Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids

C. J. Cambier1, Kevin K. Takaki2, Ryan P. Larson1,3, Rafael E. Hernandez4, David M. Tobin2, Kevin B. Urdahl1,3,4, Christine L. Cosma2 & Lalita Ramakrishnan1,2,5

The evolutionary survival of Mycobacterium tuberculosis, the cause of human tuberculosis, depends on its ability to invade the host, replicate, and transmit infection. At its initial peripheral infection site in the distal lung airways, M. tuberculosis infects macrophages, which transport it to deeper tissues1. How mycobacteria survive in these broadly microbialicidal cells is an important question. Here we show in mice and zebrafish that M. tuberculosis, and its close pathogenic relative Mycobacterium marinum, preferentially recruit and infect permissive macrophages while evading microbialicidal ones. This immune evasion is accomplished by using cell-surface-associated pthiocerol dimycoceroserate (PDIM) lipids2 to mask underlying pathogen-associated molecular patterns (PAMPs). In the absence of PDIM, these PAMPs signal a Toll-like receptor (TLR)-dependent recruitment of macrophages that produce microbialicidal reactive nitrogen species. Concordantly, the related phenolic glycolipids (PGLs)3 promote the recruitment of permissive macrophages through a host chemokine receptor 2 (CCR2)-mediated pathway. Thus, we have identified coordinated roles for PDIM, known to be essential for mycobacterial virulence4, and PGL, which (along with CCR2) is known to be associated with human tuberculosis5,6. Our findings also suggest an explanation for the longstanding observation that M. tuberculosis initiates infection in the relatively sterile environment of the lower respiratory tract, rather than in the upper respiratory tract, where resident microflora and inhaled environmental microbes may continually recruit microbialicidal macrophages through TLR-dependent signalling.

Pattern recognition receptors (PRRs) such as the TLRs enable host recognition of diverse microbes through their PAMPs. Macrophages recruited through TLR signalling can eradicate organisms invading the oropharyngeal mucosa, for example, Streptococcus pneumoniae. In contrast, pathogenic mycobacteria appear to use macrophages and myeloid dendritic cells for transport across epithelial barriers to their infection niche7,8. Mycobacteria are replete with TLR PAMPs—such as lipoproteins and bacterial cell wall peptidoglycan—that have been shown to activate cytokine responses in cultured macrophages9. Yet in vivo studies find TLR signalling to be dispensable in the early stages of infection, suggesting that mycobacteria have evolved mechanisms to circumvent the bactericidal consequences of TLR signalling.

To explore these mechanisms, we used zebrafish larvae infected with M. marinum, a close genetic relative of M. tuberculosis and the causative agent of tuberculosis in ectotherms. This model has yielded important insights into the pathogenesis and genetics of human tuberculosis10. In humans, the earliest interactions between mycobacteria and phagocytes occur at the lung epithelial surface. Such interactions can be modelled in the larva by injection of bacteria or other chemical stimuli into the hindbrain ventricle (HBV), a neuroepithelium-lined cavity to which the oropharyngeal mucosa, for example, Streptococcus pneumoniae, mucosal bacteria that can be commensal or pathogenic11–13 (Fig. 1b). Similarly, macrophage recruitment to the nonpathogenic Mycobacterium smegmatis was MyD88 dependent. In contrast, macrophage recruitment to M. marinum was MyD88 independent (Fig. 1c). This finding suggested that pathogenic mycobacteria have the ability to mask PAMPs that would otherwise induce TLR signalling during the initial infection phase. We proposed that such a factor would be a cell-surface-associated virulence determinant. In this light, PDIM seemed a likely candidate, particularly because it is present only in pathogenic mycobacteria, including M. tuberculosis and M. marinum, but absent in non-pathogenic M. smegmatis. We created a M. marinum mutant that lacks PDIM on its surface by knocking out the PDIM transporter, encoded by the mmpL7 gene, and confirmed that it was attenuated in zebrafish larvae (Fig. 1d and Extended Data Fig. 2). If PDIM is masking PAMPs, then macrophage recruitment to ΔmmpL7 bacteria should be MyD88 dependent, and this was the case (Fig. 1e). In contrast, macrophage migration remained MyD88 independent in response to M. marinum deficient in another cell-surface-associated virulence determinant, Erp (Aerp) (Fig. 1d, e and Extended Data Fig. 2)14. This result was consistent with M. smegmatis possessing a functional erp15, and suggested further that the evasion of MyD88-dependent immune detection was mediated specifically by PDIM.

Our model posits that pathogenic mycobacteria use PDIM to evade recruitment of MyD88-dependent macrophage populations detrimental to their survival. Therefore, we predicted that wild-type mycobacteria would be unaffected in MyD88 morphants, whereas the attenuation of ΔmmpL7 should be reversed. We found both to be the case (Fig. 1f). For these assays, approximately 80 M. marinum were injected into the HBV. However, MyD88 morphants were previously reported to be susceptible to higher M. marinum inocula delivered intravenously16. We confirmed these findings, showing that MyD88 deficiency increased susceptibility at later time points after intravenous administration of >300 colony forming units (c.f.u.) (Extended Data Fig. 3). It is likely that MyD88 exerts its protective responses at these later stages through mechanisms distinct from the ones we have uncovered, such as through interleukin (IL)-1-mediated responses17. Indeed, IL-1 expression was undetectable 3 h after infection, when we observed MyD88-dependent macrophage recruitment (data not shown), suggesting an IL-1-independent role for MyD88 in mediating recruitment towards PDIM-deficient mycobacteria.

Further characterization of wild-type versus PDIM-deficient bacteria revealed that both strains recruited cells expressing the macrophage-specific marker Mpeg1 (ref. 8) (Extended Data Fig. 4a and Supplementary Videos 1, 2). We next asked whether these macrophages possessed differential microbialicidal potential. We examined the expression of inducible nitric oxide synthase (iNOS) in these recruited cells because: (1) it is induced in macrophages upon TLR signalling18, and can be expressed by zebrafish19, mouse20 and human21 macrophages after mycobacterial infection; and (2) mycobacteria are known to be susceptible to reactive...
Significance testing for all panels done using one-way analysis of variance. Dor wild-type or MyD88-morphant fish at 3 dpi after HBV infection with wild-type M.marinum. Representative of four separate experiments.

Macrophages recruited into the HBV of wild-type or MyD88-morphant fish after infection with wild-type P. aeruginosa and not with wild-type or MyD88-erp M. marinum. Representative of three separate experiments.

Macrophages recruited into the HBV of wild-type or MyD88-erp M. marinum. Representative of three separate experiments.

Macrophages recruited into the HBV of wild-type or MyD88-erp M. marinum. Representative of three separate experiments.

Macrophage recruitment in response to M. marinum pks15 infection model21. Similarly, we found that PGL was required for this recruitment. We investigated CCR2, which has been implicated in macrophage migration to bacterial pathogens in mice19, including macrophages that are permissive to M. tuberculosis replication after aerosol infection15. We identified the functional zebrafish CCR2 orthologue (see Methods) and confirmed that its knockdown resulted in reduced macrophage migration in response to recombinant human chemokine ligand 2 (CCL2) and not to the closely related human macrophage chemokines CCL4 and CCL5 (Extended Data Fig. 8a). The specificity of CCL2-mediated macrophage migration was revealed by the following findings: (1) human and mouse CCL2 induced macrophage but not neutrophil migration (Extended Data Fig. 8b, c); (2) recombinant human IL-8, a neutrophil chemokine, induced neutrophil but not macrophage migration (Extended Data Fig. 8b, c); (3) human leukotriene B4 (LTB4) induced recruitment of both neutrophils and macrophages (Extended Data Fig. 8b, c), as expected16; and (4) MyD88 knockdown did not diminish CCL2-mediated macrophage migration, ruling out TLR-mediated migration in response to any endotoxin that might be contaminating the chemokine preparations (Extended Data Fig. 8b).

CCR2 morphants had reduced macrophage migration in response to wild-type M. marinum, confirming the role of this pathway in recruitment (Fig. 4a). Recruitment to PDIM-deficient M. marinum was unaffected, showing that TLR PAMPs trigger recruitment through a CCR2-independent pathway (Fig. 4a). Accordingly, we found that M. marinum infection induced CCL2, and that CCL2 morphants also had reduced macrophage recruitment in response to infection (Fig. 4b and Extended Data Fig. 9).

Turning to the question of which bacterial determinant induced the CCR2 pathway, we considered PGL, a molecule closely related to PDIM in both M. marinum and M. tuberculosis2. Although many clinical M. tuberculosis isolates have lost PGL, its presence has been linked to increased virulence. Moreover, among M. tuberculosis clinical isolates, PGL expression was linked to Ccl2 expression in a mouse lung infection model21. Similarly, we found that PGL was required for Ccl2 induction in the zebrafish larva; deletion of the M. marinum pks15 locus...
Figure 2 | Increased iNOS-dependent microbicidal activity of macrophages recruited to PDIM-deficient mycobacteria. a, b, Representative images of wild-type (WT) (a) and ΔmmpL7 (b) M. marinum-infected fish from c. N = 13 (wild-type) and 14 (ΔmmpL7) larvae per group. Scale bar, 50 μm. c, Percentage of infected macrophages that were iNOS positive in the HBV at 3 dpi with 80 wild-type, ΔmmpL7 or Δerp M. marinum. Representative of three separate experiments. d, Mean bacterial burdens of 2 dpf control (CTRL)- or RNS scavenger (CPTIO)-treated fish after HBV infection with 80 wild-type or ΔmmpL7 M. marinum. Representative of two separate experiments. NS, not significant. e-h, Mean bacterial volume of red fluorescent wild-type M. marinum (infection inoculum 30–40) when co-infected with 30–40 green fluorescent wild-type, ΔmmpL7 or Δerp M. marinum at 3 dpi in wild-type specifically abrogates PGL, but not PDIM, production (data not shown) and resulted in loss of ccl2 induction. ΔmmpL7 bacteria, which lack surface expression of both PGL and PDIM, similarly failed to induce ccl2, highlighting that this chemokine is not induced through TLR interactions, but rather is specifically induced through PGL-mediated interactions (Fig. 4b). Furthermore, Δpks15 bacteria recruited fewer macrophages upon infection of wild-type larvae, and this reduction was similar to that seen in CCR2 morphants infected with wild-type bacteria (Fig. 4c). There was no additional reduction in recruitment when CCR2 morphants were infected with PGL-deficient bacteria, suggesting that PGL recruits macrophages solely through the CCR2 pathway (Fig. 4c).

Our findings implicate PGL in bacterial virulence and, correspondingly, the CCR2 pathway in host susceptibility. Globally, a large proportion of M. tuberculosis isolates are PGL deficient due to a frameshift in pks15 (ref. 2). However, the importance of PGL in mediating virulence and/or transmission is underscored by its presence in many of the W-Beijing strains, which are becoming rapidly enriched among M. tuberculosis isolates globally5, and have predominated in outbreaks in North America, where tuberculosis is not prevalent6. Infectivity is a key requirement for transmission, and our data suggested that PGL may enhance infectivity through CCR2-mediated recruitment of permissive macrophages at the earliest stages of infection. This enhancement may be particularly relevant in the context of human infections, in which the infectious dose is thought to be as low as 1–3 bacteria22,23. To test the hypothesis that PGL enhances infectivity at low doses, we compared the ability of wild-type and PGL-deficient strains to establish infection. Confocal microscopy was used 5 hours after HBV injection to select those animals that had received 1–3 bacteria (Extended Data Fig. 10), and then again at 5 dpi to identify which animals were still infected. We found that 89% of the wild-type but only 18% of the Δpks15 infections were successful (Fig. 4d). Concurrent administration of recombinant CCL2 restored the infectivity of Δpks15 bacteria, provided the CCR2 pathway was intact (Fig. 4d). Correspondingly, we found that wild-type bacteria had a lower infectivity rate in CCR2 morphants (Fig. 4d). Consistent with our finding that PGL recruits macrophages solely through CCR2, there was no further decrease in infectivity in CCR2 morphants infected with the PGL mutant (Fig. 4d). Finally, the infectivity of wild-type bacteria in MyD88 morphants was undiminished (90% for wild type versus 83% for morphants), consistent with our finding that TLR signalling is not involved in macrophage recruitment to wild-type bacteria.

Figure 3 | Elevated frequencies of iNOS-expressing inflammatory monocytes in mice infected with PDIM-deficient M. tuberculosis. a, b, C57BL/6 mice were infected through the aerosol route with H37Rv or an isogenic PDIM-deficient mutant (ΔdrvA). Lung tissue was harvested at 21 dpi and iNOS protein expression was measured using flow cytometry. Representative fluorescence-activated cell sorting (FACS) plots (a) and graphical depiction (b) of frequencies of iNOS-expressing cells within the CD11b⁺Ly6C⁺ inflammatory monocyte population. Representative of two separate experiments. Student’s unpaired t-test.
80 wild-type or c after caudal vein infection of 2 dpf larvae with 250–300 wild-type, must reach the alveolar surfaces of the distal lung in order to initiate using Fisher’s exact test for each comparison. One-way ANOVA, with Bonferroni’s post-test for each comparison shown. *P < 0.05. b,c, Microarray analysis of gene expression in wild-type or CCR2-morphant (MO) fish after infection with M. marinum. One-way ANOVA with Tukey’s post-test. **P < 0.01, ***P < 0.001. d, Wild-type and CCR2-morphant fish, with or without the addition of 1 μg ml⁻¹ FLuc were infected in the HBV with 1–3 wild-type or ΔmmpL7 M. marinum. Graph shows the percentage of fish that were infected (black) or uninfected (grey) after 5 days. n = number of larvae per group. Representative of two separate experiments. Significance was evaluated using Fisher’s exact test for each comparison. *P < 0.01, ***P < 0.001. NS, not significant.

These findings highlight the interdependency between bacterial PGL and host CCR2 signalling in driving bacterial infectivity under the low inoculum conditions relevant to human infection. Previous investigations into the role of PGL and CCR2 may have failed to reveal these mechanisms because those studies used higher inocula and, in the study of CCR2 signalling, a PGL-deficient strain. Indeed, our finding that CCR2 signalling is a host susceptibility factor is reinforced by human tuberculosis susceptibility. Furthermore, the association appears to be stronger in east Asian populations, where clinical isolates are relatively sterile, which is relatively sterile, would favour recruitment of Mycobacterium-permissive macrophages. To test this hypothesis, we co-infected animals with M. marinum together with bacterial colonizers of the pharynx that induce TLR signalling—either S. aureus, a common Gram-positive colonizer of the nasopharynx in both adults and children, or the Gram-negative bacterium P. aeruginosa, also reported to colonize the pharynx of asymptomatic adults and children. Co-infection with P. aeruginosa resulted in the attenuation of wild-type mycobacteria by 1 dpi, and continued into 3 dpi. Mycobacterial growth was attenuated despite rapid clearing of P. aeruginosa: 56% of the animals had cleared the co-infected P. aeruginosa by 1 dpi, and 76% by 3 dpi, with only a few residual bacteria in the remaining animals. Thus, it was not the physical presence of, but rather the detrimental immunological milieu induced by P. aeruginosa that was responsible for the attenuation of M. marinum. Consistent with our hypothesis, we found that the detrimental effect of P. aeruginosa on mycobacterial survival was MyD88 dependent (Fig. 5b). S. aureus co-infection also had a MyD88-dependent detrimental effect on M. marinum survival (Fig. 5c).

Our previous work identified strategies by which intracellular mycobacteria manipulate host pathways after having traversed epithelial barriers; these involve a bacterial protein secretion system that expands the bacterial niche through macrophage recruitment to the nascent granuloma. We now describe what may be the first contact between mycobacteria and their hosts, and the manner in which mycobacteria manipulate recruitment, and potentially influence the differentiation or activation state of the first responding macrophages so as to gain access to their preferred niche. The choreographed entry involves two related mycobacterial lipids acting in concert to avoid TLR-mediated immunity in protection against M. tuberculosis infection in both human and animal studies. In contrast, PGL is dispensable for virulence, being variably present among clinical isolates. Yet its presence in the ancestral M. canettii strains as well as in M. marinum,
the closest genetic relative of the *M. tuberculosis* complex, suggests its integral role in the evolution of mycobacterial pathogenecity. Tuberculosis is an ancient disease, and the enhanced infectivity conferred by PGL may have been essential for most of its history before human crowding, with its greatly increased opportunities for transmission, made it dispensable.

Our findings suggest a central role for commensal flora in choreographing mycobacterial entry. Not only must pathogenic mycobacteria possess a physical barrier to prevent host TLR-mediated detection, but they must also evade TLR signalling initiated by other organisms, by entering through the distal lung (Extended Data Fig. 1). Our work may also explain the paradox that smaller *M. tuberculosis* droplets are more infectious than larger ones. However, the requirement placed on mycobacteria to gain entry through the distal lung makes tuberculosis less contagious than most other respiratory infections, thus assigning a protective role to the commensal flora. Conversely, the persistence of human tuberculosis for over 70,000 years attests to the effectiveness of the mycobacterial evolutionary survival kit (masking lipid, recruiting lipid and small infection droplets) to simultaneously evade and manipulate the host and its commensal flora.

**METHODS SUMMARY**

*M. marinum*, *S. aureus* and *P. aeruginosa* constitutively expressed fluorescent proteins GFP, Wasabi or tdTomato to allow visualization. Zebrafish larvae (of undetermined sex given the early developmental stages used) were infected at 36–48 h post-fertilization (hpf) via caudal vein or HBV injection. Larvae were randomly allotted to the different experimental conditions. Fluorescence images were captured and quantitative fluorescence was used as a surrogate for bacterial burdens. For the macrophage recruitment assays, macrophages and neutrophils in the HBV were enumerated using differential interference contrast microscopy 3 h after HBV infection. For determination of infection burdens in the HBV, 1 and 3 dpi larvae were enumerated using differential interference contrast microscopy 3 h after HBV injection. For infection, 2 dpi larvae were infected in the HBV with a concentration of mycobacteria that resulted in an average of 0.8 bacteria per infection. Larvae harbouring 1–3 bacteria were identified at 5 hpi using confocal microscopy, and were re-imaged at 5 dpi and scored as infected or uninfected. Antibody staining for iNOS was performed as described by confocal microscopy. CPTIO (Sigma) was used at a final concentration of 500 μM in 0.1% dimethyl-sulphoxide in fish water. Larvae were incubated immediately after infection and fresh CPTIO was added every 24 h for the duration of the experiment. For quantitative real-time PCR, complementary DNA was synthesized from pools of 20–40 larvae as previously described, cd2c RNA levels were determined using SYBR green and the primers 5′-GTCGTGTGTCCTCTCGCTTCT-3′ and 5′-TGCGAG AAGATGGCTGCTGA-3′. Ten-week-old female C57BL/6 mice were infected through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. J. Clin. Invest. 120, 1674–1682 (2010).

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**Supplementary Information** is available in the online version of the paper.

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METHODS

Bacterial strains and methods. M. marinum strain M (ATCC BAA-335) and the Δepr mutant have been described. The AmmpL7 and Apks15 mutants were generated as described in the following section. Fluorescently labelled bacterial strains were generated by transformation with the pTCE15 or pTEC27 plasmids (deposited with Addgene, plasmids 30174 and 30182, respectively), resulting in msp12-driven expression of the Wasabi or tdTomato fluorescent proteins, respectively. Mycobacteria were grown at 33 °C in Middlebrook 7H9 broth or on 7H10 agar (both by Difco) supplemented with 0.5% bovine serum albumin, 0.005% oleic acid, 0.2% glucose, 0.2% glycerol, 0.085% sodium chloride and 0.05% Tween-80 (culture broth only). 50 μg ml⁻¹ hygromycin was added as appropriate. For sucrose counter-selection, 7H10 agar was supplemented with 10% sucrose. Single-cell suspensions of bacteria were prepared as described. To prepare heat-killed cultured M. marinum, bacteria were incubated at 80 °C for 20 min and then homogenized in a Biospec Beater together with 0.1 mm silica spheres for 1 min. The expressing pOS1-SdrC-mCherry #391 was a gift from J. Bubeck Wardenburg.

Zebrafish husbandry and infections. Wild-type AB zebrafish were maintained as infected or uninfected, based on the presence or absence of fluorescent bacteria. Larvae (of undetermined sex given the early developmental stages used) were in compliance with Institutional Animal Care and Use Committee approved recommendations (eBioscience). Cells were fixed and permeabilized using eBioscience’s Cytofix/CytoPerm. In the absence of other specifications, primary antibodies were incubated with secondary antibodies for 1 h, followed by washing three times with PBS. Detection of fluorescently labelled bacterial strains was done after fixation and permeabilization, following the manufacturer’s recommendations (eBioscience). Cells were fixed and permeabilized using eBioscience’s Fix/Perm buffer for 1 h at 4 °C, followed by staining for iNOS with anti-iNOS2 Alexa Fluor 405 (C-11, Santa Cruz Biotechnology) or mouse IgG1 isotype control

Recessive transcription PCR to verify efficacy of MyD88 morpholin. RNA was extracted from pools of 15–40 embryos using TRizol reagent (Life Technologies), treated with Turbo DNA-Free Kit (Life Technologies) and CDNA synthesized with PrimerScript (TaKaRa). Primers used for PCR were as follows: actin, forward, 5'-ACCTGGACAGACTCTGATG, reverse, 5'-TGAAGTTGGTCTCTAGGATAC; myd88, forward, 5'-ATGGCATCAAGTGTAAAGTACC, reverse, 5'-AGGACAGGAGTGCTT.

Identification of candidate CCR2 orthologue in zebrafish. Basic Local Alignment Search Tool (BLAST) searches of the zebrafish genome (http://www.ensembl.org) identified two closely related CCR-like genes on chromosome 16: ENSDARG00000079829 and ENSDARG00000062999. In BLAST comparisons to the human genome, ENSDARG00000079829 was found to have the highest homology to human CCR2 (E value 8.8 × 10⁻¹¹), whereas ENSDARG00000062999 was most highly homologous to human CCR4 (E value 2 × 10⁻⁹). In addition, annotation of the zebrafish genome from NCBI annotates ENSDARG00000079829 as a CCR2-like gene. We confirmed expression of the mRNA and identified the 5' upstream exon ATGCGGGACACAAAAAGCUTA using 5' rapid amplification of cDNA ends (RACE). Identification of zebrafish CCL2 orthologue. Protein sequences of human and mouse CCL2 were used to interrogate the zebrafish genome by BLAST. Expression levels of the four most closely related zebrafish proteins were then examined at 3 hpi to identify the likely functional orthologue (Extended Data Fig. 9a). Of the four candidates, only ENSDARG00000041835 was significantly induced at 3 hpi. Knockdown of ENSDARG00000041835 resulted in a decrease in macrophage recruitment into the HBV at 3 hpi (Extended Data Fig. 9b).Quantitative real-time PCR. CDNA was synthesized from pools of 20–40 larvae as previously described. Quantitation of ccl2 RNA levels was determined using SYBR green and the following primer pair: 5'-GTCTGAGGTCTCCTGTGTTCC-3' and 5'-TGCAAGAAAAGATGCTGTA-3'. Identification of zebrafish iNOS staining. Antibody staining of larvae was performed as described. Larvae were then imaged using confocal microscopy and the number of infected macrophages that were positive for iNOS staining was determined for each larva. Morpholinos. The morpholinos described in Supplementary Table 1 were injected at the one-cell stage as previously described.

Microscopy and image-based quantification of infection level. Whole-field microscopy was performed using a Nikon Eclipse Ti-E equipped with a C-HGHTE 130W mercury light source, Chroma FITC (41001) filter, and ×2/0.10 Plan Apo chromatic objective. Fluorescent images were captured with a CoolSNAP HQ2 Monochrome Camera (Photometrics) using NIS-Elements (version 3.22). Quantification of fluorescent M. marinum infection using images of individual embryos using Fluorescent Pixel Count (FPC) was performed as previously described. For confocal imaging, larvae were imbedded in 1.5% agarose (low melting point). A series of z-stack images with a 2 μm step size was generated through the infected HBV, using the galvo scanner (laser scanner) of the Nikon A1 confocal microscope with a ×20 Plan Apo 0.75 NA objective. Bacterial burdens were determined by using the three-dimensional surface-rendering feature of Imaris (Bitplane Scientific Software).

Hindbrain assays. Macrophage recruitment assays were performed as previously described. For determination of HBV infection burdens, 1 and 3 dpi larvae were mounted in 1.5% agarose and confocal z-stacks of 2 μm were obtained.
for 30 min at 4°C. Samples were analysed on an LSR-II (BD Biosciences) and FlowJo Software (Treestar).

**Statistics.** Statistical analyses were performed using Prism 5.01 (GraphPad). For data sets requiring log_{10} transformation before ANOVA, embryos with no detectable fluorescence above background were assigned a value of 0.9, with 1 being the limit of detection, before log_{10} transformation. Post-test P values are as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

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Extended Data Figure 1 | Coordinate use of PDIM-mediated immune evasion and PGL-mediated recruitment by pathogenic mycobacteria. Models for infection with wild-type (WT) and PDIM-deficient mycobacteria are shown in the context of the relatively sterile lower airway versus the upper airway, with its higher levels of resident microflora and inhaled environmental organisms.
Extended Data Figure 2 | ΔmmpL7 bacteria are attenuated in zebrafish larvae. **a**, Kaplan–Meier graph showing daily survival of larvae infected via caudal vein injection with medium (mock), 29 wild-type or 70 ΔmmpL7 *M. marinum. N = 25 (mock), 31 (wild-type), or 29 (ΔmmpL7) larvae per group. Mean time to death (days): mock (11), wild type (7.6) and ΔmmpL7 (11.2). Survival was compared by log-rank test: wild type versus mock and wild type versus ΔmmpL7, \( P < 0.0001 \); mock versus ΔmmpL7, \( P = 0.5601 \). **b**, Infection burdens were measured by Fluorescent Pixel Count (FPC; mean ± s.e.m.). **c**, Representative images at 7 dpi. \( N = 29 \) (wild-type and ΔmmpL7) or 30 (Δerp) larvae per group. Scale bar, 500 \( \mu \text{m} \). At 3, 5 and 7 dpi, \( \log_{10} \) FPC was compared by ANOVA, with Dunnett’s post-test. ***\( P < 0.001 \). **d**, e, Representative images from wild-type (d) and ΔmmpL7 (e) *M. marinum* HBV infections quantified in Fig. 1d. \( N = 18 \) (wild-type) or 16 (ΔmmpL7) larvae per group. HBVs are outlined with a dashed white line. Scale bar, 100 \( \mu \text{m} \).
Extended Data Figure 3 | Knockdown of MyD88 results in a late, dose-dependent hypersusceptibility to *M. marinum* systemic infection.

**a**, RT–PCR for actin (top) and *myd88* (bottom), demonstrating that the majority of *myd88* transcripts at 7 dpf are abnormal in MyD88 morphants. Lanes marked ‘b’ and ‘c’ correspond to morphants from the same experiments depicted in panels b and c, respectively. The abnormal larger transcript (indicated by an asterisk) results from the inclusion of intron 2 in the final transcript, incorporating a premature stop codon that truncates the protein before the TIR (Toll/interleukin receptor) domain. **b**, Caudal vein infection of MyD88 morphants with 141 (b) or 325 (c) c.f.u. *M. marinum* larva. Bacterial burden was assessed by FPC, values plotted represent the mean ± s.e.m. Time points were compared by one-way ANOVA and Bonferroni’s post-tests. ***P < 0.001.** **d**, Representative images of larvae at 5 dpi from experiment in c, *N* = 30 control, 15 MyD88 morphant. Scale bar, 500 μm.
Extended Data Figure 4 | Characteristics of macrophages recruited to wild-type and PDIM-deficient bacteria. 

a. Mean Mpeg1-positive macrophages recruited at 3 hpi into the HBV of wild-type fish after infection with 80 wild-type or ΔmmpL7 M. marinum. 

b. Data from Fig. 2c expressed as mean numbers of total infected macrophages and iNOS-expressing infected macrophages after HBV infection with 80 wild-type, ΔmmpL7, or Δerp M. marinum. 

c. Bacterial burdens after L-NAME treatment. Mean bacterial burdens of 2 dpf control (CTRL)- or iNOS inhibitor (L-NAME)-treated fish after HBV infection with 80 wild-type or ΔmmpL7 M. marinum. NS, not significant.
Extended Data Figure 5 | Wild-type bacterial burdens after co-infection with wild-type or ΔmmpL7 bacteria. Representative images from the HBV co-infections quantified in Fig. 2e. a, b, Red fluorescent wild-type (WT) M. marinum co-infected with green fluorescent wild-type (a) or ΔmmpL7 M. marinum (b) with and without L-NAME treatment. Significance tested by one-way ANOVA with Bonferroni’s post-test for comparisons shown. Scale bar, 50 μm. c, Wild-type bacterial burdens after co-infection with wild-type or ΔmmpL7 M. marinum with and without L-NAME treatment. Significance tested by one-way ANOVA with Bonferroni’s post-test for comparisons shown.
Extended Data Figure 6 | MyD88-dependent macrophage recruitment occurs in response to PDIM deficiency rather than being due to loss of another MmpL7-exported product. a, Mean macrophage recruitment at 3 hpi into the HBV of wild-type or MyD88-morphant (MO) larvae after infection with 80 Δmas M. marinum. Student’s unpaired t-test. b, Mean surviving bacterial volume of red fluorescent wild-type M. marinum (initial infection dose of 30–40 c.f.u.) when co-infected with 30–40 green fluorescent wild-type, ΔmmpL7 or Δmas M. marinum at 3 dpi. Representative of two separate experiments. Significance tested by one-way ANOVA with Tukey’s post-test.
Extended Data Figure 7 | Gating strategy and isotype controls for iNOS staining of mouse lung. a, Representative gating strategy for isolation of inflammatory monocytes. A dump channel containing anti-CD4, CD8 and CD11c was plotted against a channel exhibiting autofluorescence and also containing anti-Ly6G. Using these markers, T cell, dendritic cell, alveolar macrophage, and neutrophil cell populations were excluded from the double-negative gate. Inflammatory monocytes were identified within the double-negative population by their co-expression of Ly6C and CD11b. These cells were then evaluated for intracellular iNOS expression. a, N = 4 per group (Fig. 3a, b) or b, with isotype control antibodies, N = 4 per group.
Extended Data Figure 8 | Specificity of CCL2-mediated macrophage recruitment in wild-type and CCR2-morphant larvae

**a.** Mean macrophage recruitment at 3 hpi into the HBV of control (ctrl), or CCR2-morphant (CCR2) larvae after injection of vehicle control (‘mock’; 0.1% BSA in PBS), human CCL2 (hCCL2), human CCL4 (hCCL4), or human CCL5 (hCCL5).

**b.** Mean macrophage recruitment at 3 hpi into the HBV of control (CTRL), CCR2-morphant (CCR2), or MyD88-morphant (MyD88) larvae after injection of vehicle control (mock), murine CCL2 (mCCL2), human CCL2 (hCCL2), human IL-8 (hIL-8), or human LTB4 (hLTB4). Representative of three separate experiments. Significance assessed by one-way ANOVA with Bonferroni’s post-test for the comparisons shown. *$P < 0.05$; ***$P < 0.001$. 

**c.** Mean neutrophil recruitment at 3 hpi into the HBV of control (CTRL), CCR2-morphant (CCR2), or MyD88-morphant (MyD88) larvae after injection of vehicle control (mock), murine CCL2 (mCCL2), human CCL2 (hCCL2), human IL-8 (hIL-8), or human LTB4 (hLTB4). Representative of three separate experiments. Significance assessed by one-way ANOVA with Bonferroni’s post-test for the comparisons shown. *$P < 0.05$; ***$P < 0.001$. 

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Extended Data Figure 9 | Identification of zebrafish CCL2 orthologue.

**a**, mRNA levels of potential CCL2 orthologues (mean ± s.e.m. of four biological replicates) induced at 3 h after caudal vein infection of 2 dpf larvae with 250–300 wild-type *M. marinum*. These assays were performed on the same cDNA pools as the data presented in Fig. 4b. **b**, Mean macrophage recruitment at 3 hpi into the HBV of wild-type or CCL2 morphant (MO) fish following infection with 80 *M. marinum*. Representative of two separate experiments.
Extended Data Figure 10 | Infectivity assay. a, b, Representative 5 hpi images from Fig. 4d following HBV infection with one (a) or three (b) M. marinum. Scale bar, 100 μm. N values for fish represented in a and b (that is, those found to be infected with 1–3 bacteria) are presented in Fig. 4d (18, 22, 28, 28, 28, 22, 22 for the respective conditions as specified in the figure). c, Mean bacterial burdens 5 h after HBV infection with 1–3 wild-type (WT), ΔmmpL7 or Δpks15 M. marinum.