Pathogenic mycobacteria induce the formation of complex cellular aggregates called granulomas that are the hallmark of tuberculosis. Here we examine the development and consequences of vascularization of the tuberculous granuloma in the zebrafish–Mycobacterium marinum infection model, which is characterized by organized granulomas with necrotic cores that bear striking resemblance to those of human tuberculosis. Using intravital microscopy in the transparent larval zebrafish, we show that granuloma formation is intimately associated with angiogenesis. The initiation of angiogenesis in turn coincides with the generation of local hypoxia and transcriptional induction of the canonical pro-angiogenic molecule Vegfaa. Pharmacological inhibition of the Vegf pathway suppresses granuloma-associated angiogenesis, reduces infection burden and limits dissemination. Moreover, anti-angiogenic therapies synergize with the first-line anti-tubercular antibiotic rifampicin, as well as with the antibacterial metronidazole, which targets hypoxic bacterial populations. Our data indicate that mycobacteria induce granuloma-associated angiogenesis, which promotes mycobacterial growth and increases spread of infection to new tissue sites. We propose the use of anti-angiogenic agents, now being used in cancer regimens, as a host-targeting tuberculosis therapy, particularly in extensively drug-resistant disease for which current antibiotic regimens are largely ineffective.

The human tuberculous granuloma, a tightly cohesive cellular structure that houses infecting mycobacteria, develops hypoxic areas around its necrotic core. In tumours, the development of hypoxia is tightly linked to angiogenesis and subsequent metastasis. In tuberculosis, attention has focused on the possible consequences of granuloma hypoxia to bacterial physiology, but relatively little attention has been paid to the functional significance of findings that tuberculous granulomas are extensively vascularized.

In its natural ectothermic hosts, M. marinum, the closest relative of the M. tuberculosis complex, causes a disease called fish tuberculosis, a systemic wasting disease with organized epithelioid granulomas with necrotic cores. In zebrafish larvae, mycobacterium-infected macrophages form early granulomas, undergo a hallmark epithelioid transformation, and activate granuloma-specific gene expression programs.

To monitor host vasculature in zebrafish, we used the Tg(kdrl:egfp) line (referred to hereafter as Tg(flk1:eGFP), in which vascular endothelial cells are fluorescently labelled with enhanced green fluorescent protein (eGFP)). Injection of mycobacteria into the most commonly used caudal vein site results in granulomas in the immediate vicinity of the richly vascularized area of the caudal haematopoietic tissue (CHT) (Fig. 1a). To determine whether a different injection site with sparser and smaller blood vessels was more suitable to detect angiogenesis, we assessed primary granulomas that typically formed dorsally after injection into the trunk (Fig. 1b, c). As with caudal vein injection, trunk injection resulted in most bacteria becoming resident in macrophages in granulomas (Extended Data Fig. 1a). Trunk granulomas progressed similarly to caudal vein granulomas, with dissemination into richly vascularized areas (Supplementary Video 1). Using Tg(mpeg1:tdTomato-<sup>caax</sup>) larvae, in which macrophages are labelled by membrane-bound Tomato, revealed similar macrophage dynamics, including the interstitial egress of infected macrophages, the transfer of M. marinum between granulomas, and coalescence of distal bacteria into existing granulomas (Supplementary Videos 2, 3, 4). Some infected macrophages invaded vasculature around the primary granuloma (Extended Data Fig. 1b and Supplementary Video 5).

Imaging of trunk-infected larvae revealed angiogenesis, with growth of vasculature around sites of infection. We observed sprouting from the existing intersegmental vessels (ISVs) starting at 4 days post-infection (dpi), just after the formation of granulomas (Fig. 1c). We assessed the features of vessel sprouting through long-term live imaging of infected larvae (Supplementary Videos 6, 7, 8, 9). Vessel growth occurred in spurs
with extended periods of quiescence or even reversed directionality (Extended Data Fig. 1c).

To examine the mode of vascular elongation, we used blue-fluorescent M. marinum to infect the transgenic zebrafish line Tg(fli1a:nls-eGFP; fkl1:mCherry
c), in which endothelial nuclei are marked by eGFP expression. At 4 dpi, nuclei left the highly organized ISVs, always towards sites of infection, and subsequently divided within the somites (Extended Data Fig. 1d and Supplementary Video 10). Vessels sprouted from both arterial and venous ISVs (Extended Data Fig. 1e).

We determined whether the new blood vessels generated around the granuloma were functional. Using DsRed-labelled erythrocytes in the transgenic line Tg(fkl1:eGFP; gata1:1DsRed
c), we found substantial blood flow through both ectopic vessels that spanned existing vessels completely and into newer blind-ending vessels (Extended Data Fig. 1f).

Angiogenesis required persistent M. marinum infection; it did not develop after injection of PBS, heat-killed M. marinum or non-pathogenic Escherichia coli (Extended Data Fig. 2a). Tumour-associated macrophages are important drivers of tumour angiogenesis upon tumour hypoxia. Since macrophages serve as a principal repository of virulent mycobacteria, we assessed whether there were differences in vascular recruitment between macrophage-resident and extracellular mycobacteria. Infection of double-transgenic Tg(fkl1:eGFP, mpeg1:tdTomato-caax) embryos with Cerulean-fluorescent M. marinum allowed us to discriminate between intracellular and extracellular bacteria (Fig. 2a). Enumeration of vascular branching revealed an elevated vascularization rate for intracellular compared with extracellular foci (odds ratio (OR) and 95% confidence interval: 4 dpi, 6.63 (2.57–17.11); 5 dpi, 6.93 (1.51–31.75); 6 dpi, 27.84 (10.18–76.18)) (Extended Data Fig. 2b).

To demonstrate a functional requirement for macrophages in recruiting vasculature we performed morpholino knockdown of the transcription factor Pu.1 (also known as Spi-1), which fully ablates all macrophages until 5 days post-fertilization (dpf) (Extended Data Fig. 2c). As previously reported, infection burden was markedly increased in the Pu.1 knockdown animals (Extended Data Fig. 2d). Despite increased burden, the total length of abnormal vasculature was decreased in morpholino-injected animals compared with controls, suggesting that macrophages specifically mediate new vessel growth in the context of inflammation (Fig. 2b).

Vascularization coincided with the formation of granulomas at around 4 dpi. We disrupted mycobacterium-driven granuloma formation using a M. marinum strain deficient in the ESX1 protein export system (AESX1), which could not form granulomas. We infected AESX1 with a 7.5-fold excess over wild type to generate equivalent bacterial burdens at 4 dpi (Extended Data Fig. 2e). As expected, both strains were predominantly macrophage resident, but AESX1 infection resulted in fewer granulomas and a marked reduction in angiogenesis (Fig. 2c). Thus, macrophage residence per se is not sufficient to induce angiogenesis. Rather, macrophages that have undergone further differentiation to form granulomas appear to be required.

When we analysed the relationship between the size of individual infection foci and the length of recruited vasculature we found a strong correlation (Extended Data Fig. 3a). This relationship is reminiscent of the angiogenic switch, in which tumour size is related directly to the requirement for vascularization due to the development of local hypoxia.

We therefore asked whether hypoxia develops within granulomas. Activation of Hif-1α increases transcription of prolyl hydroxylase 3 (phd3; also known as egln3), which serves as a reporter for hypoxic conditions in zebrafish larvae. We detected robust expression of phd3 within trunk granulomas, but not CHT granulomas, which are already proximate to the vasculature (Extended Data Fig. 3b), consistent with a lack of hypoxia in the CHT. Conversely, to assess whether mycobacteria within granulomas were experiencing hypoxia, we investigated the effect of metronidazole treatment. Metronidazole specifically kills anaerobically growing bacteria, including anaerobically growing M. tuberculosis, but is inefficient during aerobic growth. We used a nitrateducetase-expressing transgenic line Tg(lyzC:ntr-p2A-lanYFP) to titrate a biologically active dose of metronidazole (Extended Data Fig. 3c). Consistent with the phd3 staining, metronidazole treatment reduced bacterial burden in trunk-infected animals but not caudal-vein-infected larvae (Fig. 2d).

In tumours, hypoxia is known to induce VEGF expression, which in turn stimulates angiogenesis. VEGF has been associated with tuberculosis pathogenesis: it is induced in active pulmonary tuberculosis, and, in a rat corneal model, mediates neovascularization in granulomas triggered by mycobacterial trehalose-6,6'-dimycolate. VEGF has a conserved role in homeostatic blood vessel recruitment in zebrafish. We observed strong vegfaa expression around sites of mycobacterial granulomas in the trunk (Fig. 2e). Similar expression levels were also observed around CHT granulomas, indicating that initiation of pro-angiogenic signalling is not dependent on prior development of hypoxia during infection (Extended Data Fig. 4a). Analysis of stained sections revealed cells around the edge of mycobacterial granulomas expressing vegfaa, an observation consistent with a primarily macrophage-driven expression pattern that could be reduced by macrophage depletion (Extended Data Fig. 4b). In addition to its role in angiogenesis, VEGF plays an important part in driving vascular permeability. We performed microangiography on infected animals and found increased vascular leakage, suggesting a local effect of vegfaa expression around infection foci (Extended Data Fig. 4c).
Figure 3 | Inhibition of VEGFR signalling reduces M. marinum pathogenicity in zebrafish larvae. a. Bacterial burden in trunk-infected pazopanib-treated (left) and SU5416-treated (right) larvae. Student’s t-test, data are pooled from two (left graph) or three (right graph) biological replicates. DMSO, dimethylsulphoxide. b. Bacterial dissemination in untreated and pazopanib-treated larvae. Total number of granulomas and larvae analysed: untreated, 77 granulomas from 18 larvae; pazopanib, 130 granulomas from 22 larvae. Fisher’s exact test. c. Expression of phd3 hypoxia marker in untreated and pazopanib-treated infected larvae detected by in situ hybridization. Total number of larvae analysed: 52 (DMSO); 30 (pazopanib). Fisher’s exact test, data are from a single technical replicate of two pooled biological replicates. d. Bacterial burden in pazopanib-treated, metronidazole (MET)-treated, and pazopanib and metronidazole treated larvae. One-way analysis of variance (ANOVA) with Tukey’s post-test, data are pooled from three biological replicates. e. Bacterial burden in rifampicin (RIF)-treated, SU5416 and rifampicin treated, and SU5416-treated larvae. One-way ANOVA with Tukey’s post-test, data are pooled from three biological replicates. Error bars represent mean ± s.d. **P < 0.01, ***P < 0.001.

Angiogenesis and, specifically, VEGFR signalling have been targeted in cancer therapies. Our findings suggested that these therapies might also be useful for mycobacterial infections. We chose the well-characterized small molecule SU5416, a prototypical tyrosine kinase receptor, and pazopanib, a clinically relevant VEGFR inhibitor. Treatment of infected animals with SU5416 or pazopanib prevented ectopic angiogenesis around the forming granulomas in Tg(flk1:eGFP) zebrafish and reduced net bacterial burdens (Fig. 3a and Extended Data Fig. 5a). Neither compound affected in vitro growth of M. marinum, suggesting that the effect on bacterial burden was achieved through targeting host pathways (Extended Data Fig. 5b). We confirmed that the treatments were specifically targeting angiogenesis, since bacterial burdens were lowered only in trunk-infected and not in caudal-vein-infected zebrafish (Extended Data Fig. 5c). Additionally, in trunk-infected animals, growth restriction did not occur until after the initiation of angiogenesis at 4 dpi (Extended Data Fig. 5d). To determine whether VEGFR inhibition affected macrophage recruitment, we compared the association of bacterial foci with macrophages between control and pazopanib-treated larvae. We did not observe any differences in the proportion of macrophage-associated foci at time points between 4 and 6 dpi (Extended Data Fig. 5e). Together, these data suggest that VEGFR inhibition reduces bacterial burden specifically through restriction of vascularization.

To determine whether VEGFR inhibitors also reduced infection-induced vascular permeability, we measured permeability in treated and untreated animals matched for infection burden. We found a reduction in vascular leakage in zebrafish treated with a VEGFR inhibitor (Extended Data Fig. 5f).

Angiogenesis is thought to have an important role in tumour metastasis. Analogously, in long-term monitoring experiments, we observed a decreased rate of M. marinum dissemination to distal sites in zebrafish treated with a VEGFR antagonist compared with untreated animals (OR 0.27, 95% confidence interval 0.12–0.63) (Fig. 3b and Extended Data Fig. 5g). To investigate whether decreased dissemination was solely a consequence of reduced bacterial growth, we examined a hypoinflammatory state caused by knockdown of an enzyme involved in eicosanoid biosynthesis, Lta4h. lta4h knockdown increased burden, limited angiogenesis and also decreased dissemination, suggesting a role for angiogenesis in dissemination independent of burden (Extended Data Fig. 5h).

We hypothesized that reduced bacterial growth due to decreased oxygen availability may contribute to overall reduced burdens. Pazopanib treatment resulted in an increased number of phd3-positive granulomas (Fig. 3c and Extended Data Fig. 5i). Moreover, pazopanib treatment increased the effectiveness of metronidazole (Fig. 3d). These results suggest that angiogenesis is an important modulator of oxygen availability for infecting mycobacteria and that its limitation can enhance the efficacy of therapies targeting hypoxia. Metronidazole has only marginal therapeutic efficacy in human tuberculosis; our results suggest combining it or related compounds with VEGFR inhibitors that increase the hypoxic environment. Finally, we showed that targeting of VEGFR signalling could complement the first-line antitubercular drug rifampicin: a combination of rifampicin and SU5416 resulted in decreased burden compared to either drug alone (Fig. 3e).

Figure 4 | Inhibition of VEGFR signalling reduces M. marinum burden in adult zebrafish. a. Survival analysis of adult zebrafish infected with 400 c.f.u. (red lines), 4,000 c.f.u. (blue lines) or 8,000 c.f.u. (green lines) of M. marinum. Zebrafish are further grouped into control (dashed lines) or pazopanib-treated (solid lines) groups. Log-rank test: 400 c.f.u., not significant; 4,000 c.f.u., P = 0.012; 8,000 c.f.u., P = 0.029. b, Representative image of a necrotic granuloma from a 2 weeks post-infection adult Tg(flk1:eGFP) zebrafish infected with Cerulean-fluorescent M. marinum (cyan), and stained for hypoxprobe (red) and with 4',6-diamidino-2-phenylindole (DAPI; blue). Image is representative of granulomas found in 16 individual animals. c. Pooled bacterial burden in pazopanib-treated adult zebrafish. Matched Student’s t-test. d. Comparison of granulomas between control and pazopanib-treated adult zebrafish scored for M. marinum burden as less than ten or more than ten bacteria. Total number of zebrafish analysed: 4 (control), 4 (pazopanib). Scale bar, 100 μm. Error bars represent mean ± s.d.
We next addressed the therapeutic effectiveness of VEGFR inhibition in adult animals. We infected zebrafish with a range of mycobacterial doses from 400–8,000 colony-forming units (c.f.u.) via intraarterial injection, treated them with pazopanib, and observed their survival. Over 17 days, pazopanib treatment increased survival in animals infected with high doses (4,000 and 8,000 c.f.u.) of M. marinum, but there was not yet appreciable mortality in the low-dose infection group (Fig. 4a). At a dose of 500 c.f.u., where significant mortality was not observed in the first 3 weeks, we observed granulomas that were completely cellular as well as ones that had developed necrotic cores by 2 weeks post-infection (wpi) (Extended Data Fig. 6a). Many adult granulomas were hypoxic, as assessed by pimonidazole treatment and staining. Staining was largely specific to granulomas and was concentrated in the cellular rim of the necrotic area, a pattern similar to that seen in macaques9 (Fig. 4b and Extended Data Fig. 6b).

Pazopanib treatment increased the mean distance of granulomas to the nearest vasculature (Extended Data Fig. 6c). As in larvae, treatment resulted in reduced bacterial burdens, with a mean fourfold reduction relative to control animals over 2 weeks (Fig. 4c). In week-old established infections, pazopanib treatment for 1 week resulted in a mean eightfold reduction in burden (Extended Data Fig. 6d).

After 6 weeks of treatment, vascularization was still significantly reduced relative to controls (Extended Data Fig. 7a). Notably, there was an increased fraction of low-burden or sterile granulomas in the pazopanib-treated zebrafish (Fig. 4d and Extended Data Fig. 7b). In addition, the drug-treated animals displayed an increased fraction of hypoxic granulomas, and there was an association of hypoxic granulomas with low-burden lesions (Extended Data Fig. 7c, d). Many low-burden or sterilized hypoxic granulomas in the drug-treated zebrafish had acellular necrotic central areas (Extended Data Figs 6a and 7b). Studies of casedous tuberculous granulomas that are sterile in asymptomatic humans suggest that such an outcome is possible even in the normal course of tuberculosis80.

We conclude that angiogenesis triggered by mycobacterial granulomas is an important feature of mycobacterial pathogenesis and has important consequences for infection pathology and progression. We have shown that interplay of this angiogenic program using host-directed therapies can limit mycobacterial disease. These findings suggest the potential utility of host-targeting anti-angiogenic agents as adjunctive therapies.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Ernst, J. D. The immunological life cycle of tuberculosis. *Nature Rev. Immunol.* **12**, 581–591 (2012).

2. Ramakrishnan, L. Revisiting the role of the granuloma in tuberculosis. *Nature Rev. Immunol.* **12**, 352–366 (2012).

3. Freeman, C. D., Klutman, N. E. & Lamp, K. C. Metronidazole. *A therapeutic review* for adults.* Drugs** **54**, 679–708 (1997).

4. Rittershaw, E. S., Baek, S. H. & Sassetti, C. M. The normalcy of dormancy: common themes in microbial quiescence. *Cell Host Microbe* **13**, 643–651 (2013).

5. Folkman, J. Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol.* **29**, 15–18 (2002).

6. Tsai, M. C. et al. Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension. *Cell Microbiol.* **8**, 218–232 (2006).

7. Aly, S. et al. Interferon-γ-dependent mechanisms of mycobacteria-induced pulmonary immunopathology: the role of angiotensin and CXCR3-targeted chemokines for granuloma necrosis. *J. Pathol.* **212**, 295–305 (2007).

8. Urichs, T. et al. Differential organization of the local immune response in patients with active cavitary tuberculosis or with nonprogressive tuberculosis. *J. Infect. Dis.* **192**, 89–97 (2005).

9. Davis, J. M. et al. Real-time visualization of mycobacterium–macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity* **17**, 693–702 (2002).

10. Jin, S. W., Beis, D., Mitchell, T., Chen, J. N. & Stainier, D. Y. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* **132**, 5199–5209 (2005).

11. Davis, J. M. & Ramakrishnan, L. The role of the granuloma in expansion and dissemination of early tuberculosis infection. *Cell* **136**, 37–49 (2009).

12. Dirix, A. E., Oude Egbrink, M. G., Wagstaaf, J. & Griffioen, A. W. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *J. Leukoc. Biol.* **80**, 1183–1196 (2006).

13. Rhodes, J. et al. Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev. Cell* **8**, 97–108 (2005).

14. Clay, H. et al. Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish. *Cell Host Microbe* **2**, 29–39 (2007).

15. Volkman, H. E. et al. Tuberculosis formation is enhanced by a mycobacterium virulence determinant. *PLoS Biol.* **2**, e367 (2004).

16. Aprilieva, O. et al. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *J. Cell. Biochem.* **92**, 491–501 (2004).

17. Santhakumar, K. et al. A zebrafish model to study and therapeutically manipulate hypoxia signaling in tumorigenesis. *Cancer Res.* **72**, 4017–4027 (2012).

18. Elks, P. M. et al. Hypoxia inducible factor signaling modulates susceptibility to mycobacterial infection via a nitric oxide dependent mechanism. *PLoS Pathog.* **9**, e1003789 (2013).

19. Via, L. E. et al. Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect. Immun.* **76**, 2333–2340 (2008).

20. Lin, P. L. et al. Metronidazole prevents reactivation of latent Mycobacterium tuberculosis infection in macaques. *Proc. Natl Acad. Sci. USA* **109**, 14118–14193 (2012).

21. Forsythe, J. A. et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* **16**, 4604–4613 (1996).

22. Matsuyma, W. et al. Increased serum level of vascular endothelial growth factor in pulmonary tuberculosis. *Am. J. Respir. Crit. Care Med.* **162**, 1120–1124 (2000).

23. Saita, N., Fujivara, N., Yano, I., Soeijma, K. & Kobayashi, K. Trehalose 6,6′-dimycoclate (cord factor) of Mycobacterium tuberculosis induces corneal angiogenesis in rats. *Infect. Immun.* **68**, 5991–5997 (2000).

24. Nasruevicus, A., Larson, J. & Ekker, S. C. Distinct requirements for zebramice angiogenesis revealed by a VEGF-A morphant. *Yeast* **1**, 294–301 (2000).

25. Dvorak, H. F., Brown, L. F., Detmar, M. & Dvorak, A. M. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.* **155**, 413–424 (1999).

26. Podar, K. et al. The small-molecule VEGF receptor inhibitor pazopanib (GW786034B) targets both tumor and endothelial cells in multiple myeloma. *Proc. Natl Acad. Sci. USA* **103**, 19478–19483 (2006).

27. Fong, T. A. et al. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flik-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res.* **59**, 90–106 (1999).

28. Tobin, D. M. et al. The ιtα4 locus modulates susceptibility to mycobacterial infection in zebrafish and humans. *Cell* **140**, 717–730 (2010).

29. Carroll, M. W. et al. Efficacy and safety of metronidazole for pulmonary multidrug-resistant tuberculosis. *Antimicrob. Agents Chemother.* **57**, 3903–3909 (2013).

30. Opie, E. L. & Aronson, J. D. Tubercle bacilli in latent tuberculous lesions and in lung tissue without tuberculous lesions. *Arch. Pathol. Lab. Med.* **4**, 1–21 (1927).
METHODS

Zebrafish handling. All zebrafish husbandry and experimental procedures were performed in accordance and compliance with policies approved by the Duke University Institutional Animal Care and Use Committee (protocol A180-11-07). Clutches of eggs were collected from natural spawning and raised in filtered fish system water at 28°C. Pigment development was halted in 1 dpf embryos by the addition of 1-phenyl-2-thiourea (PTU; Sigma-Aldrich; final concentration 45 μg ml⁻¹). Unless otherwise indicated, all zebrafish are from the wild-type AB strain.

Infection by microinjection. Embryos were anaesthetized at 2 dpf (or 1 dpf for morphants) with tricaine (MS-222; Sigma-Aldrich; final concentration 160 μg ml⁻¹) and injected with approximately 200 c.f.u. M. marinum or E. coli in an injection bolus of 10-20 nl as shown in Fig. 1B. Infected embryos were then recovered back to filtered fish system water supplemented with PTU. Embryos that were physically damaged by injection handling were discarded and excluded from further analysis.

Live imaging. Conventional and time-lapse fluorescent microscopy was carried out on a Zeiss Observer Z1 inverted microscope. Embryos were anaesthetized with tricaine and mounted in 3% (w/v) methylcellulose for static microscopy. Embryos for time-lapse microscopy were anaesthetized with 120 μg ml⁻¹ tricaine, mounted in 0.75% low melting point agarose in 96-well plates and immersed in filtered fish system water supplemented with PTU. Confocal microscopy was performed on an Olympus FV1000 confocal microscope. Images were processed with ImageJ (NIH), Photoshop CS4 (Adobe) and Velocity 5.4 image analysis software (Improvision, PerkinElmer Life and Analytical Sciences).

Image analysis. Abnormal vascular length was measured as the two-dimensional length of vessels not seen in control embryos in Photoshop using the ruler tool. Infection burden and neutrophil units were measured as the number of pixels above background per embryo in ImageJ using binary thresholding of single channel images and the Analyze Particles function. Macrophage association was scored as having mpeg1tdTomato-caax expression around sites of Wasabi-fluorescent M. marinum expression. Disappearance was scored by comparing images of infected larvae at 4- and 6-dpi to track M. marinum infection foci.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was carried out essentially as described. Primers used for cloning vegfaa were previously described. Primers used for cloning phd3 were 5′-ATTCCTCGGTTGGCCTCTCA AC-3′ and 5′-ACAGGAACAACTGTGCACT-3′. Images of stained embryos were collected on a Nikon AZ100 microscope.

Construction of mpeg1:tdTomato-caax transgene. The previously characterized mpeg1 promoter was PCR amplified using the primers 5′-CCCAAACTCAGTGTTGAGGAACACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ and 5′-GGGGACAACTTTGTATAATAAAGTTGTTACTTGTACGTCCATGGGCG-3′. These primers introduced a silent SacI site in the 3′-UTR of mpeg1:tdTomato-caax. The product was subsequently cloned into p5E MCS using SacI and HindIII sites to generate p5E mpeg1:tdTomato-caax. The previously characterized vegfaa promoter was PCR amplified using the primers 5′-GTAAGTTCTGTGGCTGTTTTATGTTGTAGAGCTCGTCCAT-3′ and 5′-CCCAAACTCAGTGTTGAGGAACACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ to generate p5E MSCs using KpnI and XhoI sites.

Construction of mpeg1:tdTomato-caax transgene construct. The sequence of tdTomato was PCR amplified with the primers 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ and 5′-GGGGACAACTTTGTATAATAAAGTTGTTACTTGTACGTCCATGGGCG-3′. These primers introduced a silent SacI site in the 3′-UTR of tdTomato coding sequence and a BglII site downstream of the tdTomato stop codon. The PCR product was subsequently cloned by Gateway recombination (Invitrogen) into pDONR211 to generate pME tdTomato-caax.

To generate mpeg1:tdTomato–CAAX, pME tdTomato–CAAX was digested with SacI and BglII and a linker sequence encoding the human H-Ras prenylation signal was cloned into pDONR221 to generate pME tdTomato–CAAX. The native start codon was mutated using the primer 5′-CTGATGAGCAGATGATAGTGAGAGGCTGATGGATGGAGATGAGGATG-3′ and 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ to amplify off pmTurquoise2 H2A (Addgene plasmid #36202; ref. 35). The PCR product was subsequently cloned into pDONR221 by BP cloning (Invitrogen) to generate pME Turquoise2.

The mfpaturquoise transgene construct was subsequently constructed by recombining p5E mfpaturquoise and p3E polyA into pDestTo2p2a2 to generate pDestTo2; mfpaturquoise.

Construction of lycCntr-p2A-lypYFP transgene. The previously characterized lycC promoter was PCR amplified from the bacterial artificial chromosome (BAC) CH211-250A24 (BACPAC resources, Children’s Hospital of Oakland Research Institute) using the primers 5′-CCCATAGTACCCGTTGCTAGCTGTAGTTGATGTACCTC-3′ and 5′-CCCAAACTCAGTGTTGAGGAACACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ to amply the dimeric form of lypYFP from pNCS-lypYFP (Allele Biotechnologies). The PCR product was subsequently cloned into pDONR221 by BP cloning (Invitrogen) to generate pME lycCntr.

To generate p3E lycCntr-p2A-lypYFP, we used the primers 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ and 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′. The product was subsequently cloned into p5E MCS using KpnI and XhoI sites to generate p5E lycCntr.

To generate p3E Ntr, we used the primers 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ and 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′. The product was subsequently cloned into pDONR221 by BP cloning (Invitrogen) to generate pME Ntr.

To generate p3E p2A-lypYFP, we used the primers 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′ and 5′-GGGGACAACTTTGTACAAGAAAGCTGGGTCCACTTCGGTTAAGGTGATCCATGGTC-3′. The product was subsequently cloned into pDONR221 by BP cloning (Invitrogen) to generate pME lycCntr-p2A-lypYFP.

Nucleic acid microinjection. Tol2 transposase was generated from T3TS-Tol2 (ref. 37) using the mMessage mMachine T3 kit (Invitrogen). To generate transgenic zebrafish, embryos at the one-cell stage were injected with approximately 1 nl of trangenesis mixture consisting of 25 ng nl⁻¹ transposase RNA and 50 ng μl⁻¹ pDestTol2; transgenesis positive. Embryos were selected by fluorescence microscopy, raised to adulthood and transgenic founders were subsequently identified.

The Pu.1/Spi1 morpholino sequence was 5′-GATAATACAGTACCTTTGGAAGTGGTTAG-3′ and the lta4h morpholino sequence was 5′-AGCTAGGTTGCTAAACCGTACATGG-3′. Morpholinos were injected at 10–60 μM.

Drug treatments. Metronidazole (M1547; Sigma-Aldrich; final concentration 5 mM) was dissolved in water; the relatively high concentration of metronidazole seems to be a function of bioavailability in zebrafish. A higher concentrations are routinely required for nitroreductase-mediated cellular ablation studies. Paazopanib (sc-364564; Santa Cruz Biotechnology; final concentration 250 nM for larvae, 1 μM for adults) and SU5416 (S8442; Sigma-Aldrich; final concentration 250 nM) were dissolved in DMSO. Metronidazole, pazopanib and SU5416 were added immediately after injection and refreshed every 2 days for the duration of the experiment. Randomization into drug treatment groups was achieved by random selection of infected zebrafish from a single pool before addition of drugs.

Microangiography. To detect vascular leakiness in wild-type and Tg(kl:egFP) embryos, embryos were anesthetized in tricaine water and injected with a 10 μl bolus of dextran-Texas Red 70,000 MW (D-1830; Life Technologies; 1 mg ml⁻¹ final concentration) into the posterior section of the dorsal aorta or posterior cardinal vein. This injection location avoided injection-trauma-induced tissue leakage occurring near M. marinum lesions. Injected embryos were rinsed in tricaine water and immediately mounted in methylcellulose for fluorescent microscopy. Vascular leakage was calculated as a ratio of intersomitic dextran-Texas Red signal divided by aortic dextran-Texas Red signal.

Combined drug treatments. Rifampicin (R3501; Sigma-Aldrich; final concentration 30 μM) was dissolved in DMSO. To achieve a suboptimal dose of VEGFR inhibition, SU5416 was added immediately after injection at a final concentration of 200 nM and refreshed at 3 dpi. Rifampicin was added at 3 dpi and not refreshed for the duration of the experiment.

To generate p3E TOAST (final concentration 5 μM) and paazopanib (suboptimal final concentration 200 nM) were added immediately after injection and refreshed every 2 days for the duration of the experiment. Randomization into drug treatment groups was achieved by random selection of infected embryos from a single pool prior to addition of drugs; blinding was not performed for subsequent quantitation.
Adult infections. Adult zebrafish were infected with approximately 500 c.f.u. of fluorescent *M. marinum* via intraperitoneal injection. Zebrafish were maintained in beakers in a dedicated incubator at 28 °C with a 14:10 h light:dark cycle. Pazopanib was added to a final concentration of 1 μM immediately after infection and refreshed every 2 days for the duration of the experiment.

Randomization into drug treatment groups was achieved by random selection of infected fish from a single pool prior to addition of drugs; blinding was not performed for subsequent quantitation.

Bacterial recovery from infected adults. Infected adult zebrafish were pre-treated with 25 μg ml⁻¹ hygromycin to reduce microbiota load for 2 h before harvesting. Zebrafish were euthanized by tricaine overdose and homogenized by bead mill for three bursts of 15 s. Homogenate was plated on Middlebrook 7H10 (262710; Difco) supplemented with OADC, hygromycin (H0654; Sigma-Aldrich, 50 μg l⁻¹) and amphotericin B (SV3007801; Thermo Scientific, 10 mg l⁻¹). Plates were grown at 30 °C for 10–14 days until fluorescent colonies could be counted.

Hypoxpyrobe staining. Infected adult zebrafish were injected with 15 μl of a 10 mg ml⁻¹ pimonidazole solution (HP7; Hypoxyprobe) every 2 days from 8 dpi to 14 dpi. Zebrafish were euthanized by tricaine overdose at 14 dpi, fixed in 4% PFA, decalcified in 0.5 M EDTA and cyrosectioned. Frozen sections were stained with 4.3.1.3 mouse Dylight 549-MAb (HP7; Hypoxyprobe) or with unconjugated 4.3.1.3 mouse monoclonal antibody and secondary detection was carried out with goat anti-mouse Alexa-Fluor 647 (A-21235; Life Technologies) to detect hypoxic cells.

Statistics. Data are presented as mean ± s.d. Experiments were analysed with the statistical tests indicated in figure legends using Prism 5 (Graphpad). Unpaired Student’s *t*-tests were performed unless otherwise indicated. For ANOVA analyses with Tukey’s post-test, *P* values are indicated as follows: *P* < 0.05, **P** < 0.01, ***P** < 0.001.

31. Thisse, C. & Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nature Protocols* 3, 59–69 (2008).
32. Liang, D. et al. Cloning and characterization of vascular endothelial growth factor (VEGF) from zebrafish, *Danio rerio*. *Biochim. Biophys. Acta* 1397, 14–20 (1998).
33. Elliott, F., Pasa, L., Hayman, J. W., Andrianopoulos, A. & Lieschke, G. J. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood* 117, e49–e56 (2011).
34. Kwan, K. M. et al. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev. Dyn.* 236, 3088–3099 (2007).
35. Goedhart, J. et al. Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%. *Nature Commun.* 3, 751 (2012).
36. Kim, J. H. et al. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS ONE* 6, e18556 (2011).
37. Balciunas, D. et al. Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. *PLoS Genet.* 2, e169 (2006).
38. Curado, S., Stainier, D. Y. & Anderson, R. M. Nitroreductase-mediated cell/tissue ablation in zebrafish; a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. *Nature Protocols* 3, 948–954 (2008).
Extended Data Figure 1 | Angiogenesis in the zebrafish M. marinum infection model. a, Image of 6 dpi Tg(mfap4:turquoise\textsuperscript{+\textasciitilde}) larvae infected with M. marinum SM2 pMAP49::Venus. Blue arrowheads indicates site of granuloma with induced expression of Venus from phagocytosed M. marinum. White arrowheads indicate sites of extracellular M. marinum growth detected by constitutive DsRed expression but no macrophage-induced Venus expression. Image is representative of granulomas found in five individual animals. b, Time-lapse images of Cerulean-fluorescent M. marinum dissemination from an established granuloma into the adjacent intersegmental vessel in a Tg(flk1:eGFP, mpeg1:tdTomato-caax\textsuperscript{+\textasciitilde}) double-transgenic larva where bacterial are labelled blue, blood vessels are labelled green and macrophages are labelled red. Yellow arrow tracks a single infected macrophage egressing the established granuloma and entering the vasculature. Images are representative of macrophage behaviour in three individual animals. c, Plots of vessel growth kinetics from three individual branches in individual Tg(flk1:eGFP) larvae. Videos of each larva analysed are available in Supplementary Videos 6 and 7 (left), and 8 and 9 (right). d, Time-lapse images of nuclear division during vascular growth in a single Tg(flk1:eGFP-caax\textsuperscript{+\textasciitilde}) larva. Blue arrowhead indicates nucleus of interest. Images are representative of nuclear division in ten individual animals. Video of nuclear division is available in Supplementary Video 10. e, Three-dimensional rendering of recruited blood vessels in a Tg(flk1:eGFP) larva infected with Tomato-fluorescent M. marinum originating from arterial and venous ISVs as indicated by red and blue arrows, respectively. Image is representative of ten individual animals. f, Extended exposure images of blood flow in Tg(flk1:eGFP, gata1:DsRed\textsuperscript{+\textasciitilde}) larvae. Blue arrows indicate blood flow through ectopic vessels. Images are representative of blood flow in 20 individual animals. Scale bars, 100 μm.
Extended Data Figure 2 | Formation of ectopic vasculature is dependent on granuloma formation. a. Length of abnormal vasculature in Tg(flk1:eGFP) larvae injected with PBS, live M. marinum, heat-killed M. marinum and E. coli. One-way ANOVA with Tukey’s post-test, data are representative of two biological replicates. b. Recruitment of vasculature by intracellular and extracellular foci of M. marinum. Total number of foci analysed: 4 dpi, 221 intracellular, 105 extracellular; 5 dpi, 71 intracellular, 26 extracellular; and 6 dpi, 131 intracellular, 50 extracellular. Fisher’s exact test. c. Comparative images of 5 dpf control and Pu.1 morphant Tg(mpeg1:tdTomato-caax) larvae. White arrowhead indicates comparative locations within the caudal haematopoietic tissue. Blue arrowhead indicates intestinal and yolk sac autofluorescence. Scale bar, 100 μm. Images are representative of transgene expression in 20 animals per treatment group. d, e. Bacterial burden in 5 dpi control and Pu.1 morphant larvae (d), and 4 dpi larvae infected with wild-type (WT) or ΔESX1 Tomato-fluorescent M. marinum (e). Student’s t-test with Welch’s correction, all data are pooled from two biological replicates. Error bars represent mean ± s.d. **P < 0.01, ***P < 0.001.
Extended Data Figure 3 | Granuloma vascularization correlates with granuloma size. a, Plot of abnormal vasculature length and bacterial burden for individual foci of infection measured by fluorescent pixel count (FPC) in Tg(flk1:GFP) larvae. Slope significantly not zero, $P < 0.0001$ linear regression, data are pooled from three biological replicates. b, Whole-mount in situ hybridization detection of phd3 expression. Images are representative of phd3 staining in uninfected (20/20), caudal vein (CV)-infected (20/20) and trunk-infected (7/20) zebrafish. c. Left, images of Tg(lzC:ntn-p2A-lanYFP<sup>xt14</sup>) larvae treated with metronidazole as indicated. Green arrowheads indicate comparative locations within caudal hematopoietic tissue. Images are median images from experimental groups: control, $n = 21$; 100 nM, $n = 22$; 1 mM, $n = 24$; and 10 mM, $n = 19$. Right, quantification of neutrophil numbers by area of fluorescence in Tg(lzC:ntn-p2A-lanYFP<sup>xt14</sup>) larvae treated with metronidazole from 2 dpf to 6 dpf. Error bars represent mean ± s.d.
Extended Data Figure 4 | *M. marinum* infection induces expression of vegfaa. 

**a.** Whole-mount *in situ* hybridization detection of vegfaa expression in uninfected, caudal vein (CV)-injected and trunk-injected larvae. Red arrow indicates sites of infection with vegfaa expression. Images are representative of 20 animals per treatment group. **b,** Representative histological sections of whole-mount *in situ* hybridization detected vegfaa expression in control infected larvae and a Pu.1 morpholino (MO)-treated infected larva. Black arrows indicate sites of infection identified by increased nuclear fast red staining density. Images are representative of ten animals per treatment group. **c,** Microangiography of *Tg(flk1:eGFP)* larvae imaged at 1, 5 and 10 min post-injection (mpi). Top panels are representative of uninfected larvae, bottom panels are representative of larvae infected with unlabelled *M. marinum*. Images are representative of ten animals per treatment group. Scale bars, 100 μm.
Extended Data Figure 5 | Pazopanib and SU5416 reduce *M. marinum* pathogenicity in zebrafish larvae. **a**, Left, comparative images of Tg(flk1:eGFP) larvae infected with Tomato-fluorescent *M. marinum* and treated with DMSO, pazopanib or SU5416. Top panels depict Tomato-fluorescent *M. marinum* and labelled vasculature. Bottom panels depict only Tg(flk1:eGFP)-labelled vasculature. Blue arrowheads indicate somites with ectopic vasculature. Images are representative of 20 animals per treatment group. Right, length of abnormal vasculature in pazopanib- or SU5416-treated larvae. Student’s *t*-test, data are pooled from two or three biological replicates, respectively. **b**, Growth curve of Tomato-fluorescent *M. marinum* in 7H9 broth culture supplemented with pazopanib or SU5416. Data are representative of two biological replicates. **c**, Bacterial burden in caudal-vein-infected larvae treated with either pazopanib or SU5416. Student’s *t*-test, data are pooled from two biological replicates. **d**, Longitudinal bacterial burden from 2 to 6 dpi in trunk-infected larvae treated with pazopanib. One-way ANOVA with Tukey’s post-test. NS, not significant; *n* = 14 individuals per group. **e**, Comparison of *M. marinum* foci between control and pazopanib-treated larvae scored by association with macrophages. Fisher’s exact test, *n* = 40 individuals per group. **f**, Left, microangiography of larvae infected with cerulean-fluorescent *M. marinum*, injected with high-molecular-weight dextran-Texas Red at 6 dpi and imaged at 5 minutes post dextran injection (mpi). Top panels depict Cerulean-fluorescent *M. marinum* and dextran-Texas Red, bottom panels depict only dextran-Texas Red in vasculature and leakage around sites of infection. Green arrowheads indicate somites with the highest leakage signals in infected larvae. Images are median images from graph on right. Right, quantification of vascular leakage in uninfected, DMSO- and pazopanib-treated larvae. One-way ANOVA with Tukey’s post-test, data are representative of two biological replicates. **g**, Dissemination of Wasabi-fluorescent *M. marinum* in larvae treated with DMSO or pazopanib. Red arrowheads indicate contained foci of infection that remain in the same location throughout the course of infection, blue arrowheads indicate disseminated foci of infection. Images are representative of data in Fig. 3b. **h**, Bacterial burden (left), length of abnormal vasculature (middle) and dissemination (right) in 5 dpi control and Lta4h morphant larvae. **i**, Whole-mount *in situ* hybridization detection of phd3 expression in uninfected (white arrow) and *M. marinum*-infected zebrafish larvae. Blue arrows indicate phd3-expression-positive larvae with purple staining, red arrow indicates site of bacterial infection with no purple staining, indicating phd3-expression-negative larva. Image is representative of data in Fig. 3c. Scale bars, 100 μm. Error bars represent mean ± s.d. *P* < 0.05, **P** < 0.01, ***P*** < 0.001.
Extended Data Figure 6 | Effects of pazopanib treatment are reproduced in adult zebrafish infections. a, Images of non-necrotic (left) and necrotic (right) Tomato-fluorescent *M. marinum* granulomas stained with DAPI (top) and haematoxylin and eosin (bottom). White arrows indicate non-necrotic granuloma, yellow arrows indicate necrotic granuloma. Images are representative of granulomas found in eight individual animals.
b, Representative image of a necrotic granuloma from a negative control, not injected with pimonidazole, 2 wpi adult *Tg(flk1:eGFP)* zebrafish infected with cerulean-fluorescent *M. marinum* (cyan), and stained for hypoxyprobe (red) and with DAPI (blue). Images are representative of granulomas found in two individual animals. c, Left, representative image of Tomato-fluorescent *M. marinum* granuloma in *Tg(flk1:eGFP)* zebrafish stained with DAPI. White arrow indicates granuloma, yellow line indicates path measured for distance between granuloma and nearest vasculature (indicated by green arrow). Image is representative of data presented on the right, in panel d and Extended Data Fig. 7a. Right, distance between granulomas and nearest vasculature measured in 2 wpi adult *Tg(flk1:eGFP)* zebrafish. Total number of zebrafish analysed: 4 (control), 4 (pazopanib). d, Left, distance between granulomas and nearest vasculature measured in 2 wpi adult *Tg(flk1:eGFP)* zebrafish treated with pazopanib for 1 week. Total number of zebrafish analysed: 2 (control), 2 (pazopanib). Right, bacterial burden in 2 wpi adult zebrafish treated with pazopanib for 1 week. Student’s t-test, data are pooled from three biological replicates.
Extended Data Figure 7 | Pazopanib increases the frequency of hypoxic and low-burden granulomas. a, Distance between granulomas and nearest vasculature measured in 6 wpi adult Tg(flk1:eGFP) zebrafish. Total number of zebrafish analysed: 4 (control), 4 (pazopanib). Green dot indicates outlier that was omitted from statistical analysis. b, Images of low burden/hypoxic (left) and high burden/non-hypoxic (right) granulomas in zebrafish that were injected with pimonidazole. Asterisks indicate Tomato-fluorescent M. marinum, arrows indicate areas of hypoxia in granuloma. Images are representative of data in c, d and Fig. 4d. c, Comparison of granulomas between control and pazopanib-treated adult zebrafish scored for pimonidazole staining. Total number of zebrafish analysed: 4 (control), 4 (pazopanib). d, Comparison of granulomas between non-hypoxic and hypoxic granulomas in control and pazopanib-treated adult zebrafish scored for M. marinum burden. Total number of zebrafish analysed: 4 (control), 4 (pazopanib). Scale bars, 100 μm. Error bars represent mean ± s.d.