Universiteit
Leiden
The Netherlands

Innate host defense against intracellular pathogens

Vaart, M. van der

Citation
Vaart, M. van der. (2013, November 14). *Innate host defense against intracellular pathogens*. Retrieved from https://hdl.handle.net/1887/22208

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/22208

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/22208 holds various files of this Leiden University dissertation

Author: Vaart, Michiel van der
Title: Innate host defense against intracellular pathogens
Issue Date: 2013-11-14

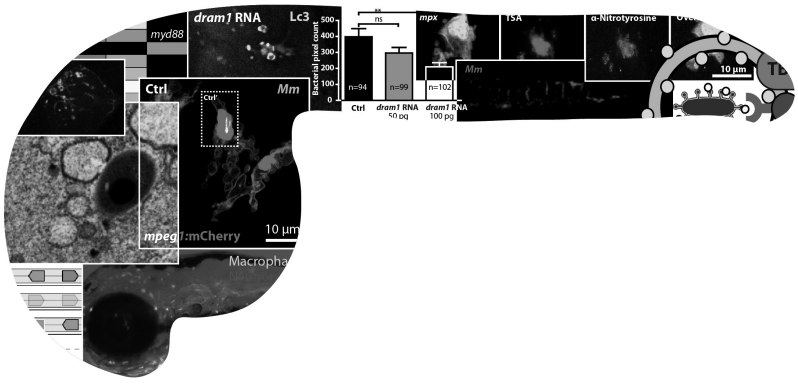
Chapter 4

Mycobacteria counteract a nitrosative host defense mechanism initiated upon TLR/IL1R-MyD88 signaling

Michiel van der Vaart[1], Philip M. Elks[1], Vincent van Hensbergen, Esther Schutz, Herman P. Spaink, and Annemarie H. Meijer

[1] These authors contributed equally to this work

Manuscript in preparation



Abstract

Pulmonary tuberculosis (TB), caused by the intracellular bacterial pathogen *Mycobacterium tuberculosis* (*Mtb*), is a major world health problem. The production of reactive nitrogen species (RNS) is a potent cytostatic and cytotoxic defense mechanism against intracellular pathogens. Nevertheless, the protective role of RNS during *Mtb* infection remains controversial. Here we use a nitrotyrosylation readout for nitrosative stress to study RNS production by the zebrafish host during early mycobacterial pathogenesis. We found that recognition of *Mycobacterium marinum*, a close relative of *Mtb*, was sufficient to induce a nitrosative defense mechanism in a TLR/IL1R-MyD88 dependent fashion. However, this host response was attenuated by mycobacteria via a virulence mechanism independent of the well-characterized RD1 virulence locus. Our results indicate a mechanism of pathogenic mycobacteria to circumvent host defense *in vivo*. Shifting the balance of host-pathogen interactions in favor of the host by targeting this virulence mechanism may help overcome the problem of *Mtb* strains that are resistant to multiple drug treatments.

Introduction

Pulmonary tuberculosis (TB) is a common and often lethal infectious disease that represents a major global health threat. The pathogenic bacterium *Mycobacterium tuberculosis* (*Mtb*) is the causative agent of TB and the high incidence and associated mortality worldwide is partly explained by the increasing occurrence of *Mtb* strains with resistance to multiple drug treatments. This makes TB a key priority for infectious disease research, since understanding of the host-pathogen interactions during *Mtb* pathogenesis is necessary to develop novel therapeutic strategies[1, 2]. Mycobacteria phagocytosed by macrophages evade the majority of leukocyte bacterial killing mechanisms and create a niche for themselves in which they can survive and proliferate[3]. Recruitment of macrophages, neutrophils, and T-cells to the primary infected cells leads to the formation of highly organised structures known as granulomas[4, 5]. During latent TB infection, a condition estimated to affect one-third of the human population, *Mtb* can persist for many years within granulomas before its reactivation

The combined efforts of the innate and adaptive arms of the immune system are relatively efficient in containing and killing *Mtb* in immunocompetent individuals. It is estimated that only 5-10 out of a 100 individuals newly infected with *Mtb* develop active TB during their lifetime[7]. One of the most effective host-defense mechanisms against intracellular pathogens is the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[8, 9]. The production of ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($OH^\bullet$), is catalyzed by the NADPH-oxidase complex during respiratory burst[8]. In vertebrates nitric oxide (NO) is formed when the guanidine nitrogen of L-arginine is oxidized by nitric oxide synthases (NOS). There are two constitutively expressed NOS enzymes, neuronal NOS (nNOS or NOS1)

and endothelial NOS (eNOS or NOS3), and one inducible NOS enzyme (iNOS or NOS2). Regulation of *iNOS* plays an important role in the inflammatory response and many cells of the immune system are capable of producing NO upon infection[10].

Although a mutation in the components of the NADPH-complex increased susceptibility to *Mtb*[11-13], ROS production itself was found to be relatively ineffective in killing *Mtb*[14]. In contrast, exposure to low concentrations of NO is cytotoxic to mycobacteria, killing more than 99% of *Mtb* in culture[15]. However, a significant part of the cytostatic and cytotoxic antimicrobial effects of NO *in vivo* are attributed to RNS, the various reaction products of $NO^{\bullet}$[16]. The reaction of NO radicals with O_2 radicals produced during the respiratory burst results in the generation of peroxynitrite ($ONOO^-$), a potent RNS causing lipid peroxidation, DNA damage, oxidation of thiols, and nitration of tyrosine residues[17, 18]. The addition of a nitrogen-group to tyrosine residues is also catalyzed by the neutrophil specific enzyme myeloperoxidase (Mpx), using H_2O_2 and NO_2^- as substrates[19]. Tyrosine nitration can lead to disruption of tyrosine phosphorylation-dependent signaling pathways[20]. The bactericidal activity of peroxynitrite has been demonstrated against *Salmonella enterica* serovar typhimurium[21], and *Escherichia coli*[22]. Peroxynitrite is also required for the fungicidal activity of macrophages infected with *Candida albicans*[23].

The murine TB model was used to demonstrate an essential role for NO in the killing of *Mtb* by mononuclear phagocytes[24, 25], and iNOS deficient mice lacking inducible NO production were highly susceptible to *Mtb* infection[12, 26, 27]. In contrast, the cytostatic and cytotoxic role of NO during *Mtb* infection in humans is debated, since early mycobacteriostatic activity of human macrophages was found to be independent of NO[20, 28]. Nevertheless, there is an increasing amount of evidence that NO produced by macrophages and epithelial cells also provides protection against *Mtb* in humans [27 29-32 33]. In agreement, polymorphisms in the gene for iNOS have been associated with susceptibility to TB in different populations[34]. The conflicting results on the role of NO and iNOS in defense against mycobacterial infection may, in part, be explained by the range of mechanisms utilized by *Mtb* to counteract ROS and RNS produced by the host[35]. Oxidative and nitrosative stresses induce a transcriptional response in *Mtb* that can lead to direct scavenging of reactive species and repair and protection of proteins and DNA[35, 36].

The natural fish pathogen *Mycobacterium marinum* (*Mm*) is a close relative of human *Mtb* and displays similar pathogenesis, including the formation of caseating granulomas and development of latency[37, 38]. The zebrafish embryo model, which allows for detailed observation of host-pathogen interactions, is well-established for the *in vivo* study of host signaling and mycobacterial virulence determinants during early stages of TB disease[39, 40]. In this study we used an antibody against nitration of tyrosine residues as readout for the oxidative and nitrosative response of the zebrafish host to infection with *Mm*[41-43]. We found that mycobacterial infection led to an increase of nitrosative stress in leukocytes which was dependent on functional IL1R/TLR-Myd88 signaling. During the course of infection, this host response was attenuated by live mycobacteria

via a virulence mechanism independent of the well-characterized mycobacterial RD1 virulence locus[3]. Our results shed light on the intriguing host-pathogen interactions that occur during the onset of mycobacterial disease and can help understand the relevance of RNS as anti-mycobacterial defense mechanisms *in vivo*.

Results

Tyrosine nitration levels are detectable in neutrophils and macrophages

Tyrosine nitration is a hallmark of nitrosative stress caused by ROS and RNS produced by macrophages and neutrophils[41]. An antibody against nitrated tyrosine residues has been successfully used in common carp (*Cyprinus carpio*) to study the differential contribution of macrophages and neutrophils to nitrosative stress in response to a parasitic infection[41]. We have previously used a similar approach as readout for nitrosative stress induced by *Mm* infection in zebrafish embryos[42, 43]. In the absence of infection, tyrosine nitration was mainly detectable in a cellular pattern in the caudal hematopoietic tissue (figure 1A). Neutrophils display high basal levels of tyrosine nitration, since the neutrophilic peroxidase Mpx (myeloperoxidase) catalyzes tissue nitration without peroxynitrite present[41, 42]. Indeed, levels of anti-nitrotyrosine staining were detectable in *mpx:GFP*-labelled neutrophils of uninfected zebrafish embryos (figure 1B), but not or in rare cases in *mpeg1:GFP*-macrophages (figure 1D)[42]. Mpx activity can be visualized in zebrafish embryos using an immunohistochemical peroxidase detection protocol[44]. Mpx activity co-localized with anti-nitrotyrosine staining in neutrophils of wild type embryos, while both the TSA and anti-nitrotyrosine staining were undetectable in neutrophils of embryos mutated in Mpx (Figure 1B and C). These data confirm our previous observation that the basal levels of tyrosine nitration in neutrophils were independent of iNOS activity[42]. Nitration of tyrosine residues was occasionally observed in *mpeg1:EGFP*-labelled macrophages when embryos were infected with *Mm* (Figure 1D and E). Furthermore, a significant increase in anti-nitrotyrosine staining was observed in neutrophils surrounding infected macrophages, confirming the infection inducible nature of NO production by iNOS (figure 2)[42].

RNS production is an early host response to detection of live, heat-killed, or ΔRD1 mutant mycobacteria

Mycobacteria are rapidly phagocytosed by macrophages when injected into the bloodstream of zebrafish embryos[45]. Tyrosine nitration and iNOS levels were greatly increased in leukocytes, predominantly neutrophils, one day post infection (dpi) with *Mm* and the increased NO output of these immune cells was dependent on iNOS[42]. To better understand the dynamics of this process, we looked at nitrosative stress levels in leukocytes at early time points after infection with *Mm*. At 4 and 8 hours post infection (hpi) we could not detect increased levels of anti-nitrotyrosine staining compared to uninfected controls, but the levels of tyrosine nitration in neutrophils were significantly higher at 12 hpi (figure 2A and B). The lag between the onset of infection

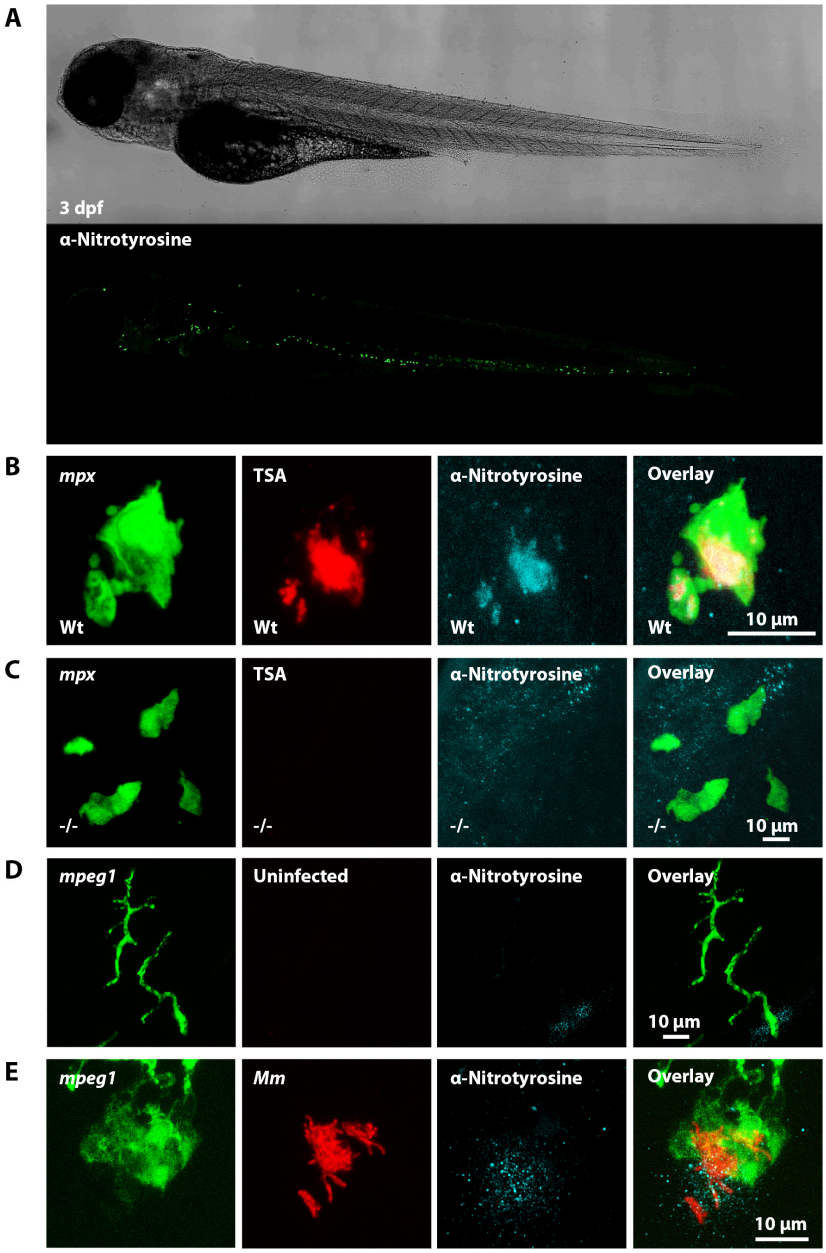


Figure 1: Tyrosine nitration is detectable in leukocytes (A) Fluorescence micrographs of confocal images stitched together to show the location of anti-nitrotyrosine staining in a whole-mount wild type zebrafish embryo of 3 dpf. (B and C) Fluorescence micrographs of confocal images showing the co-localization of *mpx:GFP*, TSA staining, and anti-nitrotyrosine staining for an embryo with normal Mpx activity (B) or an inactive mutant version of Mpx (C). (D) Fluorescent micrographs of confocal images showing no co-localization between *mpeg1:GFP* and anti-nitrotyrosine staining in the absence of infection, (C) overlap between *mpeg1:GFP* and anti-nitrotyrosine staining was observed when embryos were infected with *Mm*.

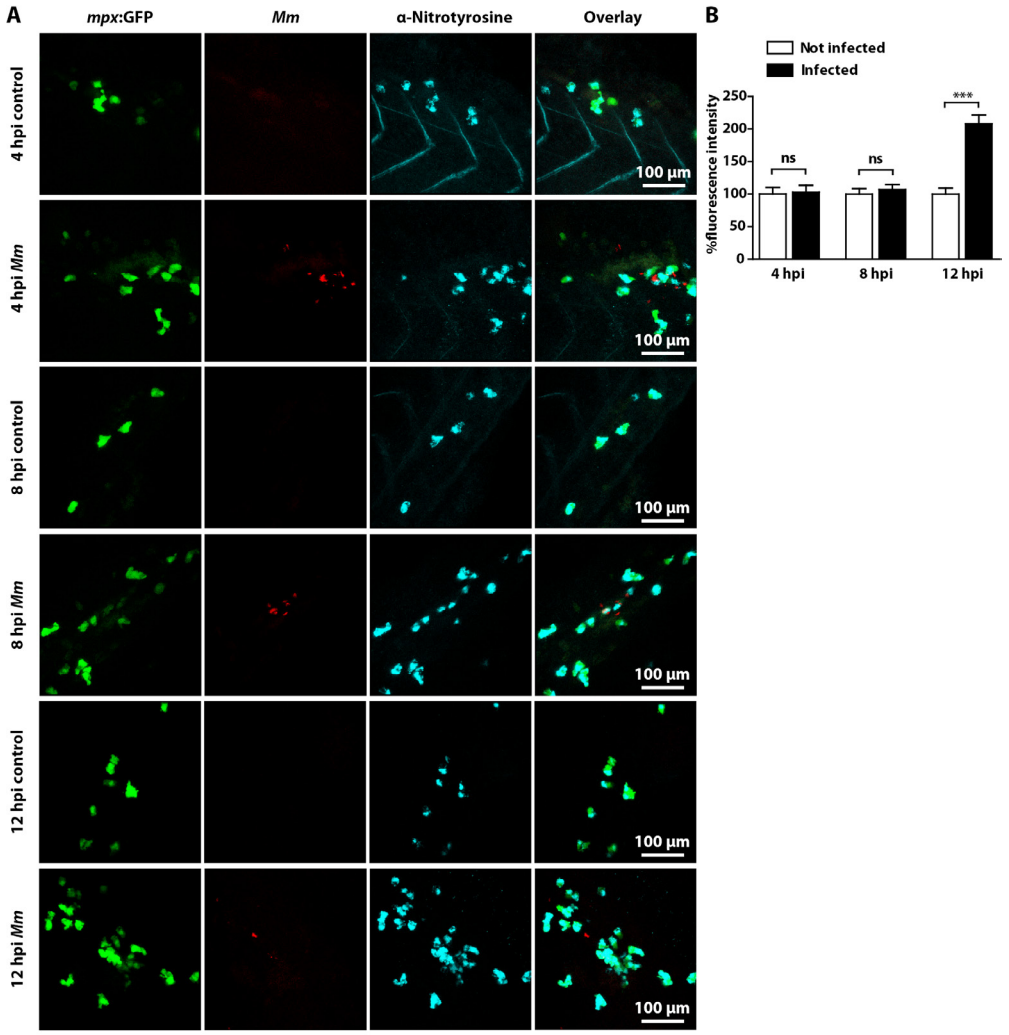


Figure 2: *Mm* infection significantly increased tyrosine nitration levels at 12 hpi (A) Example fluorescence confocal z-stacks of the caudal vein region of embryos stained with anti-nitrotyrosine antibody, imaged at 4, 8 or 12 hpi in the presence or absence of *Mm* infection. (B) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks at 4, 8 or 12 hours after *Mm* infection relative to the control group per time point. Data shown are mean ± SEM, n= 50-85 cells from 5 embryos representative of 3 independent experiments.

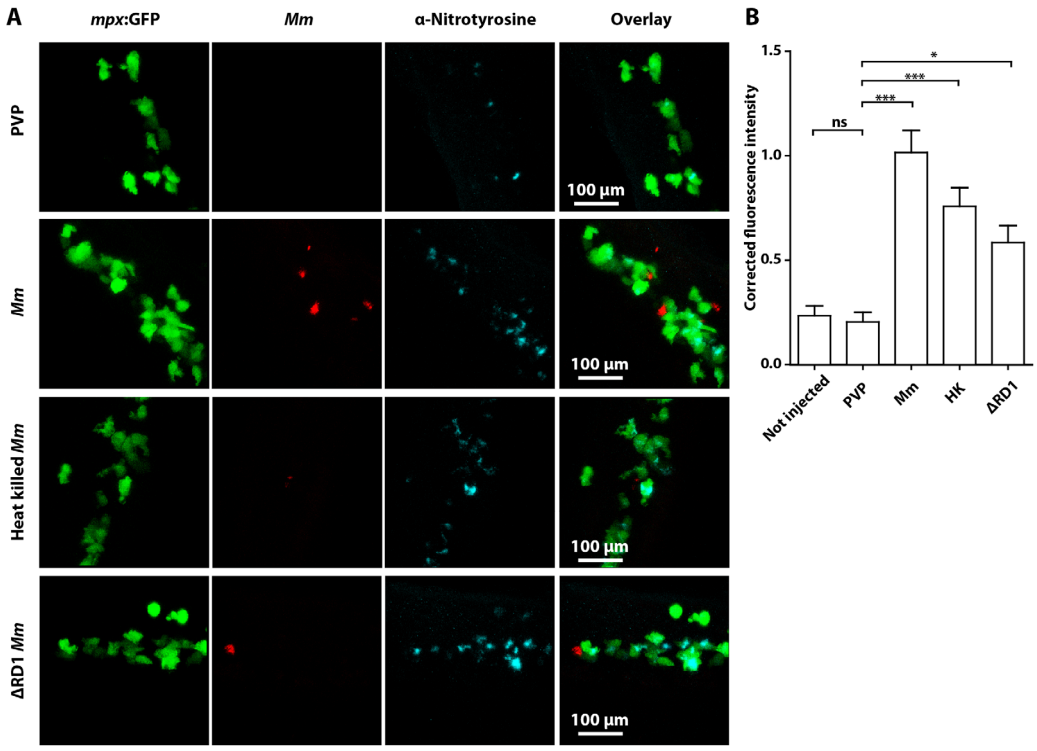


Figure 3: Injection with live, heat killed or ΔRD1 *Mm* significantly increased tyrosine nitration levels (A) Example fluorescence confocal z-stacks of the caudal vein region of embryos stained with anti-nitrotyrosine antibody, imaged 1 day after injection with live, heat killed or ΔRD1 *Mm*. (B) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks 1 day after injection with live, heat killed or ΔRD1 *Mm*. Data shown are mean ± SEM, n= 58-99 cells from 5 embryos representative of 3 independent experiments.

and detectable increased tyrosine nitration is consistent with the fact that infection-induced RNS production first requires *de novo* synthesis of iNOS[27]. Increased nitration levels in leukocytes were observed after injection with live, heat-killed, or *Mm* with a deleted RD1 (ΔRD1) locus (figure 3A and B, supplementary figure 1). The RD1 virulence locus of pathogenic mycobacteria contains genes for the ESX-1 secretion system and secreted factors required for survival and replication inside macrophages[46, 47]. These results demonstrate that the presence of *Mm* is sufficient to induce a leukocyte RNS response shortly after encountering bacteria, irrespective of their infectivity.

TLR/IL1R-MyD88 signaling is required for *Mm* induced RNS production

Pattern recognition receptors, like Toll-like receptors (TLRs), can recognize pathogen associated molecular patterns (PAMPs) present on the exterior of mycobacteria and initiate an appropriate immune response[43, 48]. Stimulation of mouse macrophages with

TLR ligands increased the expression of *iNOS in vitro*[49], and we have previously shown that a zebrafish mutant for the important TLR/IL1R signaling adaptor MyD88 (*myd88*$^{-/-}$) was more susceptible to mycobacterial infection[50]. The basal level of anti-nitrotyrosine staining in neutrophils was unchanged in uninfected *myd88*$^{-/-}$ embryos compared to *myd88*$^{+/+}$ embryos (figure 4A). Anti-nitrotyrosine staining was significantly increased in infected *myd88*$^{+/+}$ embryos, whereas infected *myd88*$^{-/-}$ embryos did not show an increase compared to uninfected controls (figure 4B and C). Therefore, we conclude that MyD88-dependent signaling is required for mycobacterial infection induced RNS production, most likely following TLR recognition of the pathogen.

Nitrotyrosine levels are attenuated during *Mm* pathogenesis in a live-bacteria dependent mechanism

We evaluated RNS production at later stages of mycobacterial pathogenesis by performing anti-nitrotyrosine staining on zebrafish embryos with clustered bacteria. At 3 days post infection (dpi) anti-nitrotyrosine staining co-localized with, or was in proximity of, bacterial clusters, demonstrating that nitrosative defense mechanisms remain active at a later time point of pathogenesis (figure 5A). Interestingly, we observed that the anti-nitrotyrosine staining that co-localizes with heavily infected areas was less intense than the staining at the periphery of these areas, or in their direct proximity (figure 5B). The same phenomenon was observed for larger and more organized mycobacteria-containing developing granulomas at 5 dpi (supplementary figure 2). Thus, while *Mm* infection initially increased nitrotyrosine levels of infected and bystander leukocytes, the host RNS response was attenuated in developing granulomas.

Signaling via the hypoxia inducible factor α (HIF-α) was shown to be involved in leukocyte activation and host defense[51]. Our previous results showed that activated HIF-1α signaling due to injection of dominant active *hif-1α* RNA was able to upregulate anti-nitrotyrosine staining in zebrafish embryos prior to infection[42]. However, when HIF-1α signaling was activated, *Mm* infection led to a decrease in tyrosine nitration levels, in contrast to the increase that occurs following *Mm* infection of wild type embryos (figure 6A and B)[42]. By injecting live, heat killed, or ΔRD1 *Mm* into embryos with activated HIF-1α signaling we found that the decrease in anti-nitrotyrosine levels is dependent on live bacteria (figure 6A and B). Exposure to heat killed *Mm* further increased tyrosine nitration levels of leukocytes, while infection with living wild type or ΔRD1 *Mm* significantly decreased tyrosine nitration levels compared to mock-injected controls (figure 6B). These data confirmed that recognition of mycobacteria increased host NO output, even when RNS production of leukocytes was genetically increased prior to infection. However, live *Mm* lowered the efficacy of this nitrosative defense via a mechanism independent of the RD1 virulence locus.

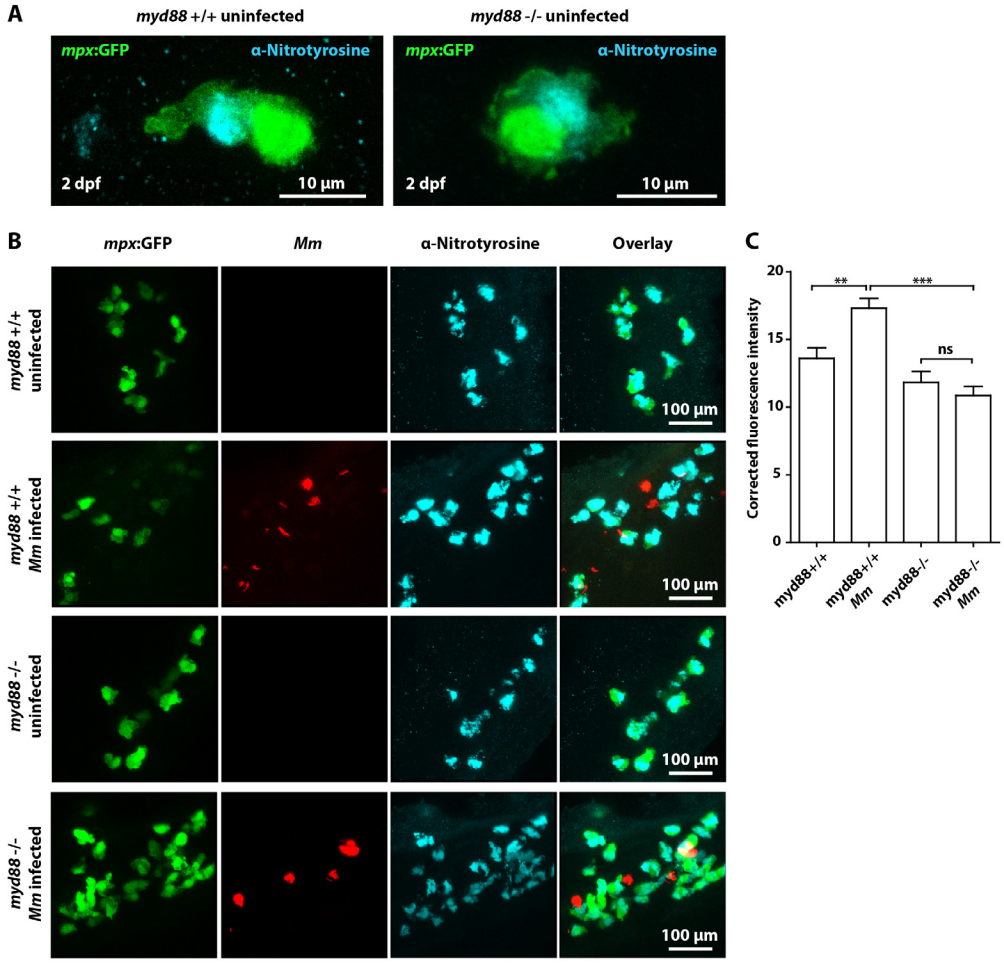


Figure 4: Increased tyrosine nitration following *Mm* infection is dependent on MyD88 (A) Fluorescent micrographs of confocal images showing the overlap between *mpx:GFP* and anti-nitrotyrosine staining in *myd88*$^{+/+}$ or *myd88*$^{-/-}$ embryos. (B) Example fluorescence confocal z-stacks of the caudal vein region of *myd88*$^{+/+}$ or *myd88*$^{-/-}$ embryos stained with anti-nitrotyrosine antibody, imaged at 1 dpi in the presence or absence of *Mm* infection. (C) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks 1 day after injection with *Mm* in *myd88*$^{+/+}$ or *myd88*$^{-/-}$ embryos. Data shown are mean ± SEM, n= 45-52 cells from 5 embryos representative of 3 independent experiments.

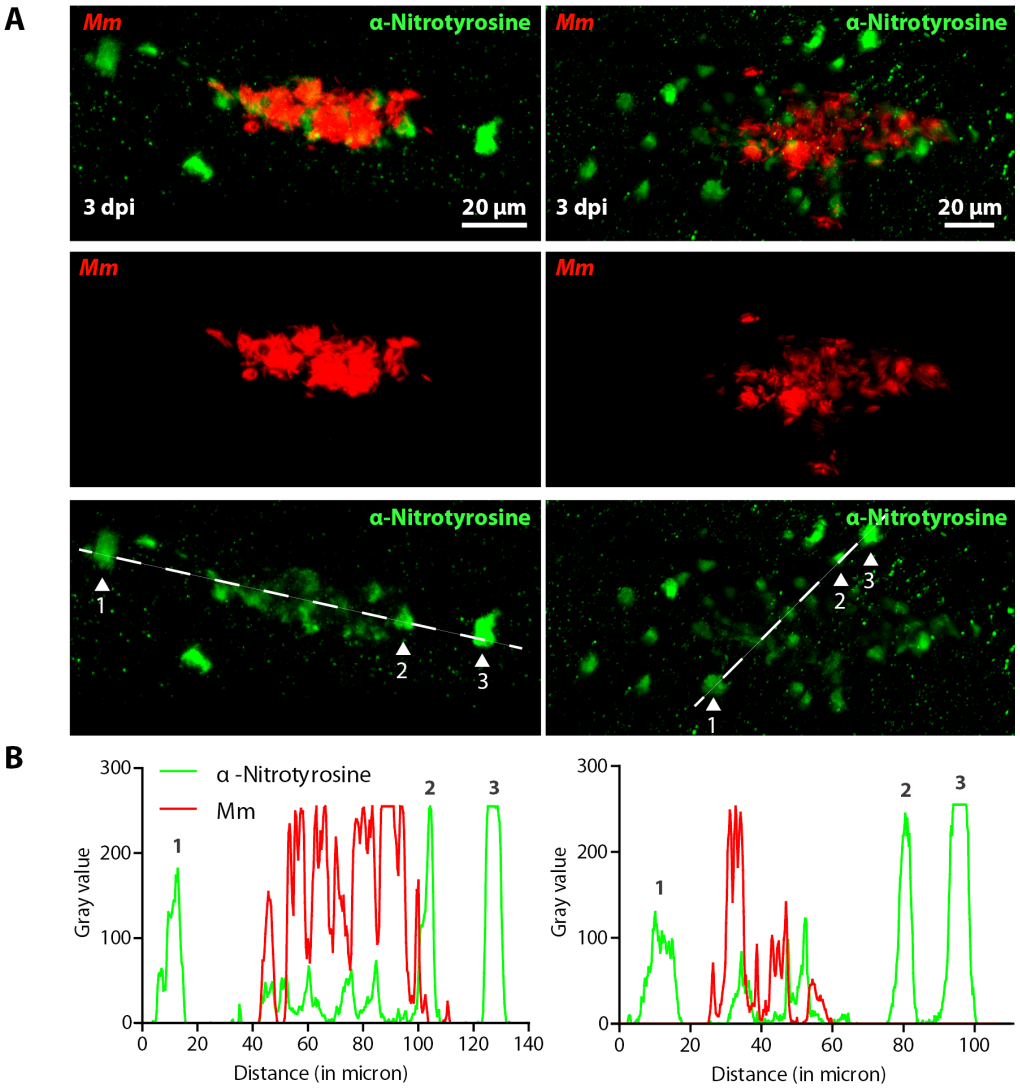


Figure 5: Anti-nitrotyrosine staining levels are lower when co-localizing with bacteria (A) Example fluorescence confocal micrographs of anti-nitrotyrosine staining performed on 3 dpi granuloma structures in wild type embryos infected with *Mm*. (B) Gray values of the bacterial and anti-nitrotyrosine fluorescence signals measured along a straight line through the center of the granuloma (see white lines in (A)), intensity of the fluorescent signal was measured using ImageJ. Numbered peaks in the graphs correspond to numbered patches of tyrosine nitration in (A).

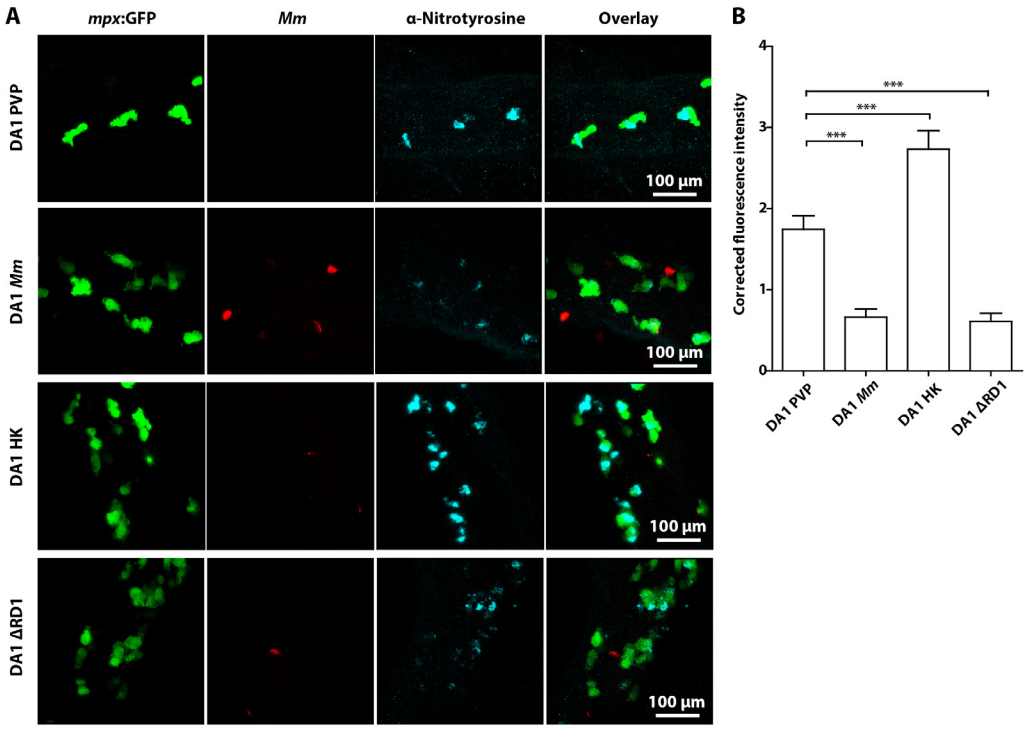


Figure 6: Genetically increased tyrosine nitration in leukocytes due to activated HIF-1α signaling is attenuated by live wild type or ΔRD1 *Mm*, but not heat killed *Mm* (A) Example fluorescence confocal z-stacks of the caudal vein region of dominant active *hif-1α* (DA1) mRNA injected embryos stained with anti-nitrotyrosine antibody, imaged 1 day after injection with live, heat killed or ΔRD1 *Mm*. (B) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks on dominant active *hif-1α* (DA1) mRNA injected embryos, 1 day after injection with live, heat killed or ΔRD1 *Mm*. Data shown are mean ± SEM, n= 74-108 cells from 5 embryos representative of 3 independent experiments.

Discussion

In this study we observed that TLR/IL1R-MyD88 signaling was required for the induction of a nitrosative defense mechanism when zebrafish embryos were infected with *Mm*. We found earlier that this mechanism is dependent on iNOS, which is expressed at higher levels in both infected and bystander leukocytes, predominantly neutrophils[42, 52]. However, neither a morpholino targeting iNOS, encoded by the *nos2a* gene in zebrafish[52], nor the use of pan-NOS or iNOS specific inhibitors resulted in significantly increased *Mm* bacterial burdens[42]. Similarly, treatment with ROS scavengers did not increase *Mm* bacterial burdens of wild type zebrafish embryos[52], despite that we find components of the NADPH oxidase complex (including *nox1*, *noxo1a*, *cyba)* to be induced during *Mm* infection in a TLR/IL1R-MyD88-dependent manner (RNASeq dataset GSE49188). Apparently, the activation of these potent anti-microbial defense mechanisms has

little or no effect on the survival of *Mm* inside the zebrafish host. Indeed, in this study we found that live *Mm* are capable of attenuating the nitrosative stress generated by leukocytes during the innate immune response.

Pathogenic mycobacteria have evolved as metabolically flexible bacteria that can sense and adapt to the continuously changing host environment during infection[53]. *Mtb* is continuously exposed to endogenous ROS produced by normal aerobic respiration, but also exogenous ROS and RNS generated by the host immune system in the often hypoxic human granuloma[53, 54]. The *Mtb* WhiB4 protein can sense O_2 and NO levels and activate the oxidative stress response, which maintains the redox balance and modulates virulence[55]. Exposure of *Mtb* to H_2O_2 or NO initiated a robust transcriptional response, including the expression of cellular repair mechanisms and upregulation of genes that perform ROS and RNS scavenging functions[35, 36]. The presence of macrophages and neutrophils expressing NOS proteins was demonstrated for *Mtb* granulomas in human and macaque tissue samples[56]. However, the presence of NOS proteins does not guarantee a cytostatic or cytotoxic effect of NO production. We found that attenuation of the host RNS response occurred in developing granulomas of *Mm*-infected zebrafish embryos. Similarly, the host RNS response to *Mm* infection was attenuated in embryos with activated HIF-1α signaling, an experimental strategy utilized to genetically increase RNS production of leukocytes prior to infection. This attenuation was independent of the RD1 virulence locus required for survival and replication inside macrophages, but could not be induced by heat-killed bacteria. Together, the data suggest that Mm infection initially induces increased NO production in bystander neutrophils and that this response is attenuated during the course of infection by a bacterial virulence mechanism (figure

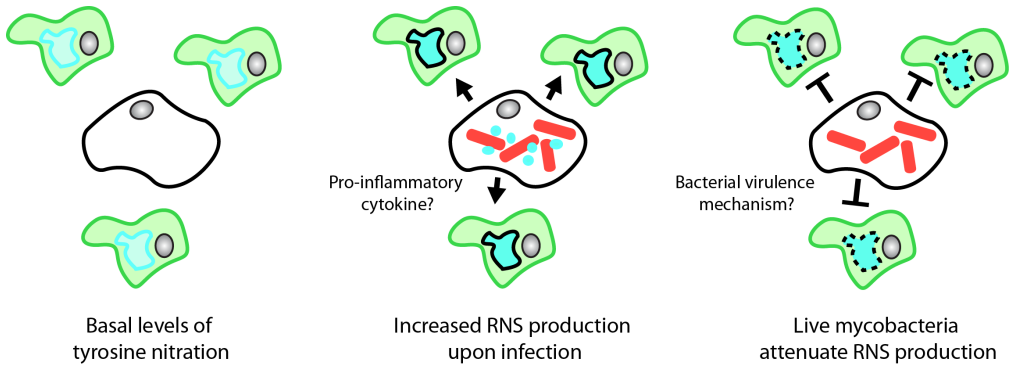


Figure 7: Model of RNS production and attenuation during mycobacterial infection Leukocytes have basal levels of tyrosine nitration, most commonly caused by the neutrophil specific enzyme Mpx. Tyrosine nitration levels resulting from RNS production are increased in infected and bystander leukocytes following mycobacterial infection, possibly via pro-inflammatory cytokine secretion. The effectivity and/or production of RNS is then attenuated by live mycobacteria, via a presently unknown virulence mechanism. Priming leukocytes for defense against mycobacteria by inducing RNS production via the hypoxia pathway reduced bacterial burdens.

7). A bacterial protein potentially involved in regulating this oxidative stress response is OxyR[57]. Additionally, mycobacteria can directly protect themselves against oxidative stress via AhpC and MsrA and B, by catalyzing the breakdown of peroxynitrite or protecting *Mtb* against its detrimental effects[58-60]. These virulence mechanisms of *Mtb* and *Mm* have evolved to specifically counteract the oxidative defenses of their natural human and zebrafish hosts, which may partly explain why *Mtb* is more susceptible to RNS production in the non-natural mouse model of TB disease[61, 62].

The presence of heat killed, live or ΔRD1 *Mm* was sufficient to increase the host nitric oxide output 12 hours after encountering the microbes. This demonstrated that the recognition of mycobacterial PAMPs, rather than virulence or viability of the bacteria was responsible for this effect. Since the increase in tyrosine nitration was absent in *myd88*$^{-/-}$ embryos infected with *Mm*, we concluded that TLR recognition of mycobacteria was required to initiate this defense mechanism. The mycobacterial cell wall contains lipoproteins and lipoglycans that serve as TLR ligands[63]. Recognition of the cell wall component lipoarabinomannan (LAM) by macrophages resulted in iNOS-NO production directly[62], or via induction of the *iNOS* expression regulating cytokines TNF-α and IL-1β [64, 65]. It is possible that production of IL-1β played a role in the increased RNS production in leukocytes following *Mm* infection, since the interleukin 1 receptor (IL1R) also relies on MyD88 for its downstream signaling [50, 66]. Furthermore, we found that most leukocytes near bacteria, particularly neutrophils, displayed increased tyrosine nitration, not just those leukocytes directly interacting with *Mm*, suggesting a potential role for a locally secreted pro-inflammatory cytokine like IL-1β in increased RNS production. Likewise, the dampening of RNS production at later stages of infection may be caused by secretion of anti-inflammatory cytokines. NO produced by iNOS is also required for the infection-responsive expansion of hematopoietic stem and progenitor cells and controls immunopathology of TB by preventing overproduction of IL-1β by limiting inflammasome activity[52, 67].

The search for new host- or pathogen-based anti-TB therapies has intensified with the increasing occurrence of *Mtb* strains that are resistant to multiple drug treatments[1, 2]. The attenuation of RNS activity described here is directly linked to this problem, since resistance of clinical *Mtb* strains to first-line antibiotics was shown to be associated with decreased susceptibility to NO[68]. We have previously shown that dominant active expression of the hypoxia inducible transcription factor Hif-1α primed leukocytes for defense against mycobacterial disease by increasing iNOS-dependent RNS production prior to infection, resulting in a decrease in bacterial burden[42]. This suggested that mycobacteria were more susceptible to host nitrosative defenses before they had a chance to sense and adapt to the high levels of RNS present in primed phagocytes. Our current data indicate that targeting the bacterial response to nitrosative stress represents a potential therapeutic option for the treatment of multiple drug resistant TB strains.

Materials and methods

Fish husbandry

Zebrafish strains (wildtype strain used was AB/TL) were maintained according to standard protocols. Transgenic and mutant strains used were Tg(*mpx:GFP*)^{i114} [69], Tg(*mpeg1:EGFP*)^{gl22} [70], *myd88*^{hu3568}/Tg(*mpx:GFP*)^{i114} [50], and *mpx*^{NL144}/Tg(*mpx:GFP*)^{i114}. The *mpx*^{NL144} mutant line contains a C to T point mutation at nucleotide position 1126 of the *mpx* RefSeq mRNA sequence (NM_212779) resulting in a premature stop codon (M.J. Redd, E. Murayama, W. Horsley, P. Herbomel, and N. Trede, unpublished). Adult fish were maintained on a 14 hour light and 10 hour dark cycle at 28°C in accordance with local animal welfare regulations.

Bacterial culture preparation

Infection experiments were performed using *M. marinum* strain M (ATCC #BAA-535)[71], containing the pSMT3-mCherry vector[72]. Liquid cultures were prepared from bacterial plates as previously described[72] with 50 µg/ml hygromycin. Injection inoculum was prepared from overnight liquid cultures (at OD600<1) by washing three times in sterile 0.05% Tween80/PBS solution (BD Difco), assessing optical density at 600 nm and resuspending in a 2% polyvinylpyrrolidone40 (PVP40) solution (CalBiochem) in PBS (Benard et al., 2012).

Intravenous injection of bacteria into one-day old embryos

Injection of bacteria was performed into the blood-forming region above the caudal vein at 28hpf as previously described[73]. An inoculum volume of 1nl and 100 colony-forming units (CFU) of bacteria were injected. CFU was checked by plating out the injection volume.

RNA injections of dominant active Hif-1α

Embryos were injected with dominant active Hif-1α (ZFIN: *hif1ab*) RNA at the one to two cell stage as previously described[74]. Phenol red (Sigma Aldrich) was used as an injection control.

Stereoimaging and bacterial pixel quantification

Embryos were imaged at 4dpi on a Leica MZ16FA Fluorescence Stereo Microscope. Brightfield and fluorescence images were generated with a Leica DC500 (DFC420C) camera. Bacterial loads were analysed using dedicated pixel counting software as previously described [75].

Anti-nitrotyrosine antibody staining and TSA staining

Larvae were fixed in 4% paraformaldehyde in PBS overnight at 4°C and nitrotyrosine levels were immune-labelled using a rabbit polyclonal anti-nitrotyrosine antibody (Merck Millipore 06-284, MA, USA) at a 1:200 dilution of primary antibody, and were detected using an Alexa Fluor (Invitrogen Life Technologies, NY, USA) secondary antibody. Mpx activity was labelled with TSA (TSAplus kit, Fluorescence Systems, Perkin

Elmer Inc., Waltham, MA) as previously described [44].

Confocal microscopy and quantification of corrected cell fluorescence of anti-nitrotyrosine levels

Embryos were imaged at 1dpi, in the presence or absence of infection, embedded in 1% low melting point agarose (Sigma Aldrich) and transferred to a Leica DMIRBE inverted microscope with a Leica SP1 confocal scanhead for imaging with 40 or 63 times lenses. For quantification purposes acquisition settings and area of imaging (in the caudal vein region) were kept the same across the groups. Corrected total cell fluorescence was calculated for each immune-stained cell using Image J measurements as previously described [42]. The GFP of the Tg(*mpx:GFP*)^{i114} [76] was used to assess the leukocyte cell boundaries.

Statistical analysis

All data were analysed (Prism 5.0, GraphPad Software, San Diego, CA) using unpaired, two-tailed t-tests for comparisons between two groups and one-way ANOVA (with Bonferroni post-test adjustment) for multiple group comparisons.

Acknowledgements

The authors would like to thank Ulrike Nehrdich, Laura van Hulst, and Davy de Witt for fish care, and Gerrit Onderwater and Kiki Spaninks for help with experimental procedures. We gratefully thank Maria Forlenza (University of Wageningen) for expertise in detection of nitrotyrosine and Michael J. Redd and Nikolaus S. Trede (University of Utah, Huntsman Cancer Institute) for making available their zebrafish *mpx* mutant line. We also thank Steve Renshaw (University of Sheffield) and Graham Lieschke (Monash University, Melbourne) for zebrafish transgenic lines. This work was supported by the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Ministry of Education, Culture and Science, the European Commission 7th framework projects ZF-HEALTH (HEALTH-F4-2010-242048) and FishForPharma (PITN-GA-2011-289209), and a European Respiratory Society Fellowship (LTRF *fellowship n°176*-2011).

References

1. Goldberg, D.E., Siliciano, R.F. & Jacobs, W.R., Jr. Outwitting evolution: fighting drug-resistant TB, malaria, and HIV. *Cell* **148**, 1271-1283 (2012).
2. Koul, A., Arnoult, E., Lounis, N., Guillemont, J. & Andries, K. The challenge of new drug discovery for tuberculosis. *Nature* **469**, 483-490 (2011).
3. Vergne, I., Chua, J., Singh, S.B. & Deretic, V. Cell biology of mycobacterium tuberculosis phagosome. *Annual review of cell and developmental biology* **20**, 367-394 (2004).
4. Ulrichs, T. & Kaufmann, S.H. New insights into the function of granulomas in human tuberculosis. *The Journal of pathology* **208**, 261-269 (2006).
5. Flynn, J.L., Chan, J. & Lin, P.L. Macrophages and control of granulomatous inflammation in tuberculosis. *Mucosal immunology* **4**, 271-278 (2011).

6. Gengenbacher, M. & Kaufmann, S.H. Mycobacterium tuberculosis: success through dormancy. *FEMS Microbiol Rev* **36**, 514-532 (2012).

7. Toossi, Z. The inflammatory response in Mycobacterium tuberculosis infection. *Archivum immunologiae et therapiae experimentalis* **48**, 513-519 (2000).

8. Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* **4**, 181-189 (2004).

9. Peranzoni, E. *et al.* Role of arginine metabolism in immunity and immunopathology. *Immunobiology* **212**, 795-812 (2007).

10. Bogdan, C. Nitric oxide and the immune response. *Nat Immunol* **2**, 907-916 (2001).

11. Lau, Y.L., Chan, G.C., Ha, S.Y., Hui, Y.F. & Yuen, K.Y. The role of phagocytic respiratory burst in host defense against Mycobacterium tuberculosis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **26**, 226-227 (1998).

12. Adams, L.B., Dinauer, M.C., Morgenstern, D.E. & Krahenbuhl, J.L. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. *Tuber Lung Dis* **78**, 237-246 (1997).

13. Cooper, A.M., Segal, B.H., Frank, A.A., Holland, S.M. & Orme, I.M. Transient loss of resistance to pulmonary tuberculosis in p47(phox-/-) mice. *Infection and immunity* **68**, 1231-1234 (2000).

14. Jones, G.S., Amirault, H.J. & Andersen, B.R. Killing of Mycobacterium tuberculosis by neutrophils: a nonoxidative process. *The Journal of infectious diseases* **162**, 700-704 (1990).

15. Long, R., Light, B. & Talbot, J.A. Mycobacteriocidal action of exogenous nitric oxide. *Antimicrobial agents and chemotherapy* **43**, 403-405 (1999).

16. Wu, G. & Morris, S.M., Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J* **336 (Pt 1)**, 1-17 (1998).

17. Pfeiffer, S., Lass, A., Schmidt, K. & Mayer, B. Protein tyrosine nitration in cytokine-activated murine macrophages. Involvement of a peroxidase/nitrite pathway rather than peroxynitrite. *J Biol Chem* **276**, 34051-34058 (2001).

18. Chakravortty, D. & Hensel, M. Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes and infection / Institut Pasteur* **5**, 621-627 (2003).

19. Sampson, J.B., Ye, Y., Rosen, H. & Beckman, J.S. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Archives of biochemistry and biophysics* **356**, 207-213 (1998).

20. Nathan, C. & Shiloh, M.U. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8841-8848 (2000).

21. De Groote, M.A. & Fang, F.C. NO inhibitions: antimicrobial properties of nitric oxide. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **21 Suppl 2**, S162-165 (1995).

22. Pacelli, R. *et al.* Nitric oxide potentiates hydrogen peroxide-induced killing of Escherichia coli. *The Journal of experimental medicine* **182**, 1469-1479 (1995).

23. Vazquez-Torres, A., Jones-Carson, J. & Balish, E. Peroxynitrite contributes to the candidacidal activity of nitric oxide-producing macrophages. *Infection and immunity* **64**, 3127-3133 (1996).

24. Chan, J., Xing, Y., Magliozzo, R.S. & Bloom, B.R. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *The Journal of experimental medicine* **175**, 1111-1122 (1992).

25. Chan, J., Tanaka, K., Carroll, D., Flynn, J. & Bloom, B.R. Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. *Infection and immunity* **63**, 736-740 (1995).

26. MacMicking, J.D. *et al.* Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 5243-5248 (1997).

27. Yang, C.S., Yuk, J.M. & Jo, E.K. The role of nitric oxide in mycobacterial infections. *Immune Netw* **9**, 46-52 (2009).

28. Aston, C., Rom, W.N., Talbot, A.T. & Reibman, J. Early inhibition of mycobacterial growth by human alveolar macrophages is not due to nitric oxide. *American journal of respiratory and critical care medicine* **157**, 1943-1950 (1998).

29. Rich, E.A. *et al.* Mycobacterium tuberculosis (MTB)-stimulated production of nitric oxide by human alveolar macrophages and relationship of nitric oxide production to growth inhibition of MTB. *Tuber Lung Dis* **78**, 247-255 (1997).

30. Rockett, K.A. *et al.* 1,25-Dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of Mycobacterium tuberculosis in a human macrophage-like cell line. *Infection and immunity* **66**, 5314-5321 (1998).

31. Nicholson, S. *et al.* Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *The Journal of experimental medicine* **183**, 2293-2302 (1996).

32. Jagannath, C., Actor, J.K. & Hunter, R.L., Jr. Induction of nitric oxide in human monocytes and monocyte cell lines by Mycobacterium tuberculosis. *Nitric Oxide* **2**, 174-186 (1998).

33. Nathan, C. Inducible nitric oxide synthase in the tuberculous human lung. *American journal of respiratory and critical care medicine* **166**, 130-131 (2002).

34. Azad, A.K., Sadee, W. & Schlesinger, L.S. Innate immune gene polymorphisms in tuberculosis. *Infection and immunity* **80**, 3343-3359 (2012).

35. Ehrt, S. & Schnappinger, D. Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cellular microbiology* **11**, 1170-1178 (2009).

36. Voskuil, M.I., Bartek, I.L., Visconti, K. & Schoolnik, G.K. The response of mycobacterium tuberculosis to reactive oxygen and nitrogen species. *Frontiers in microbiology* **2**, 105 (2011).

37. Swaim, L.E. *et al.* Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect.Immun.* **74**, 6108-6117 (2006).

38. Parikka, M. *et al.* Mycobacterium marinum Causes a Latent Infection that Can Be Reactivated by Gamma Irradiation in Adult Zebrafish. *Plos Pathogens* **8** (2012).

39. Berg, R.D. & Ramakrishnan, L. Insights into tuberculosis from the zebrafish model. *Trends in molecular medicine* **18**, 689-690 (2012).

40. Meijer, A.H. & Spaink, H.P. Host-pathogen interactions made transparent with the zebrafish model. *Curr Drug Targets* **12**, 1000-1017 (2011).

41. Forlenza, M. *et al.* Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host-parasite animal model. *Mol Immunol* **45**, 3178-3189 (2008).

42. Elks, P.M. *et al.* Hypoxia Inducible Factor (Hif) Signaling Modulates Susceptibility to Mycobacterial Infection Via a Nitric Oxide Dependent Mechanism. *under review* (2013).

43. van der Vaart, M., Spaink, H.P. & Meijer, A.H. Pathogen recognition and activation of the innate immune response in zebrafish. *Adv Hematol* **2012**, 159807 (2011).

44. Loynes, C.A. *et al.* Pivotal Advance: Pharmacological manipulation of inflammation resolution during spontaneously resolving tissue neutrophilia in the zebrafish. *Journal of leukocyte biology* **87**, 203-212 (2010).

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

46. Gao, L.Y. *et al.* A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Molecular microbiology* **53**, 1677-1693 (2004).

47. Guinn, K.M. *et al.* Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. *Molecular microbiology* **51**, 359-370 (2004).

48. Mogensen, T.H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* **22**, 240-273, Table of Contents (2009).

49. Buxade, M. *et al.* Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFAT5. *The Journal of experimental medicine* **209**, 379-393 (2012).

50. van der Vaart, M., van Soest, J.J., Spaink, H.P. & Meijer, A.H. Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. *Disease models & mechanisms* **6**, 841-854 (2013).

51. Cramer, T. *et al.* HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* **112**, 645-657 (2003).

52. Hall, C.J. *et al.* Infection-responsive expansion of the hematopoietic stem and progenitor cell compartment in zebrafish is dependent upon inducible nitric oxide. *Cell Stem Cell* **10**, 198-209 (2012).

53. Kumar, A. *et al.* Redox homeostasis in mycobacteria: the key to tuberculosis control? *Expert reviews in molecular medicine* **13**, e39 (2011).

54. Via, L.E. *et al.* Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infection and immunity* **76**, 2333-2340 (2008).

55. Chawla, M. *et al.* Mycobacterium tuberculosis WhiB4 regulates oxidative stress response to modulate survival and dissemination in vivo. *Molecular microbiology* **85**, 1148-1165 (2012).

56. Mattila, J.T. *et al.* Microenvironments in tuberculous granulomas are delineated by distinct populations of macrophage subsets and expression of nitric oxide synthase and arginase isoforms. *Journal of immunology* **191**, 773-784 (2013).

57. Pagan-Ramos, E., Song, J., McFalone, M., Mudd, M.H. & Deretic, V. Oxidative stress response and characterization of the oxyR-ahpC and furA-katG loci in Mycobacterium marinum. *Journal of bacteriology* **180**, 4856-4864 (1998).

58. Trivedi, A., Singh, N., Bhat, S.A., Gupta, P. & Kumar, A. Redox biology of tuberculosis pathogenesis. *Adv Microb Physiol* **60**, 263-324 (2012).

59. Bryk, R., Lima, C.D., Erdjument-Bromage, H., Tempst, P. & Nathan, C. Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein. *Science* **295**, 1073-1077 (2002).

60. Lee, W.L. *et al.* Mycobacterium tuberculosis expresses methionine sulphoxide reductases A and B that protect from killing by nitrite and hypochlorite. *Molecular microbiology* **71**, 583-593 (2009).

61. Cardona, P.J. *et al.* Towards a 'human-like' model of tuberculosis: intranasal inoculation of LPS induces intragranulomatous lung necrosis in mice infected aerogenically with Mycobacterium tuberculosis. *Scandinavian journal of immunology* **53**, 65-71 (2001).

62. Chan, E.D., Chan, J. & Schluger, N.W. What is the role of nitric oxide in murine and human host defense against tuberculosis?Current knowledge. *American journal of respiratory cell and molecular biology* **25**, 606-612 (2001).

63. Krutzik, S.R. & Modlin, R.L. The role of Toll-like receptors in combating mycobacteria. *Seminars in immunology* **16**, 35-41 (2004).

64. Zhang, Y., Doerfler, M., Lee, T.C., Guillemin, B. & Rom, W.N. Mechanisms of stimulation of interleukin-1 beta and tumor necrosis factor-alpha by Mycobacterium tuberculosis components. *The Journal of clinical investigation* **91**, 2076-2083 (1993).

65. Moreno, C. *et al.* Lipoarabinomannan from Mycobacterium tuberculosis induces the production of tumour necrosis factor from human and murine macrophages. *Clinical and experimental immunology* **76**, 240-245 (1989).

66. Janssens, S. & Beyaert, R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends Biochem Sci* **27**, 474-482 (2002).

67. Mishra, M.N. & Daniels, L. Characterization of the MSMEG_2631 gene (mmp) encoding a multidrug and toxic compound extrusion (MATE) family protein in Mycobacterium smegmatis and exploration of its polyspecific nature using biolog phenotype microarray. *Journal of bacteriology* **195**, 1610-1621 (2013).

68. Idh, J. *et al.* Resistance to first-line anti-TB drugs is associated with reduced nitric oxide susceptibility in Mycobacterium tuberculosis. *PLoS One* **7**, e39891 (2012).

69. Renshaw, S.A. *et al.* A transgenic zebrafish model of neutrophilic inflammation. *Blood* **108**, 3976-3978 (2006).

70. Ellett, F., Pase, L., Hayman, J.W., Andrianopoulos, A. & Lieschke, G.J. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood* **117**, e49-56 (2011).

71. Ramakrishnan, L. & Falkow, S. Mycobacterium marinum persists in cultured mammalian cells in a temperature-restricted fashion. *Infection and immunity* **62**, 3222-3229 (1994).

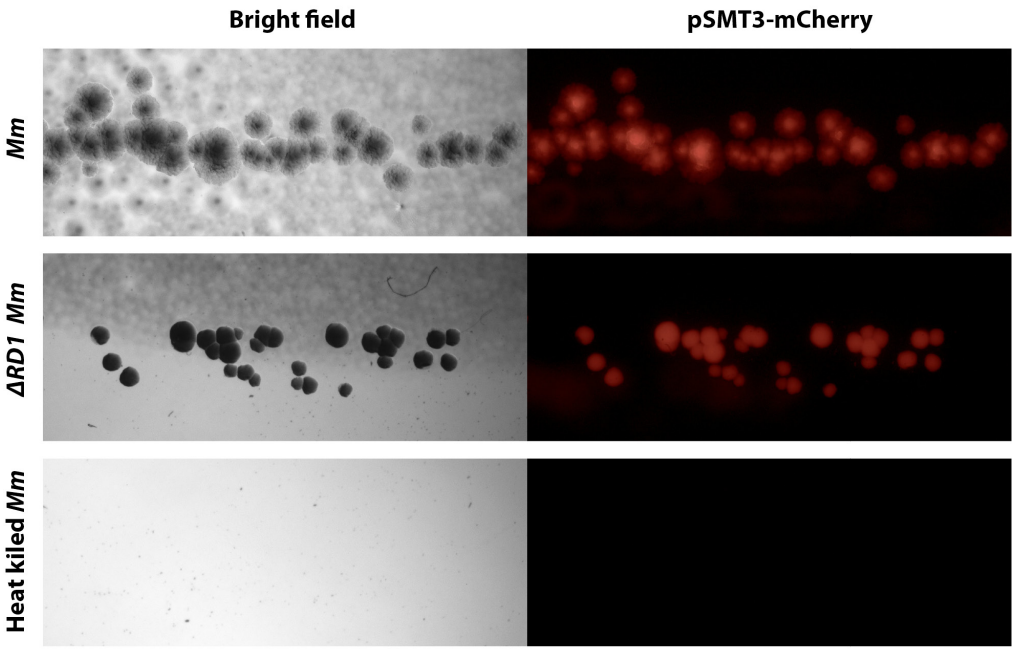
72. van der Sar, A.M., Spaink, H.P., Zakrzewska, A., Bitter, W. & Meijer, A.H. Specificity of the zebrafish host transcriptome response to acute and chronic mycobacterial infection and the role of innate and adaptive immune components. *Mol Immunol* **46**, 2317-2332 (2009).

73. Benard, E.L. *et al.* Infection of zebrafish embryos with intracellular bacterial pathogens. *Journal of visualized experiments : JoVE* (2012).

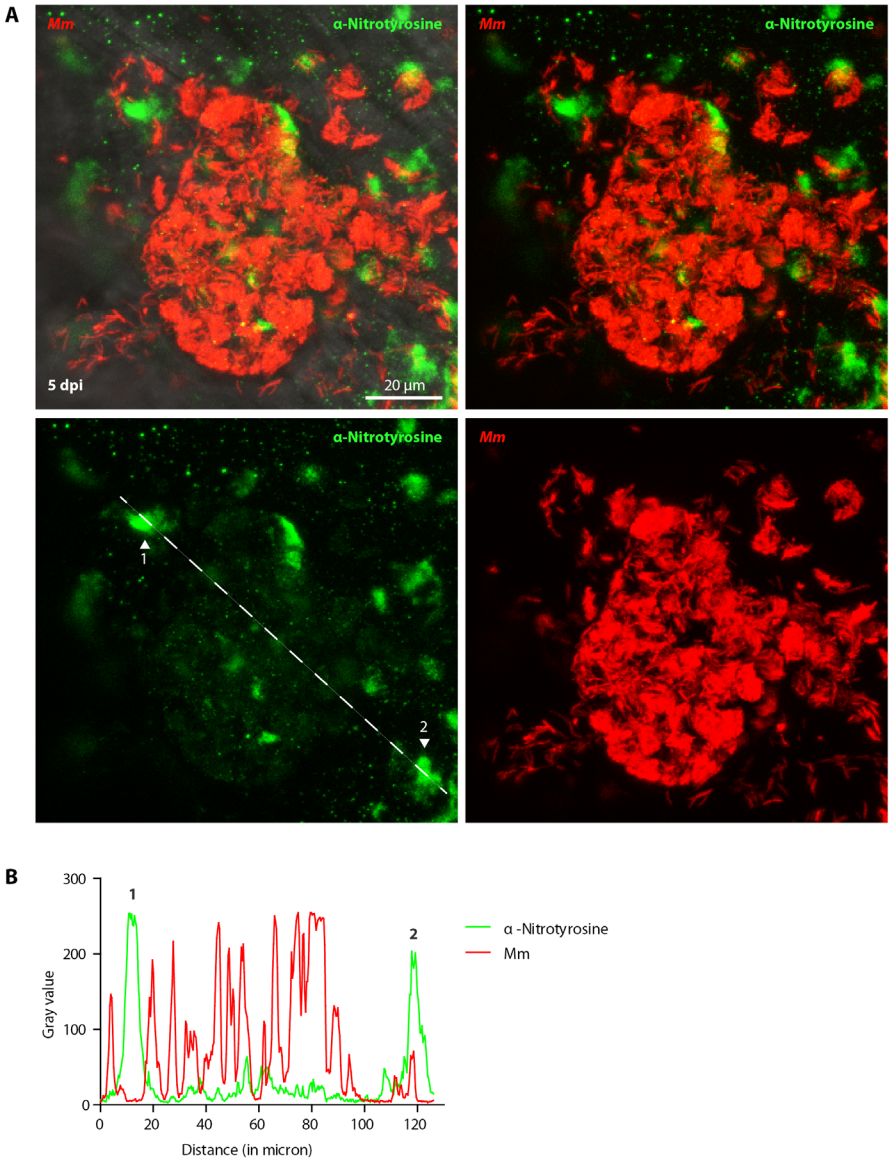
74. Elks, P.M. *et al.* Activation of hypoxia-inducible factor-1alpha (Hif-1alpha) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model. *Blood* **118**, 712-722 (2011).

75. Stoop, E.J. *et al.* Zebrafish embryo screen for mycobacterial genes involved in the initiation of granuloma formation reveals a newly identified ESX-1 component. *Disease models & mechanisms* **4**, 526-536 (2011).

76. Renshaw, S.A. *et al.* A transgenic zebrafish model of neutrophilic inflammation. *Blood* (2006).



Supplementary figure 1: Heat killed *M. marinum* The injection dose of live, heat killed or ΔRD1 *Mm* were plated for CFU counts. As expected, heat killed bacteria did not grow.



Supplementary figure 2: Anti-nitrotyrosine staining levels in progression granulomas are lower when co-localized with bacteria (A) Example fluorescence confocal micrographs of anti-nitrotyrosine staining performed on 5 dpi granuloma structures in wild type embryos infected with *Mm*. (B) Gray values of the bacterial and anti-nitrotyrosine fluorescence signals measured along a straight line through the center of the granuloma (see white lines in (A)), intensity of the fluorescent signal was measured using ImageJ. Numbered peaks in the graphs correspond to numbered patches of tyrosine nitration in (A).