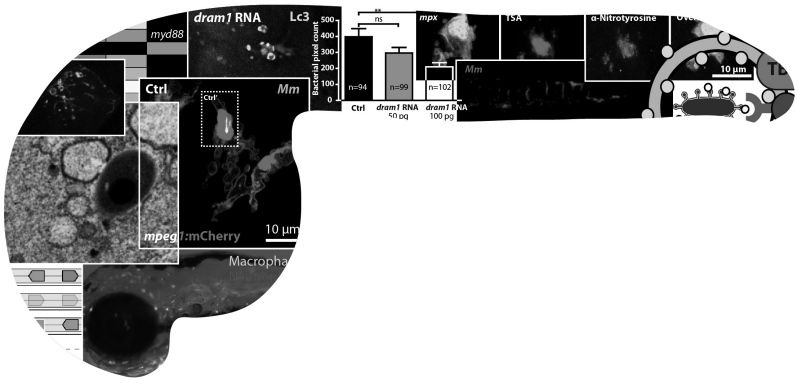
Chapter 6

Summary and discussion



Humans and other animals are constantly exposed to attacks by various potential pathogenic microorganisms. The discovery of Toll-like receptors (TLRs) as gatekeepers of the immune response triggered an explosion of research in the field of innate immunity and was later awarded with a Nobel Prize in medicine[1, 2]. TLRs are an important class of pattern recognition receptors (PRRs) that detect the presence of pathogenic microorganisms via evolutionary conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Pathogen recognition by the innate immune system activates anti-microbial defense mechanisms, such as production of defense proteins, and reactive oxygen and nitrogen species (ROS and RNS), but is also indispensible for activation of adaptive immunity[3]. The discovery of TLRs paved the way for identification of other families of PRRs: NOD-like receptors for cytoplasmatic detection of PAMPs and DAMPs; RIG-I-like receptors for detection of viruses; C-type lectin receptors that recognize and take up carbohydrates and other macromolecules; and sequestosome-like receptors (SLRs) that recognize ubiquinated microbes and microbial proteins (**chapter 2**). The subsequent discovery of the inflammasome demonstrated how cytokines are processed into excreted pro-inflammatory signals upon pathogen recognition by PRRs[4]. The most recent paradigm-shift in immunity came from the discovery that autophagy functions as an anti-microbial defense mechanism, alongside its function in cellular homeostasis[5]. With its extensive opportunities for genetic manipulation and the unparalleled *in vivo* imaging possibilities during embryonic and larval stages, the zebrafish has become an important model organism for the study of innate immunity and infectious diseases[6].

Zebrafish embryos with defective TLR/IL1R-MyD88 signaling are more susceptible to bacterial infection

Myeloid differentiation factor 88 (MYD88) is an essential signaling adaptor used for downstream signaling by all TLRs, except for TLR3[7]. MYD88 also functions downstream of the interleukin 1 receptor (IL1R) family, and has been associated with IFN-γ receptor signal transduction, further establishing it as a signaling adaptor with a central role in inflammation and innate immunity[8-10]. In humans, MYD88-deficiency causes a primary immunodeficiency syndrome characterized by an increased susceptibility to pyogenic bacteria, including *Salmonella* species[11]. Mice deficient in MyD88 were unresponsive to the common bacterial endotoxin LPS and interleukin 1 (Il1) stimulation, and were more susceptible to infection by several bacterial pathogens, including *Mycobacterium tuberculosis* (*Mtb*)[7, 12].

The major classes of innate immune receptors, their downstream signaling pathways, and innate effector mechanisms are conserved between human and zebrafish (**chapter 2**). We used well-established zebrafish embryo infection models to further examine the role of MyD88 in innate immune defense against bacterial pathogens[6]. We found that zebrafish embryos mutated in *myd88* (*myd88*$^{-/-}$) were more susceptible to infection by the acute bacterial pathogens *Salmonella enterica* serovar Typhimurium and *Edwardsiella tarda*, as well as the chronic bacterial pathogen *Mycobacterium marinum*

(*Mm*) (**chapter 3**), similar to the effect of MYD88 deficiency in humans and in the mice model. The increased infection susceptibility of *myd88*$^{-/-}$ embryos cannot be explained by a defect in a single innate immune effector mechanism, since microarray analysis revealed that infection induced gene expression downstream of MyD88 is involved in a plethora of defense mechanisms (**chapter 3**). This included the expression of genes encoding transcription factors central to innate immunity, such as NFκB and AP-1; genes encoding pro-inflammatory cytokines such as Il1b and Tnfa; genes for proteins involved in the respiratory burst; and genes for matrix metalloproteinases, such as Mmp9, which play a role in various aspects of immunity and inflammation. Based on the work presented in this thesis, we conclude that MyD88-deficiency during bacterial infection affects several innate defense mechanisms, including production of cytokines, ROS and RNS, and initiation of autophagic defense (figure 1).

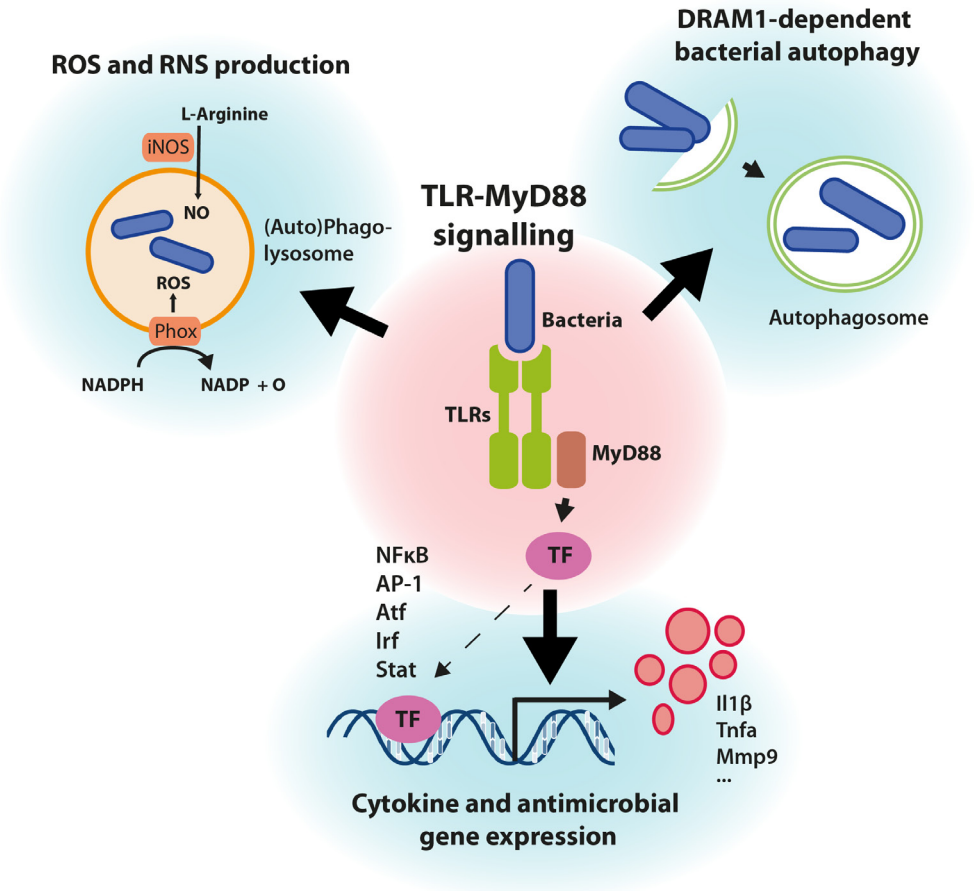


Figure 1: TLR-MyD88 signaling is involved in several innate immune defense mechanisms. These mechanisms include the activation of pro-inflammatory transcription factors and cytokines (chapter 3); the production of ROS and RNS to directly kill microorganisms or to modulate the immune response (chapter 4); and the DRAM1 regulated initiation of autophagic defense (chapter 5).

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

The production of ROS by the NADPH-oxidase complex (or Phox, for phagocytic oxidase) during the respiratory burst plays an important role in innate immune processes[13]. Nitric oxide (NO) is produced by inducible nitric oxide synthase (iNOS) in response to infection, and RNS resulting from the reaction between O_2 and NO radicals are important in the inflammatory response of innate immune cells[14]. Although ROS and RNS production are potent defense mechanisms against intracellular pathogens[13, 14], their role in defense against mycobacterial infection remains controversial. Mutations in components of the NADPH-complex increased susceptibility to *Mtb*[15-17], but ROS production itself was relatively ineffective in killing *Mtb*[18]. A recent study using the zebrafish model for mycobacterial infection investigated the effects of TNF-induced ROS production under conditions where TNF was produced in excess due to overexpression of an enzyme involved in the production of proinflammatory leukotrienes (LTA4H). ROS production due to excess TNF increased macrophage microbicidal activity initially, but, subsequently caused necrosis releasing mycobacteria into the extracellular environment[19]. This study also suggested that *Mm* can counter the effects of ROS production when TNF levels are normal. We did not study the effect of ROS production on mycobacterial infection directly, but found *nox1*, *noxo1a,* and *cyba*, components of the NADPH-oxidase complex, to be upregulated during *Mm* infection in a MyD88-dependent manner (**chapter 3**).

NO was shown to be essential for the killing of *Mtb* by phagocytes in the mouse model and *Mtb* susceptibility was increased in iNOS deficient mice[16, 20-23]. However, the role of NO during *Mtb* infection in humans is debated, since mycobacteriostatic activity of human macrophages functioned independent of NO[24, 25]. Nevertheless, evidence for a protective role for NO during mycobacterial infection in humans is accumulating and polymorphisms in the gene encoding iNOS have been associated with susceptibility to tuberculosis[23 26-31].

As described in **chapter 4**, we found that mycobacterial infection of zebrafish embryos led to an increase of nitrosative stress in leukocytes which was dependent on functional IL1R/TLR-Myd88 signaling. This host nitrosative defense mechanism was attenuated during the course of infection by live mycobacteria. The bacterial virulence mechanism used for adaptation to NO produced in host leukocytes functioned independent of the well-characterized mycobacterial RD1 virulence locus[32]. Interestingly, increasing RNS production in leukocytes via genetic upregulation of the hypoxia pathway prior to infection decreased mycobacterial burdens[33]. Together, this suggested that mycobacteria were more susceptible to host nitrosative defenses before they could sense and adapt to the high levels of RNS present in primed phagocytes. We therefore hypothesize that mycobacteria use oxidative stress sensors, such as OxyR, to upregulate bacterial proteins that can scavenge or protect against the detrimental effects of RNS[34, 35]. Elevated oxidative stress caused by ROS production in phagocytes might induce a bacterial response analogous to the attenuation of nitrosative defense mechanisms, since increasing ROS production via genetically upregulated levels of TNF prior to infection was required for increased anti-mycobacterial activity of macrophages[19].

Autophagy regulator DRAM1 functions downstream of MYD88 in defense against tuberculosis

Autophagy, the process well-known for its role during starvation and cellular stress, has emerged as a defense mechanism against intracellular pathogens[36]. Studies on the autophagic control of *Mtb* infections have demonstrated how the host deals with pathogens that otherwise circumvent host defenses[37]. In fact, selective autophagy receptors such as p62/sequestosome 1 (SQSTM1) and NDP52 are collectively referred to as a novel group of PRRs, called sequestosome like receptors (SLRs)[5]. SLRs recognize ubiquitinated targets for autophagy, in this case mycobacteria, and connect them to a forming autophagic isolation membrane interacting directly with the autophagosome marker LC3[38]. The resulting autophagosome eventually fuses with a lysosome to degrade its pathogenic contents. Furthermore, the same SLRs can target ubiquitinated proteins to the autophagosome, which endows it with unique microbicidal properties[39]. Several intracellular pathogens have evolved mechanism to evade autophagy, notably *Listeria* and *Shigella* species that escape into the cytosol to spread from cell to cell by actin-based motility[40, 41]. Some bacteria, for example *Staphylococcus aureus*, exploit the autophagosome to their advantage to facilitate intracellular survival[42]. Most likely, mycobacteria can also evade or exploit autophagy to a certain extent, but several studies show that autophagy is an effective defense mechanism against these pathogens[39, 43-49].

The immunological function of autophagy is underscored by the fact that pathogen detection by TLRs can induce the formation of autophagosomes or Lc3-associated phagosmes[50]. However, the signaling events that connect TLR recognition with autophagic defense against mycobacterial infection remained unclear. One link between TLRs and autophagy is that the TLR adaptors MYD88 and TRIF were shown to directly trigger autophagy via interaction with the autophagy modulator Atg6/Beclin[51]. In **chapter 5**, we have discovered a new connection between pattern recognition and autophagy. We have used zebrafish embryos and human primary marcophages to demonstrate that anti-mycobacterial autophagy is mediated by DNA-damage regulator of autophagy 1 (DRAM1) in an IL1R/MYD88-NFκB dependent fashion (figure 2). Previously, the only known function of DRAM1 was mediating autophagy-dependent cell death in response to cellular stress such as DNA-damage, cancer, or HIV infection[52-54]. As a regulator of cell death and survival, DRAM1 expression was induced by the tumor suppressor protein p53. By imaging of autophagy in GFP-Lc3 transgenic zebrafish, we found that autophagosomes targeting to mycobacteria requires the selective autophagy receptor p62 (**chapter 5**; figure 3A and B). This dependency on p62 is similar to a previous observation concerning autophagy induction by DRAM1 in gliobastoma stem cells[55]. The requirement of p62 in autophagic defense against tuberculosis fits perfectly with recent literature on the initiation and maturation of *Mtb* containing phagosomes[48, 49]. When mycobacteria permeabilize the bacteria containing phagosome, their extracellular DNA is sensed by the STING-pathway, which ultimately leads to ubiquitination of the bacteria and autophagosome formation via p62[48] (figure 3C). The non-canonical IKK family member TBK1 then phosphorylates p62 and regulates the maturation of *Mtb*

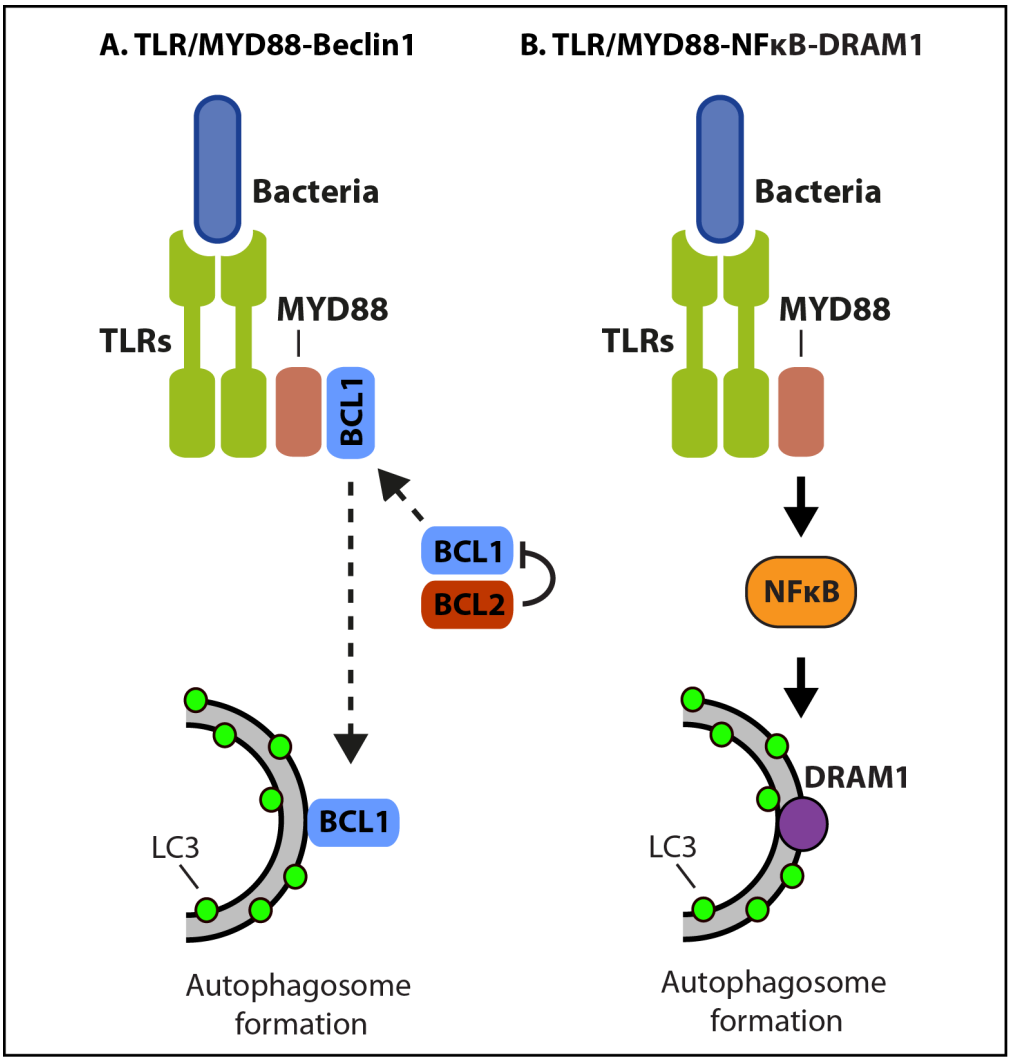


Figure 2: MyD88-dependent routes to autophagy induction. (A) MYD88 can interact directly with autophagy inducer Beclin1 (BCL1), removing it from an inhibitory complex also containing Beclin2 (BCL2). A similar function has been reported for another TLR adaptor protein, TRIF (Shi and Kehrl, 2008). (B) We now found that anti-mycobacterial autophagy is initiated when TLR-MYD88-NFκB signaling increases expression of the autophagy regulator DRAM1 (chapter 5).

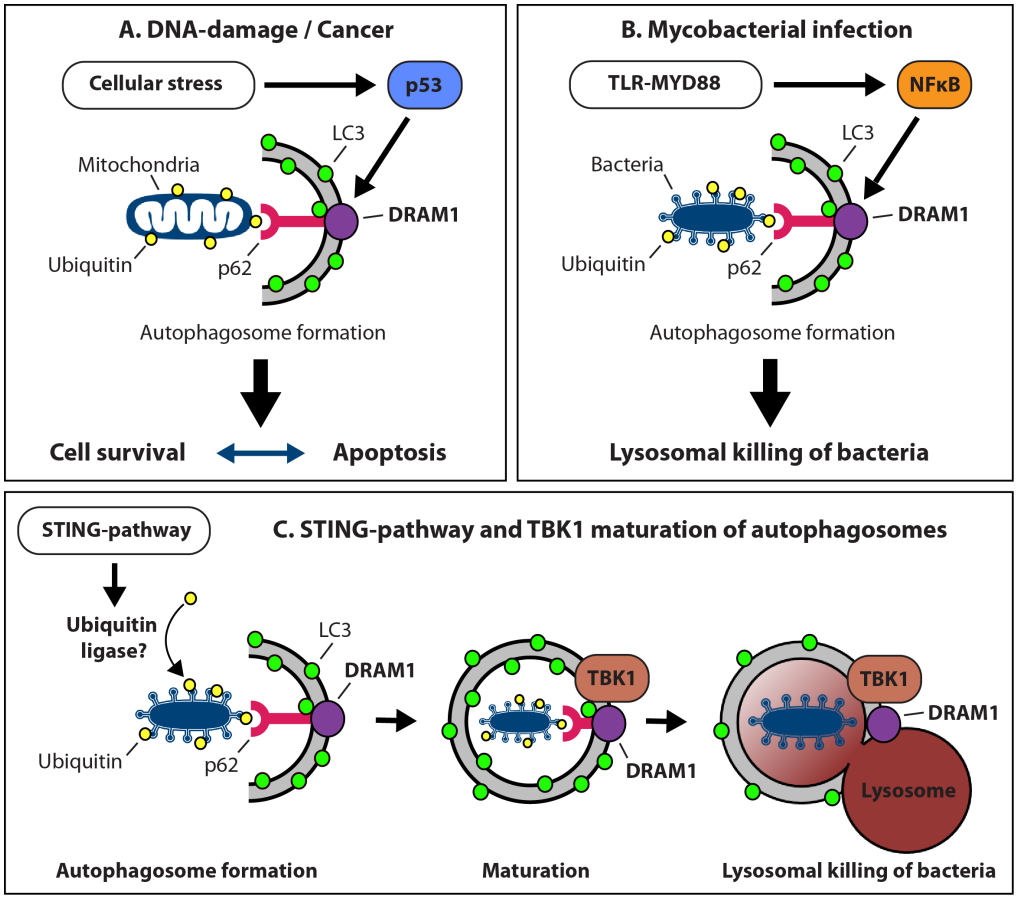


Figure 3: Autophagy initiation by DRAM1. (A) In the context of cellular stresses like DNA-damage or cancer, DRAM1 induces autophagy downstream of p53 activation (Crighton et al., 2006; Galavotti et al., 2013); (B) we now place p62-dependent induction of autophagy by DRAM1 downstream of NFκB signaling for defense against tuberculosis (chapter 5). (C) The STING DNA sensing pathway recognizes membrane permeabilization of the *Mtb* containing phagosome by detecting extracellular bacterial DNA (Watson et al., 2012). We propose that subsequent autophagosome formation via p62 requires DRAM1. TBK1 then phosphorylates p62, which leads to autophagosome maturation and lysosomal destruction of its contents (Pilli et al., 2012).

containing autophagosomes[49]. We have shown that hyper-activation of this DRAM1-dependent autophagy pathway significantly lowered mycobacterial burden *in vivo,* while knockdown of this pathway increased infection levels and resulted in extracellular mycobacterial growth (**chapter 5**).

Conclusion

Tuberculosis remains a worldwide health problem and the increasing occurrence of multiple drug resistant *Mtb* strains calls for novel therapeutic strategies which can complement current antibiotic therapies[56-58]. A broad understanding of innate immunity in general, and especially those processes providing protection against tuberculosis disease, is required for the development of host-targeted approaches for the treatment of tuberculosis. The *myd88*$^{-/-}$ mutant zebrafish line described in **chapter 3** proved highly useful for the study of innate immune mechanisms underlying tuberculosis (**chapters 4 and 5**). In view of the central role of MYD88 in the innate immune system, we expect that this *myd88*$^{-/-}$ mutant zebrafish line will also facilitate the study of other infectious diseases, inflammatory disorders, and cancer. The work presented in this thesis has added to the understanding of the host-pathogen interactions that occur during mycobacterial infections and may also contribute to the development of novel therapeutic strategies. The role of DRAM1 as an inducer of anti-mycobacterial autophagy makes this pathway a highly interesting target for host-directed therapeutic strategies against human tuberculosis disease (**chapter 5**), and therapeutically deactivating the mycobacterial oxidative stress response might sensitize *Mtb* to phagocytic killing (**chapter 4**).

References

1. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085-2088 (1998).
2. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. & Hoffmann, J.A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973-983 (1996).
3. Mogensen, T.H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* **22**, 240-273, Table of Contents (2009).
4. Martinon, F., Burns, K. & Tschopp, J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular cell* **10**, 417-426 (2002).
5. Deretic, V. Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. *Current opinion in immunology* **24**, 21-31 (2012).
6. Meijer, A.H. & Spaink, H.P. Host-pathogen interactions made transparent with the zebrafish model. *Curr Drug Targets* **12**, 1000-1017 (2011).
7. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat.Rev.Immunol.* **4**, 499-511 (2004).
8. Adachi, O. *et al.* Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity.* **9**, 143-150 (1998).

9. Sun, D. & Ding, A. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. *Nat Immunol* **7**, 375-381 (2006).

10. Lin, S.C., Lo, Y.C. & Wu, H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* **465**, 885-890 (2010).

11. Netea, M.G., Wijmenga, C. & O'Neill, L.A. Genetic variation in Toll-like receptors and disease susceptibility. *Nat Immunol* **13**, 535-542 (2012).

12. Ryffel, B. *et al.* Innate immunity to mycobacterial infection in mice: critical role for toll-like receptors. *Tuberculosis (Edinb)* **85**, 395-405 (2005).

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

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

15. 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).

16. 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).

17. 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).

18. 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).

19. Roca, F.J. & Ramakrishnan, L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. *Cell* **153**, 521-534 (2013).

20. 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).

21. 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).

22. 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).

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

24. 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).

25. 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).

26. 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).

27. 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).

28. 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).

29. 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).

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

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

32. 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).

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

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

35. 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).

36. Levine, B., Mizushima, N. & Virgin, H.W. Autophagy in immunity and inflammation. *Nature* **469**, 323-335 (2011).

37. Deretic, V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. *Immunological reviews* **240**, 92-104 (2011).

38. Youle, R.J. & Narendra, D.P. Mechanisms of mitophagy. *Nature reviews. Molecular cell biology* **12**, 9-14 (2011).

39. Ponpuak, M. *et al.* Delivery of cytosolic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. *Immunity* **32**, 329-341 (2010).

40. Lam, G.Y., Czuczman, M.A., Higgins, D.E. & Brumell, J.H. Interactions of Listeria monocytogenes with the autophagy system of host cells. *Adv Immunol* **113**, 7-18 (2012).

41. Lee, M.S. *et al.* Shiga toxins induce autophagy leading to differential signalling pathways in toxin-sensitive and toxin-resistant human cells. *Cellular microbiology* **13**, 1479-1496 (2011).

42. Mestre, M.B. & Colombo, M.I. Staphylococcus aureus promotes autophagy by decreasing intracellular cAMP levels. *Autophagy* **8**, 1865-1867 (2012).

43. Alonso, S., Pethe, K., Russell, D.G. & Purdy, G.E. Lysosomal killing of Mycobacterium mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 6031-6036 (2007).

44. Biswas, D. *et al.* ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/macrophages. *BMC immunology* **9**, 35 (2008).

45. Delgado, M.A., Elmaoued, R.A., Davis, A.S., Kyei, G. & Deretic, V. Toll-like receptors control autophagy. *The EMBO journal* **27**, 1110-1121 (2008).

46. Gutierrez, M.G. *et al.* Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766 (2004).

47. Singh, S.B., Davis, A.S., Taylor, G.A. & Deretic, V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* **313**, 1438-1441 (2006).

48. Watson, R.O., Manzanillo, P.S. & Cox, J.S. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* **150**, 803-815 (2012).

49. Pilli, M. *et al.* TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. *Immunity* **37**, 223-234 (2012).

50. Lerena, M.C. & Colombo, M.I. Mycobacterium marinum induces a marked LC3 recruitment to its containing phagosome that depends on a functional ESX-1 secretion system. *Cellular microbiology* **13**, 814-835 (2011).

51. Shi, C.S. & Kehrl, J.H. MyD88 and Trif Target Beclin 1 to Trigger Autophagy in Macrophages. *Journal of Biological Chemistry* **283**, 33175-33182 (2008).

52. Crighton, D. *et al.* DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* **126**, 121-134 (2006).

53. Ryan, K.M. p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome. *European journal of cancer* **47**, 44-50 (2011).

54. Laforge, M. *et al.* DRAM triggers lysosomal membrane permeabilization and cell death in CD4(+) T cells infected with HIV. *PLoS pathogens* **9**, e1003328 (2013).

55. Galavotti, S. *et al.* The autophagy-associated factors DRAM1 and p62 regulate cell migration and invasion in glioblastoma stem cells. *Oncogene* **32**, 699-712 (2013).

56. Koul, A., Arnoult, E., Lounis, N., Guillemont, J. & Andries, K. The challenge of new drug discovery for tuberculosis. *Nature* **469**, 483-490 (2011).

57. Kuijl, C. *et al.* Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. *Nature* **450**, 725-730 (2007).

58. Kim, S., Cho, S.N., Oh, T. & Kim, P. Design and synthesis of 1H-1,2,3-triazoles derived from econazole as antitubercular agents. *Bioorganic & medicinal chemistry letters* **22**, 6844-6847 (2012).