1 **Analysis of mycobacterial infection-induced changes to host lipid metabolism in a** 2 **zebrafish infection model reveals a conserved role for LDLR in infection susceptibility**

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16

17 **Abstract**

18 Changes to lipid metabolism are well-characterised consequences of human tuberculosis
19 infection but their functional relevance are not clearly elucidated in these or other host-
20 mycobacterial systems. The zebrafish-*Mycobacterium marinum* infection model is used
21 extensively to model many aspects of human-*M. tuberculosis* pathogenesis but has not been
22 widely used to study the role of infection-induced lipid metabolism. We find mammalian
23 mycobacterial infection-induced alterations in host Low Density Lipoprotein metabolism are
24 conserved in the zebrafish model of mycobacterial pathogenesis. Depletion of LDLR, a key
25 lipid metabolism node, decreased *M. marinum* burden, and corrected infection-induced

26 altered lipid metabolism resulting in decreased LDL and reduced the rate of macrophage
27 transformation into foam cells. Our results demonstrate a conserved role for infection-
28 induced alterations to host lipid metabolism, and specifically the LDL-LDLR axis, across
29 host-mycobacterial species pairings.

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31 **Keywords**

32 zebrafish; mycobacterium; lipid; granuloma; pathogenesis

33

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42

43 **1. Introduction**

44 Mycobacterial manipulation of host lipid metabolism and the subsequent transformation of
45 macrophages into foam cells are important pathways in the pathogenesis of *Mycobacterium*
46 *tuberculosis* (*Mtb*) (Lovewell et al., 2016). Mycobacteria drive the transformation of infected
47 macrophages into foam cells through the imbalance of low density lipoprotein (LDL) influx
48 and efflux mechanisms (Cardona et al., 2009). Given the significant proportion of the
49 mycobacterial genome that is allocated to lipid metabolism, and the utilisation of lipids such
50 as cholesterol for growth *in vitro*, the abundance of neutral lipids contained within the

51 granuloma microenvironment may supply a carbon source for intracellular mycobacterial
52 survival and persistence (Pandey and Sasseti, 2008).

53

54 Dyslipidaemia and foam cell differentiation are known to contribute to disease pathology and
55 prognosis in human *Mtb* infection where foamy macrophages may inhibit lymphocyte access
56 to infected macrophages, increase the build-up of caseum at the center of granulomas, and
57 provide lipids utilized during evasion of phagocytosis (Dong et al., 2017; Russell et al.,
58 2009). However, the involvement of dyslipidemia in the pathogenesis of non-tubercular
59 mycobacterial infections such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP)
60 or *Mycobacterium marinum*, and in non-rodent animals, has not been well explored. Our
61 group has identified infection-induced changes to host lipid metabolism genes in MAP-
62 infected cattle and aberrant host lipid metabolism gene expression and intracellular
63 cholesterol accumulation within MAP-infected macrophages (Johansen et al., submitted;
64 Thirunavukkarasu et al., 2014). Here, we have turned to the natural host-pathogen pairing of
65 the zebrafish-*M. marinum* platform to investigate the conservation and consequences of
66 altered host lipid metabolism as a conserved motif across mycobacterial infections.

67

68 Zebrafish (*Danio rerio*) have functional innate immune and digestive systems from early in
69 embryogenesis with a high degree of homology to other vertebrates and are adaptable to the
70 study of early host-pathogen interactions (Traver et al., 2003). *M. marinum* is a natural
71 pathogen of fish and amphibian species and genomic analysis has identified *M. marinum* as
72 the closest genetic relative of the *Mtb* complex, sharing 85% of orthologous regions with *Mtb*
73 (Stinear et al., 2008). Zebrafish models of *M. marinum* infection have provided unique
74 insight into host-pathogen interplay responsible for granuloma formation, exquisitely
75 reproducing the cellular structure of the human-*M. tuberculosis* granulomas, including the

76 presence of lipid-rich foam cells (Cronan et al., 2016; Johansen et al., 2018b; Oehlers et al.,
77 2017; Oehlers et al., 2015).

78

79 The aim of this study was to explore the biological significance of infection-induced changes
80 to lipid metabolism in a model non-tubercular mycobacterial infection. Our findings
81 demonstrate the key lipid metabolism node Low Density Lipoprotein Receptor (LDLR)
82 mediates the uptake of lipids into the mycobacterial granuloma niche and aids the
83 transformation of macrophages into foam cells.

84

85 **2. Materials and Methods**

86 **2.1. Zebrafish breeding and ethics statement**

87 Adult zebrafish were housed at the Garvan Institute of Medical Research Biological Testing
88 Facility (St Vincent's Hospital AEC Approval 1511). Zebrafish embryos were obtained by
89 natural spawning and embryos were raised at 28°C in E3 media. All experiments and
90 procedures were completed in accordance with Sydney Local Health District animal ethics
91 guidelines for zebrafish embryo research.

92

93 **2.2. Morpholino design and microinjection**

94 Splice-blocking morpholino oligonucleotide for *LDLR* (gene variant *ldlra*) was designed
95 from Ensembl transcript sequences (<http://www.ensembl.org/index.html>) and purchased from
96 Gene Tools. The *LDLR* morpholino (5'-ATCACATTTCATTTCTTACAGCAGT-3') was
97 targeted at the exon 4/intron 4 and 5 splice junction that was expected to cause the excision of
98 exon 4. The control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') is a
99 negative control targeting a human beta-globin intron known to cause little phenotype
100 variations in zebrafish.

101

102 Embryos at the 1-4 cell stage were injected with 2 nL of morpholino into the yolk. Embryos
103 were raised at 28°C in E3 media.

104

105 **2.3. Molecular analysis of *ldlra* knockdown**

106 RNA was extracted in TRIZOL (Thermofisher) by precipitation and cDNA was synthesised
107 with an Applied Biosystems High Capacity cDNA reverse transcription kit (Thermofisher).
108 PCR was performed with *ldlra*-specific primers (5'-AGAGCTGGAAATGTGACGGA-3'
109 and 3'-CTCATCTGGACGGCATGTTG-5') and visualised by gel electrophoresis.

110

111 **2.4. Oil Red O staining**

112 Oil Red O lipid staining on whole mount embryos was completed as previously described
113 (Johansen et al., 2018b; Passeri et al., 2009). Briefly, embryos were individually imaged for
114 bacterial distribution by fluorescent microscopy, fixed, and stained in Oil Red O (0.5% w/v in
115 propylene glycol). Oil Red O staining intensity at sites of infection were quantified in ImageJ
116 and calculated as the pixel density above background tissue.

117

118 **2.5. Infection of zebrafish embryos**

119 At 30 hours post-fertilisation, zebrafish embryos were anaesthetised with 160 µg/mL tricaine
120 (Sigma-Aldrich) and infected with approximately 200 CFU fluorescent M strain *M. marinum*
121 via caudal vein injection.

122

123 **2.6. Lipoprotein assay**

124 Low density lipoprotein abundance was measured using the HDL & LDL/VLDL Cholesterol
125 Assay Kit (Cell Biolabs) as per manufacturer's instructions and based on previously

published methods (O'Hare et al., 2014). Briefly, 10 embryos per treatment group were terminally anaesthetised and placed into BHT solution (10 µg/mL, Sigma-Aldrich) and homogenised. Homogenates were centrifuged at 4°C at 2000 x g for 5 mins to pellet debris. The supernatant was mixed with an equal volume of precipitation solution to separate the high density lipoprotein (HDL) and low density lipoprotein (LDL) fractions. Each lipoprotein fraction was mixed with an equal volume of reaction mix and quantified by fluorescent plate reader.

A standard curve was included in each run to calculate the concentration of lipoproteins within each treatment, however the measured quantities of LDL varied considerably between replicates (eg control experiment 1 vs control experiment 2) so we present data as arbitrary normalised units to facilitate fold change comparisons between experiments.

2.7. Imaging

Live zebrafish embryos were anaesthetized in M-222 (Tricaine) and mounted in 3% methylcellulose for imaging on a Leica M205FA fluorescence stereomicroscope. Further image manipulation and/or bacterial quantification was carried out with Image J Software Version 1.51j.

2.8. Quantification of *M. marinum* burden by fluorescent pixel count

Infection burden was measured as the number of pixels in each embryo above background fluorescence in ImageJ (National Institutes of Health) and pixels counted using the 'Analyse particles' function (Matty et al., 2016).

2.9. Quantitative PCR

Quantitative PCR (qPCR) reactions were completed as previously described (Taylor et al., 2008) using Mx3000P real-time PCR systems (Stratagene). Gene-specific primers were generated for *abca1* (5'-AGCTGCTGGTCGAGATCATA-3' and 3'-CTGTCTCACTGCATGCTTGG-5') for comparison against housekeeping gene *actb* (5'-CCTTCCAGCAGATGTGGATT-3' and 3'-CACCTTCACCGTTCCAGTTT-5'). Samples were run in duplicate with reaction specificity confirmed based on the post-amplification dissociation curve.

2.10. Statistical analysis

Graphpad Prism was used to perform statistical testing as indicated.

3. Results

3.1. Infection-induced altered host dyslipidemia is recapitulated in the zebrafish-*M. marinum* infection model

Previous reports have identified hypercholesterolemia as a correlate of disease pathology during human-*Mtb* and ruminant-*M. avium* paratuberculosis infections (Dong et al., 2017). To determine whether these findings were conserved within the zebrafish-*M. marinum* platform, we infected zebrafish embryos with *M. marinum* then analysed the total embryo LDL content at 5 dpi. Calculation of lipoprotein content revealed an increase in LDL demonstrating conservation of the infection-induced dyslipidemia phenotype in our zebrafish embryo model (Figure 1A).

3.2. Knockdown of *Ldlra* reduces *M. marinum* burden in zebrafish embryos

Infection of macrophages with various species of mycobacteria, including in the zebrafish-*M. marinum* system, increases *LDLR* transcription (Cronan et al., 2016; Johansen et al.,

submitted; Kim et al., 2010). Take together with our observation of infection-induced changes in the ligand LDL in infected zebrafish embryos, we tested the functional relevance of LDLR by morpholino knockdown of the zebrafish *ldlra* gene (O'Hare et al., 2014). As previously reported by O'Hare et al., knockdown of *Ldlra* had no effect on embryo development and editing of the *ldlra* transcript was observed (Figures 1B and 1C). Oil Red O staining for lipids in *Ldlra*-depleted embryos revealed marked vascular lipid accumulation consistent with previous reports of *Ldlra* depletion being atherogenic (Figure 1D) (O'Hare et al., 2014).

Following *Ldlra*-depletion, embryos were infected with fluorescent *M. marinum* and bacterial burden was analysed by fluorometry. Zebrafish embryos depleted of *Ldlra* displayed a significantly decreased bacterial burden at 3 and 5 dpi when compared to control embryos (Figure 2).

3.3. Knockdown of *Ldlra* corrects infection-induced altered host lipid metabolism

Host lipid metabolism is a tightly controlled biological pathway, responsible for addressing the cellular lipid requirements through the regulation of influx and efflux mechanisms. Of the many surface receptors involved in lipid metabolism, LDLR is arguably the most important, playing a key role in driving the uptake of circulating LDL (Cardona et al., 2009). We hypothesised knockdown of *Ldlra* would thus restore homeostatic lipid metabolism.

Following *M. marinum* infection, expression of the LDLR-network and lipid efflux gene *ABCA1* was markedly increased (Figure 3A). Depletion of *Ldlra* significantly reduced the magnitude of infection-induced *abca1* transcription (Figure 3A).

Next, we hypothesised that depletion of LDLR would prevent infection-induced hypercholesterolemia. Following *M. marinum* infection, *Ldlra*-deficient embryos displayed a decreased LDL compared to infected controls at 5 dpi (Figure 3B). Together, these data demonstrate conservation and correction of an infection-induced lipid metabolism network in our model.

At the cellular-to-tissue level, we hypothesised that the combination of reduced potential for LDL uptake and correction of total cholesterol levels would reduce granuloma lipid accumulation within granuloma foam cells in *Ldlra*-depleted embryos. Granuloma Oil Red O lipid staining density was reduced in infected *Ldlra*-deficient embryos compared to infected controls demonstrating a functional role for LDLR in directing bacterial-beneficial lipid accumulation during mycobacterial infection (Figure 4).

4. Discussion

The elevation of host lipids following mycobacterial infection has been well documented in both human and murine *Mtb* infections (Dong et al., 2017; Martens et al., 2008). Recently, we have shown that elevated serum cholesterol is associated with histopathological lesions in sheep and cattle infected with MAP (Johansen et al., 2018a). Here we show results in agreement with previous findings and suggest that the induction of host hypercholesterolemia is a conserved motif of pathogenic mycobacteria outside of mammalian hosts. Our findings in this study demonstrate 1) infection-induced altered host lipid metabolism is a conserved feature of non-tuberculous mycobacterial infection, and 2) host expression of LDLR is co-opted by mycobacterial infection to increase susceptibility to mycobacterial infection. Furthermore, our results demonstrate that *LDLR* knockdown significantly alters the availability of lipoproteins and decreases granuloma Oil Red O staining density, providing

strong evidence to suggest that the abundance of neutral lipids and therefore foam cells, was largely decreased as a consequence of *LDLR* depletion.

Host lipid metabolism is a complex pathway involving many transcription factors, surface molecules and feedback mechanisms. In the absence of key components, compensatory pathways are utilised to achieve cellular homeostasis. Expression of *abca1* was increased following *M. marinum* infection, in agreement with previous studies identifying the upregulation of *ABCA1* by mycobacterial infection across multiple host species (Long et al., 2016). Interestingly, there was a notably lower expression in *ldlra*-deficient embryos, presumably due to the decreased efflux of lipids from mycobacteria-infected macrophages as a consequence of the altered lipoprotein abundance.

An important limitation of our study is the use of a single morpholino targeting the *ldlra* transcript in the embryo stage of development. Additional work with mutant adult zebrafish is necessary to fully elucidate the role of the LDL-LDLR axis in mycobacterial infection in the context of the adaptive immune system (Liu et al., 2018). The adult zebrafish platform will also allow finer control of available lipid pools through regulation of diet.

Collectively, this study provides evidence of conserved infection-induced altered host lipid metabolism in the zebrafish-*M. marinum* model, and highlights a crucial role of *LDLR* in infection susceptibility across host and pathogen species.

Author contributions

M.D.J. Planned experiments, performed experiments, analysed data, wrote the paper; E.H. Planned experiments, performed experiments; J.A.K. Performed experiments; A.R.

Performed experiments, contributed reagents; B.N. Performed experiments, contributed reagents; A.F. Contributed reagents; W.J.B. Analysed data, contributed reagents; K.dS. Planned experiments, analysed data, contributed reagents; A.C.P. Planned experiments, analysed data, contributed reagents; S.H.O. Planned experiments, performed experiments, analysed data, contributed reagents, wrote the paper.

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Figure Legends

Figure 1: LDL is increased by infection and validation of *Ldlra* knockdown.

A, Quantification of LDL concentration in wild-type zebrafish embryos at 5 days following *M. marinum* infection. Each data point represents a pooled sample of 10 embryos. Scale bars represent 500 μ m. Statistical tests were performed using unpaired Student's t-tests. Experiments were completed with 4 independent biological replicates. *B*, Normal morphology and phenotypes at 5 days post-fertilisation as visible by light microscopy. *C*, Molecular validation of *ldlra* morpholino efficacy at 3 and 5 days post-treatment demonstrating depletion of native 464 bp amplicon and appearance of aberrant 378 bp amplicon. *D*, Oil Red O staining demonstrates visible lipid abundance within the vasculature of *Ldlra*-deficient embryos. White arrows represent expansive lipid accumulation within the vasculature.

Figure 2: Knockdown of *Ldlra* reduces *M. marinum* bacterial burden.

A, Representative images of *M. marinum* bacterial burden at 5 days-post infection. Scale bars represent 500 μ m. *B*, Quantification of *M. marinum* bacterial burden at 3 days post infection. *C*, Quantification of *M. marinum* burden at 5 days post infection. Error bars represent standard deviations, each data point represents a single embryo. Analysis of *M. marinum* bacterial burden was unmatched T tests. Experiments were completed with 4 independent biological replicates for each panel.

Figure 3: Mycobacterial infection-induced modulation of host lipid metabolism is corrected in *Ldlra*-deficient embryos.

A, *abca1* transcript fold change expression at 5 days post-infection measured by qPCR, normalised to control uninfected sample. Each column represents two independent biological

replicates analysed with technical replicates. *B*, Quantification of LDL concentration in *Ldlra*-deficient embryos at 5 days-post infection. Each data point represents a pooled sample of 10 embryos completed with four independent replicates.

Gene expression analysis was completed using one-way ANOVA. Analysis of LDL concentration was completed using matched Student's *t*-tests.

Figure 4: Infection-induced granuloma lipid accumulation is reduced in *Ldlra*-deficient embryos.

A, Representative images of Oil Red O staining density at 5 days post-infection. Red circles indicate areas of embryos with largest foci of infection. Scale bar represents 500 μ m. *B*, Quantification of Oil Red O density units at 3 days post-infection with *M. marinum*. *C*, Quantification of granuloma Oil Red O staining density units at 5 days post-infection. Each data point represents an individual granuloma. Error bars represent standard deviation. Statistical tests were performed using unpaired Student's *t*-tests.

A

B