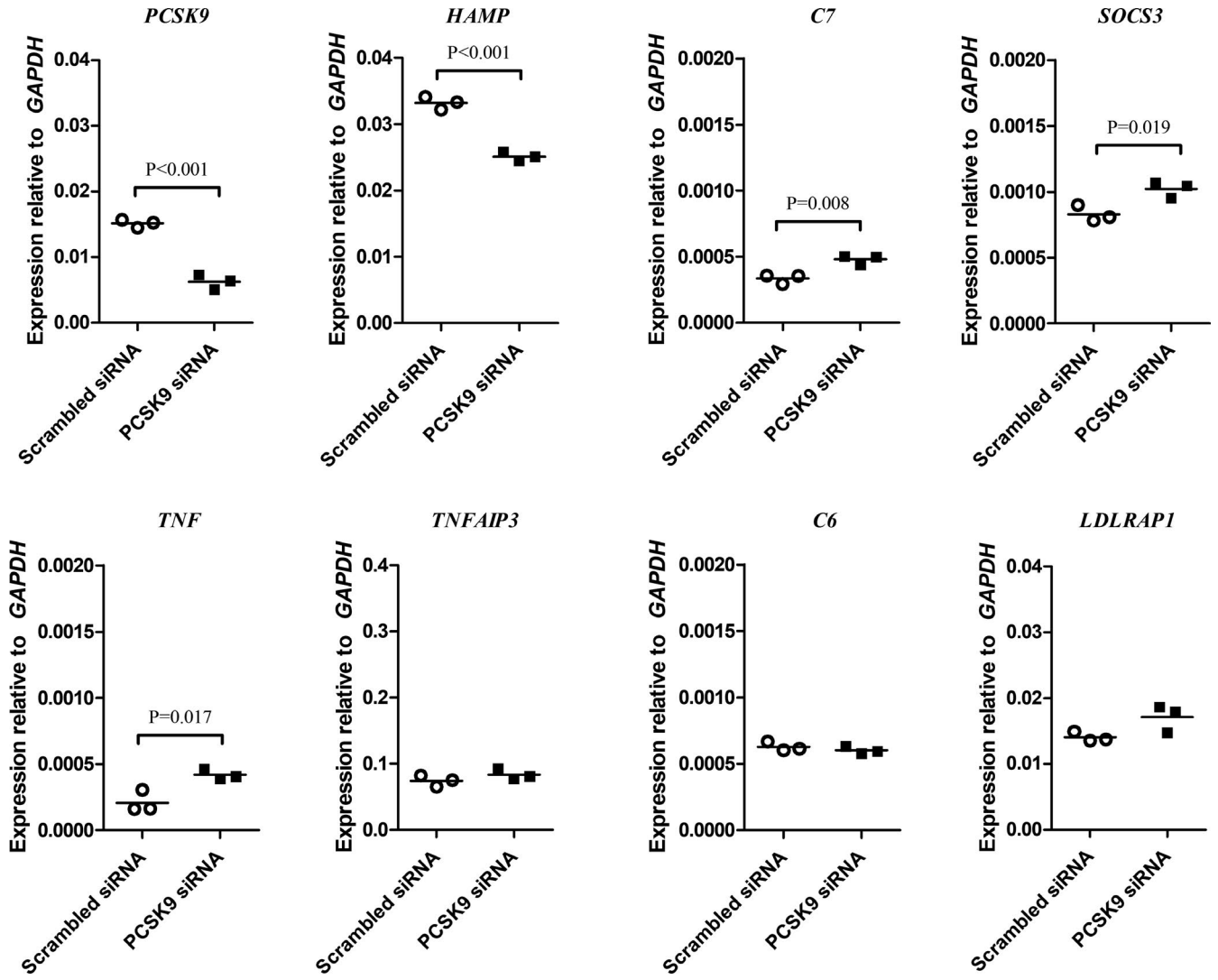

FIGURE 4 Silencing *PCSK9* influences the expression of genes of the innate immune response in HepG2 cells. The relative expression levels of *PCSK9*, *HAMP*, *C7*, *SOCS3*, *TNF*, *TNFAIP3*, *C6* and *LDLRAP1* were determined in control (n = 3) and *PCSK9* siRNA (n = 3) transfected HepG2 cells using qPCR. Gene expression levels were normalized to *GAPDH* expression and target genes were run once as technical duplicates. A 2-tailed *t* test was used for statistics

of immunological genes among the down-regulated transcripts in *pcsk9* KOs. From these genes, two complement system protein coding genes *c6* and *c7b* had significantly lower levels of mRNA in the *pcsk9*$^{t-pu-13/tpu-13}$ mutants in comparison with the WT controls. Additionally, we identified down-regulation of well-known immunoregulatory genes such as *tnfaip2b*, *nuclear factor kappa B subunit 2* (*nfkb2*), *irak3*, *socs3a* and *tnfaip3* [49-51] in the *pcsk9* KO zebrafish. In a human hepatocellular carcinoma cell line, we demonstrate that silencing *PCSK9* directly impacts the expression of innate immunity genes such as *C7*, *SOCS3* and *TNF*. However, there were notable differences between the zebrafish and HepG2 data, which can probably be attributed to the facts that the liver contains several different cell types,[13] and that there was no infectious insult in our in vitro model system.

HAMP encodes a liver-produced antimicrobial peptide, also known as hepcidin, that can reduce the concentration of iron in the blood and subsequently deprive it from micro-organisms.[52] Our RNA sequencing data of zebrafish liver indicated that *hamp* expression is induced on a *S pneumoniae* challenge in zebrafish, and that *hamp* mRNA levels in the infected *pcsk9* KO zebrafish were reduced compared with the WT controls at 1 dpi. Furthermore, in line with previously published data that the in vitro administration of recombinant PCSK9 can regulate the expression of pro-inflammatory cytokine genes in the absence of inflammatory stimuli,[13] silencing *PCSK9* in vitro in HepG2 cells reduced *HAMP* expression levels. Since HAMP is a major regulator of iron homeostasis and its levels are linked to different forms of anaemias,[53] the positive correlation between *PCSK9* and *HAMP* expression also at steady-state could have clinical implications. In fact, a recent study in mice demonstrated that non-hematopoietic anaemia, independent of LDLR expression, was more severe in *Pcsk9* KO mice.[54] It is important to note that conventional PCSK enzymes FURIN, PCSK5, PCSK6 and PCSK7 have been demonstrated to directly process pro-hepcidin,[55] whereas FURIN has been reported to process PCSK9.[56] It remains to be confirmed whether there is a direct causal relationship between lowered HAMP levels and PCSK9 or if this is also affected by the other PCSK family members. Collectively, our

gene expression analyses unravel that the lack of *pcsk9/PCSK9* transcriptionally impacts APR, arguing for an immunostimulatory role for PCSK9 on a bacterial challenge and independent of infection.

The dual role of PCSK9 in regulating hepatocytic lipoprotein uptake [3,4] and the inflammatory response [13,14] has made this proprotein convertase an attractive candidate for studying sepsis. Although some data indicate that normal PCSK9 levels are associated with a better prognosis,[20,21] and that this effect could be age dependent,[19] other studies support the idea that blocking PCSK9 activity could improve the outcome of treatment in septic patients.[14,15,17] In fact, the PCSK9 inhibiting antibodies alirocumab (Praluent, Sanofi-Regeneron) and evolocumab (Repatha, Amgen) are being tested in clinical trials to treat septic patients (NCT03634293 and NCT03869073 respectively). Although we did not see any difference in the survival of *S pneumoniae* infected *pcsk9* KO and WT zebrafish, our transcriptomic data show that PCSK9 regulates the production of acute-phase reactants such as hepcidin and complement components. Although the direct role of LDLR-signalling remains to be investigated, inhibition of PCSK9 may cause immunodeficiency by dampening APR. Further studies are clearly warranted to decipher the role of PCSK9 in *S pneumoniae* host responses in tissue-specific infections such as pneumonia as well as in the context of infections in which an optimal liver immune response is necessary.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

RNA sequencing data has been submitted to Gene Expression Omnibus (GEO) repository (identifier code: GSE165508). Other generated and analyzed data are available on reasonable request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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