EPFL

Thèse n° 10 170

Single-cell analysis of the interactions between Mycobacterium tuberculosis and macrophages

Présentée le 26 janvier 2024

Faculté des sciences de la vie
Unité du Prof. McKinney
Programme doctoral en biotechnologie et génie biologique

pour l'obtention du grade de Docteur ès Sciences

par

Ophélie RUTSCHMANN

Acceptée sur proposition du jury

Prof. D. M. Suter, président du jury
Prof. J. McKinney, directeur de thèse
Prof. P. Broz, rapporteur
Prof. M. Gutierrez, rapporteur
Prof. M. Blokesch, rapporteuse

École
polytechnique
fédérale
de Lausanne

2024

Acknowledgements

First and foremost, I would like to thank my thesis director, **Prof. McKinney**, for giving me the opportunity to join the laboratory. I highly appreciated the trust you had in me and the freedom you gave me to pursue my research on the fascinating topic of host-pathogen interactions in Tuberculosis.

Thank you also to the members of my PhD thesis committee, **Prof. Blokesch**, **Prof. Gutierrez, Prof. Broz,** and **Prof. Suter**, for your valuable feedback and the interesting discussion during my thesis defense. I really enjoyed sharing my work with you.

A huge thank you to **Dr. Chiara Toniolo** who supervised me and helped me out at the beginning of my PhD journey. This thesis would not have been the same without you, you are an amazing scientist, and I could not have hoped for a better mentor.

I would also like to thank all of the members of the McKinney lab, past and present, for creating such a nice and supportive lab atmosphere. The work environment we have in the lab is very special, and I cherished the fun moments we had together both inside and outside the lab. Thank you in particular to **Richa** and **Vivek** for sharing your deep knowledge about *Mycobacterium tuberculosis* and your enthusiasm about research. Thank you to the current UPEC team, **Kathrin**, **Gauri**, **Ashim**, **Théo**, **Kristina**, and **Fred** for always being interested in my work and providing valuable feedback on my project. I admire the way you all work together in a strong collaborative and supportive team and wish you all the best for the future of your respective projects! Thank you to **Léa** and **Valentin** for being the best technicians a lab could hope for. You have both been extremely helpful and it is always a pleasure working with you. Thank you **Esther** for being not only a great scientist but an amazing friend. This thesis has been easier knowing that I can always count on you for support, and I really appreciate our discussions about science, life in general and mountains. Thank you also to **Suzanne** for your efficient administrative support, and **Thomas** for teaching me about UPEC and your supportive discussions throughout my thesis. Thank you also to **Kunal**, **Gaëlle**, **Sofia**, and **François** for being great and supportive colleages!

Thank you as well to the Bachelor and Master students I had the opportunity to supervise during the PhD, I learned a lot while working with you. I would also like to extend my gratitude to all of the members of the GHI, I always appreciated our interesting discussions about science and valued the helpful advice I received from you.

During my work here at EPFL, I also had the opportunity to interact with many brilliant scientists in the SV core facilities. Thank you in particular to the flow cytometry core facility, the BIOP and the protein production and structure core facility without whom this thesis would not have been possible. Thank you as well to Vivianne Padrun for taking care of the P3 and maintaining it in such good condition.

Finally, thank you Schatz for your support, both emotional and scientific. You are an amazing scientist and the ideas and feedback you give me never cease to amaze me. I am grateful to have found you and to be sharing my life with you!

Abstract

Mycobacterium Tuberculosis (M. tuberculosis) is a highly effective pathogen infecting nearly a third of the world's population. An *M. tuberculosis* infection starts when droplets containing bacteria enter an individual's lungs. The first host cells to contact the bacteria are alveolar macrophages residing in the lung alveoli. These cells are usually in charge of phagocyting and killing pathogens; however, *M. tuberculosis* can survive and even replicate inside of them, leading to disease progression.

In this thesis, we investigated how *M. tuberculosis* interacts with macrophages in two projects. In a first project, we used time-lapse microscopy and fluorescent reporters to study how diversity in the bacteria and the host cells influences the outcome of single-cell infections. By using *M. tuberculosis* strains expressing different fluorescent proteins, we showed that bacteria inside a same macrophage behave more similarly than bacteria in different host cells, suggesting that some macrophages are more permissive to bacterial growth than others. Interestingly, these differences disappeared when inhibiting inducible nitric oxide synthase (iNOS), a known macrophage defense mechanism against *M. tuberculosis*. By using a macrophage reporter cell line for iNOS expression, we demonstrated that iNOS levels fluctuate in time in single cells, and that pre-existing heterogeneity in iNOS expression is linked to the control of intracellular *M. tuberculosis* by macrophages. As an infection with *M. tuberculosis* may initiate from a single bacterium coming in contact with a single macrophage, heterogeneity in the macrophage population could explain part of the variability observed in the outcome of tuberculosis infections. Indeed, the level of iNOS a macrophage expresses when it is infected could influence how well it controls the initial infection and how the disease progresses.

In a second project, we investigated the mechanisms leading to IL-1β secretion in macrophages infected with *M. tuberculosis.* IL-1β is a pro-inflammatory cytokine that plays a crucial role in host defense against *M. tuberculosis* by promoting inflammation and recruiting immune cells to the site of infection. As over-production of IL-1β is deleterious to the host, its secretion is tightly regulated and requires the activation of a molecular platform called the inflammasome inside infected cells. Previous studies have demonstrated that IL-1β secretion can be mediated by the NLRP3 and AIM2 inflammasomes upon *M. tuberculosis* infection. In this study, we demonstrated that IL-1β can also be secreted upon activation of an alternative pathway, called the non-canonical inflammasome, in macrophages infected with *M. tuberculosis*. We showed that Caspase-11 (in mice), and Caspase-4 and Caspase-5 (in humans), the main proteins involved in non-canonical inflammasome activation, are activated in infected macrophages and contribute to IL-1β secretion. Furthermore,

we demonstrated that secreted IL-1β can enhance the anti-mycobacterial properties of neighboring macrophages by promoting autophagy, a known defense mechanism against intracellular pathogens. By analyzing a publicly available sc-RNAseq dataset, we showed that Caspase-11 expression is induced in macrophages from the lungs of infected mice, suggesting a role for the control of *M. tuberculosis* infection *in vivo*. Our findings identify a novel mechanism for IL-1β secretion in *M. tuberculosis*-infected cells and suggest a role for the non-canonical inflammasome in defense against mycobacterial infections.

Keywords: *M. tuberculosis,* macrophages, time-lapse microscopy, single-cell heterogeneity, inducible nitric oxide synthase (iNOS), non-canonical inflammasome.

Zusammenfassung

Mycobacterium tuberculosis (*M. tuberculosis*) ist ein bakterieller Erreger, der fast ein Drittel der Weltbevölkerung infiziert. Eine *M. tuberculosis*-Infektion beginnt mit bakterienhaltigen Tröpfchen, die in die Lunge einer Person gelangen. In den Lungenbläschen sind Alveolarmakrophagen die ersten Zellen, die mit den Bakterien in Kontakt kommen. Diese Zellen sind normalerweise für die Phagozytierung und Abtötung von Krankheitserregern zuständig, aber *M. tuberculosis* kann in ihnen überleben und sich sogar vermehren, was zum Fortschreiten der Krankheit führt.

In dieser Arbeit haben wir in zwei verschiedenen Projekten untersucht, wie *M. tuberculosis* mit Makrophagen interagiert. In einem ersten Projekt untersuchten wir mit Hilfe von Zeitraffermikroskopie und Fluoreszenzreportern, wie die Diversität der Bakterien und der Wirtszellpopulationen das Ergebnis einer Einzellinfektion beeinflusst. Durch die Verwendung von *M. tuberculosis* Bakterien, die verschiedene fluoreszierende Proteine exprimieren, zeigen wir, dass sich verschiedene Bakterien in demselben Makrophagen ähnlicher verhalten als Bakterien in verschiedenen Makrophagen, was darauf hindeutet, dass einige Makrophagen für das Bakterienwachstum permissiver sind als andere. Interessanterweise verschwinden diese Unterschiede, wenn die induzierbare Stickstoffmonoxid-Synthase (iNOS) gehemmt wird, einen bekannten Abwehrmechanismus der Makrophagen gegen *M. tuberculosis*. Durch die Verwendung einer Makrophagen-Reporterzelllinie für iNOS-Expression zeigen wir ausserdem, dass iNOS-Spiegel auf Einzelzellebene zeitlich fluktuieren und dass die Heterogenität der iNOS-Expression mit der Kontrolle der intrazellulären *M. tuberculosis* durch Makrophagen zusammenhängt. Da eine Infektion mit *M. tuberculosis* durch ein einzelnes Bakterium, das mit einem einzelnen Wirtsmakrophagen in Kontakt kommt ausgelöst werden kann, könnte die Heterogenität der Makrophagenpopulation einen Teil der Variabilität erklären, die beim Ausgang von Tuberkuloseinfektionen beobachtet wird. So könnte die Menge an iNOS, die ein Makrophage bei der Infektion exprimiert, einen Einfluss darauf haben, wie gut die anfängliche Infektion kontrolliert werden kann und wie und ob die Krankheit schliesslich fortschreitet.

In einem zweiten Projekt untersuchten wir die Mechanismen, die zur Sekretion von IL-1β in *M. tuberculosis*-infizierten Makrophagen führen. IL-1β ist ein proinflammatorisches Zytokin, das eine entscheidende Rolle bei der Abwehr von *M. tuberculosis* spielt, indem es die Entzündung fördert und andere Immunzellen an den Infektionsherd rekrutiert. Da eine Überproduktion von IL-1β schädlich sein kann, wird seine Sekretion streng reguliert und erfordert die Aktivierung einer molekularen Plattform, des so genannten Inflammasoms, innerhalb der infizierten Zellen. Frühere

Studien haben gezeigt, dass die IL-1β-Sekretion bei einer *M. tuberculosis*-Infektion sowohl durch das NLRP3- als auch das AIM2-Inflammasom vermittelt werden kann. In dieser Studie zeigen wir, dass IL-1β auch durch die Aktivierung eines alternativen Weges, des so genannten nicht-kanonischen Inflammasoms, in mit M. tuberculosis infizierten Makrophagen ausgeschüttet werden kann. Wir zeigen, dass Caspase-11 (bei Mäusen) und Caspase-4 und Caspase-5 (bei Menschen), die wichtigsten an der nicht-kanonischen Inflammasom-Aktivierung beteiligten Proteine in infizierten Makrophagen aktiviert werden, was zur Sekretion von IL-1β führt. Darüber hinaus zeigen wir, dass IL-1β die antimykobakteriellen Eigenschaften benachbarter Makrophagen verstärken kann, indem es die Autophagie fördert, einen bekannten Abwehrmechanismus gegen intrazelluläre Krankheitserreger. Durch die Analyse eines öffentlich zugänglichen scRNAseq-Datensatzes zeigen wir dann, dass die Expression von Caspase-11 in Makrophagen aus den Lungen infizierter Mäuse induziert wird, was auf eine Rolle bei der Kontrolle der *M. tuberculosis*-Infektion in vivo hindeutet. Unsere Ergebnisse identifizieren somit einen neuen Mechanismus für die IL-1β-Sekretion in *M. tuberculosis*-infizierten Zellen und deuten auf eine mögliche Rolle des nicht-kanonischen Inflammasoms bei der Abwehr von Mykobakterieninfektionen hin.

Schlüsselwörter: *M. tuberculosis*, Makrophagen, Zeitraffermikroskopie, Einzelzellheterogenität, induzierbare Stickstoffmonoxid-Synthase (iNOS), nicht-kanonisches Inflammasom.

Résumé

Mycobacterium Tuberculosis (*M. tuberculosis*) est un agent pathogène très efficace qui infecte près d'un tiers de la population mondiale. Une infection par *M. tuberculosis* commence lorsque des gouttelettes contenant des bactéries pénètrent dans les poumons d'un individu. Les premières cellules hôtes à entrer en contact avec les bactéries sont les macrophages alvéolaires qui résident dans les alvéoles pulmonaires. Ces cellules sont généralement chargées de phagocyter et de tuer les agents pathogènes; cependant, *M. tuberculosis* peut survivre et même se répliquer à l'intérieur de ces macrophages, ce qui entraîne une progression de la maladie.

Dans cette thèse, nous avons étudié comment *M. tuberculosis* interagit avec les macrophages dans deux projets différents. Dans un premier projet, nous avons utilisé la microscopie et des rapporteurs fluorescents pour étudier comment la diversité des bactéries et des cellules hôtes influence le déroulement d'une infection. En utilisant des souches de *M. tuberculosis* exprimant différentes protéines fluorescentes, nous avons démontré que les bactéries à l'intérieur d'un même macrophage se comportent de manière plus similaire que les bactéries dans différentes cellules hôtes, ce qui suggère que certains macrophages sont plus permissifs à la croissance bactérienne que d'autres. Il est intéressant de noter que ces différences disparaissent lorsque nous inhibons l'oxyde nitrique synthase inductible (iNOS), un mécanisme de défense connu des macrophages contre *M. tuberculosis*. En utilisant une lignée de cellules rapporteuses pour l'expression de iNOS, nous avons en outre démontré que les niveaux d'iNOS fluctuent dans le temps dans chaque cellule, et que des différences dans l'expression de iNOS sont corrélées au contrôle de *M. tuberculosis* par les macrophages. Étant donné qu'une infection par *M. tuberculosis* peut être déclenchée par une seule bactérie entrant en contact avec un seul macrophage, des différences dans la population de macrophages pourrait expliquer une partie de la variabilité observée dans l'issue des infections par tuberculeuses. En effet, le niveau d'iNOS exprimé par un macrophage lorsqu'il est infecté pourrait influencer la façon dont il contrôle cette infection initiale et la façon dont la maladie évolue.

Dans un second projet, nous avons étudié les mécanismes conduisant à la sécrétion d'IL-1β dans les macrophages infectés par *M. tuberculosis*. IL-1β est une cytokine pro-inflammatoire qui joue un rôle crucial dans la défense contre *M. tuberculosis* en favorisant l'inflammation et en recrutant d'autres cellules immunitaires. Comme la surproduction d'IL-1β peut être délétère pour l'hôte, sa sécrétion est étroitement régulée et nécessite l'activation d'une plateforme

moléculaire appelée inflammasome à l'intérieur des cellules infectées. Des études précédentes ont démontré que la sécrétion d'IL-1β peut être régulée par les inflammasomes NLRP3 et AIM2 lors d'une infection par *M. tuberculosis*. Dans cette étude, nous avons démontré que IL-1β peut également être sécrété lors de l'activation d'une voie alternative appelée inflammasome non canonique lors d'une infection par *M. tuberculosis*. Nous avons démontré que Caspase-11 (chez la souris), ainsi que Caspase-4 et Caspase-5 (chez l'homme), les principales protéines impliquées dans l'activation de l'inflammasome non canonique, sont activées dans des macrophages infectés, ce qui entraîne la sécrétion d'IL-1β. En outre, nous avons démontré que l'IL-1β sécrété peut renforcer les propriétés anti-mycobactériennes des macrophages voisins en favorisant l'autophagie, un mécanisme de défense contre les pathogènes intracellulaires. En analysant un ensemble de données scRNAseq accessible au public, nous avons également montré que l'expression de Caspase-11 est induite par les macrophages dans les poumons de souris infectées par *M. tuberculosis*, ce qui suggère un rôle dans le contrôle des infections *in vivo*. Nos résultats identifient donc un nouveau mécanisme de sécrétion d'IL-1β dans les cellules infectées par *M. tuberculosis* et suggèrent un rôle potentiel de l'inflammasome non canonique dans la défense contre les infections mycobactériennes.

Mots-clés : *M. tuberculosis*, macrophages, microscopie, hétérogénéité, oxyde nitrique synthase inductible (iNOS), inflammasome non canonique.

Table of Contents

List of figures

Chapter 1. Introduction

Chapter 2. Preexisting Heterogeneity of Inducible Nitric Oxide Synthase Expression Drives Differential Growth of *Mycobacterium tuberculosis* in Macrophages

Chapter 3. Activation of the Non-Canonical Inflammasome by *Mycobacterium tuberculosis* Promotes IL-1β Secretion from Infected Macrophages

Chapter 1. Introduction

Tuberculosis is the leading cause of death by an infectious disease worldwide and is estimated to have caused 1.6 million deaths in 2021 alone (WHO, 2022). Up to a quarter of the world's population is thought to be infected with *Mycobacterium tuberculosis*, the pathogen responsible for causing the disease (WHO, 2022).

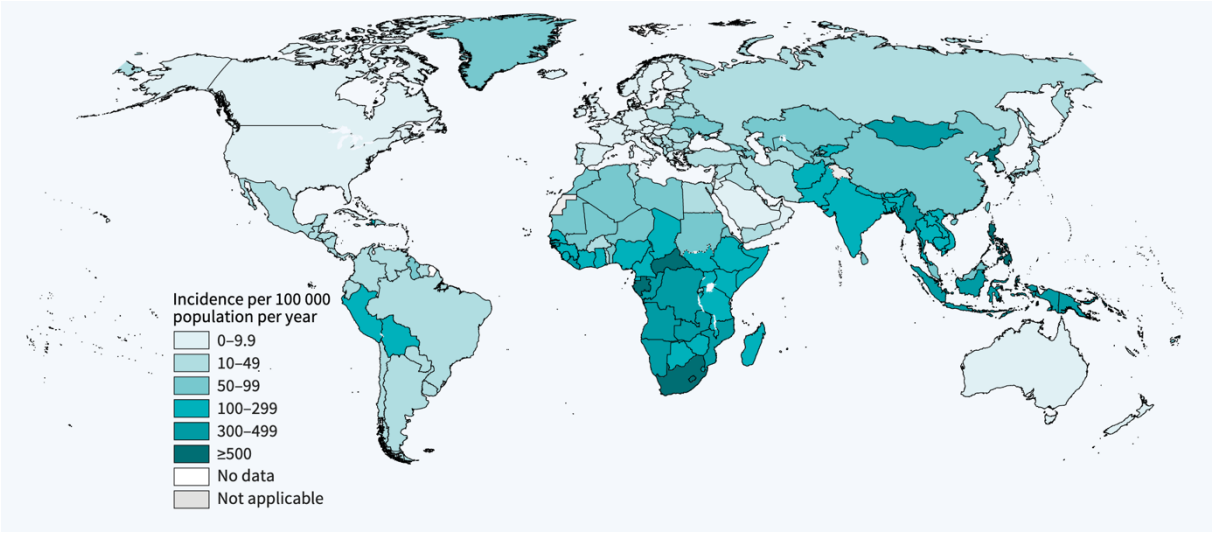


Figure 1: Estimated tuberculosis incidence rates (WHO 2022 global tuberculosis report)

While treatment options for drug-susceptible Tuberculosis exist, they involve the administration of multiple antibiotics over extended periods of time, often lasting up to six months (WHO, 2020). Furthermore, cases of drug resistant *M. tuberculosis*, for which the treatment success rate is alarmingly low, are increasingly frequent around the world. Unfortunately, development of new antibiotics against *M. tuberculosis* is challenging due to the slow growing nature of the pathogen, its waxy cell wall that limits drug penetration, and the requirement for containment in a biosafety level 3 (BSL3) facility (Boogaard et al., 2009; Koul et al., 2011). Moreover, as *M. tuberculosis* is an intracellular pathogen, drugs need to first enter the host cells without being degraded before reaching the bacteria (Day et al., 2023). Finally, disease progression is very diverse, ranging from sterilization of the bacteria to latent infection or active disease, further complicating the development of new therapeutic approaches. Therefore, a comprehensive understanding of tuberculosis is essential for the development of new antibiotics as well as alternative treatment options such as host-directed therapies.

During my thesis, I studied the interactions between *M. tuberculosis* and macrophages, immune cells responsible for phagocytosing pathogenic bacteria, at the single cell level. One focus of my research was to understand how phenotypic heterogeneity in the pathogens and host cells can influence the outcome of single-

cell *M. tuberculosis* infections. Since an *M. tuberculosis* infection can originate from a single bacterium interacting with a single host macrophage, studying these initial interactions can provide valuable insight into disease pathophysiology, and help shed light on why some individuals are able to sterilize an *M. tuberculosis* infection while others develop severe symptoms. In a second project, I focused on studying the activation of the non-canonical inflammasome in macrophages infected with *M. tuberculosis*. Studying these early innate defense mechanisms is important to elucidate how macrophages circumcise bacterial growth before the involvement of the adaptive immune system, which could pave the way for the development of new therapies.

1.1 Tuberculosis

1.1.1 Tuberculosis pathophysiology

Mycobacterium tuberculosis, the causative agent for tuberculosis, was first discovered in 1882 by Robert Koch. It is an acid-fast bacterium that is part of the actinomycete family (Talbot & Raffa, 2014). Most Mycobacteria species, such as *M. smegmatis* are found in the soil and are opportunistic pathogens. Mycobacteria belonging to the *M. tuberculosis* complex however, such as M*. africanum*, *M. bovis*, *M. microti*, *M. canetti* and *M. tuberculosis*, are obligatory pathogens that infect humans and cause tuberculous lesions in the lungs (Smith et al., 2009).

An *M. tuberculosis* infection is initiated by cough droplets containing bacteria entering an individual's lungs. The first host cells to contact the bacteria are alveolar macrophages residing in the lung alveoli. Strikingly, even as little as a single bacterium coming into contact with a single macrophage is thought to be enough to initiate an infection (Dean et al., 2005; Donald et al., 2018; Saini et al., 2012). Alveolar macrophages are normally in charge of phagocytosing and killing pathogens (Delogu et al., 2013). However, *M. tuberculosis* has evolved strategies, such as inhibition of phagosomal maturation and acidification, that allow it to survive, replicate and sometimes even kill these cells (Cambier, Falkow, et al., 2014). As the disease progresses, other immune cells such as interstitial macrophages, dendritic cells, natural killer (NK) cells and neutrophils are recruited to the site of infection. This can lead to the formation of a granuloma, a structure comprising several different cell types able to contain *M. tuberculosis* growth (Delogu et al., 2013; Ramakrishnan, 2012) **(Figure 2)**. Granulomas can be broadly classified in three groups: solid (non-necrotic), caseous (necrotic) and cavitary. Bacteria can survive inside these lesions for years without the infected individual exhibiting any symptoms nor being able to transmit the disease, a stage called latent tuberculosis infection (Cadena et al., 2017; Ramakrishnan, 2012). It is thought that in some cases, the bacteria inside these granulomas can be killed and the lesions sterilized (Behr et al., 2018; Ramakrishnan, 2012). Indeed, some patients display healed granulomas without any evidence of ever having developed active tuberculosis. In 5-10% of cases however, necrotic granulomas can cavitate, allowing the bacteria to gain access to the airway. The released bacteria can also spread via the lymphatic system to more distant tissues and organ. This stage of the disease is called active tuberculosis, and patients exhibit symptoms such as coughing, fever and weight loss, and can transmit the disease to a new host.

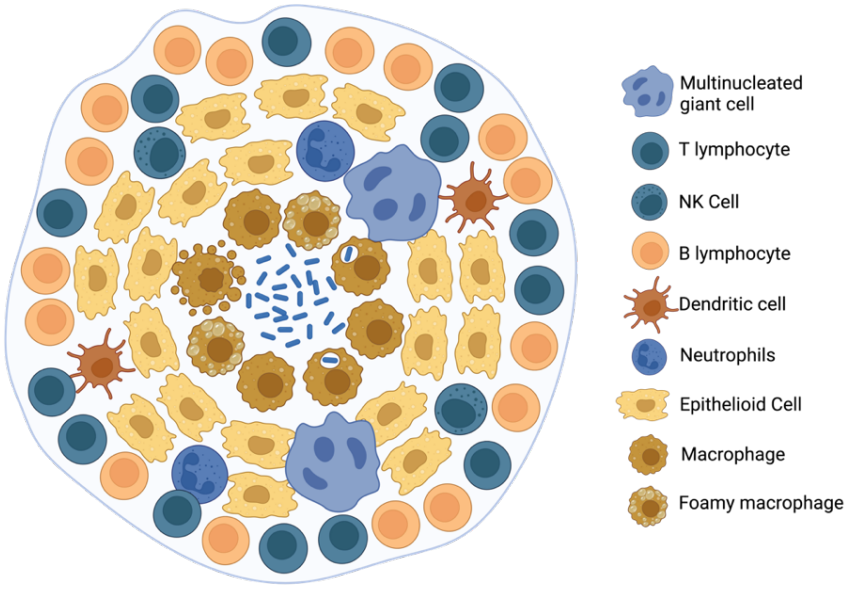


Figure 2: The tuberculosis granuloma
Granulomas have a macrophage-rich center surrounded by a lymphocytic cuff made of B and T cells. Macrophages present in granulomas can fuse into multinucleated giant cells or differentiate into other specialized cell types, such as foamy macrophages or epithelioid cells. Many other cell types are also present in granulomas, including neutrophils, dendritic cells, natural killer (NK) cells, and fibroblasts.

1.1.2 Tuberculosis treatments

Antibiotic regimens

Tuberculosis is treated by the administration of a combination of different first-line drugs. Different treatment regimens are recommended for drug-susceptible tuberculosis around the world, which all take between 4 to 9 months. The RIPE TB treatment consists of a 6 to 9-month administration of four the first-line tuberculosis drugs Rifampin, Isoniazid, Ethambutol and Pyrazinamide. Isoniazid is a pro-drug that is converted into its active form by the mycobacterial catalase-peroxidase katG and that targets mycolic acid synthesis (Timmins & Deretic, 2006). Ethambutol inhibits the synthesis of arabinogalactan, also a cell wall component (Goude et al., 2009). Pyrazinamide inhibits energy production and is important to shorten the duration of tuberculosis therapy as it can also target non-growing bacteria (Zhang et al., 2015). Finally, Rifampin inhibits bacterial RNA polymerase (Wehrli, 1983). The RIPE TB treatment regimen has an intensive phase of 2 months during which all four drugs are administered daily, followed by a continuation phase of 4 to 7 months during which only Isoniazid and Rifampin are administered (WHO, 2022). In some cases, a shorter treatment regimen of 4 months can be administered instead. This regimen consists in

the administration of Rifampin, Moxifloxacin (a topoisomerase II and IV inhibitor), Isoniazid and Pyrazinamide for 2 months daily, followed by a continuation phase of another 9 weeks during which only Rifampin, Moxifloxacin and Isoniazid are administered (WHO 2022). Unfortunately, cases of multidrug-resistant tuberculosis (MDR, resistant to at least the two first-line drugs isoniazid and rifampicin) and extensively drug-resistant tuberculosis (XDR, resistant to at least two first-line drugs and two second-line drugs) are becoming increasingly common. Indeed, it is estimated that around 3.3% of new tuberculosis cases and 18% of previously treated cases are resistant to both isoniazid and rifampicin (WHO, 2022). In particular, eastern European countries, Russia, central Asian countries and China exhibit an alarmingly high rate of drug resistance (Salari et al. 2023). In these cases, the treatment is very long and the drugs used can have severe side effects.

Research is ongoing to develop new drugs against *M. tuberculosis*, and several promising antibiotics have recently been approved for the treatment of MDR and XDR Tuberculosis (Dartois & Rubin, 2022). Bedaquiline, a diarylquinoline targeting the mycobacterial ATP synthase, was approved in 2012 and was the first drug with a novel mechanism of action to be approved since rifampicin in 1971 (Khoshnood et al., 2021). Pretomanid and Delamanid, two nitroimidazoles targeting the mycobacterial cell wall, have also been recently approved for the treatment of MDR and XDR-tuberculosis (Blair & Scott, 2015). Other promising candidates that are currently in phase I or II clinical trials include the oxazolidinones Sutezolid, Delpazolid and Contezolid that target the 23S ribosome and inhibit protein synthesis, DprE1 inhibitors that target cell wall synthesis, the Imidazopyridine Q203 that targets cell respiration, the Riminophenazine TBI-166 that targets ion transport and cell respiration and the oxaborole GSX 070 that targets protein synthesis (Tiberi et al., 2018). Research is also being done to develop new and shorter treatment regimens against susceptible and drug resistant forms of tuberculosis. One such example is the NiX-TB trial which assessed a 6-month regimen of bedaquiline, pretomanid and linezolid for the treatment of MDR Tuberculosis (Conradie et al., 2020). The results of this trial were published in 2020 and showed favorable outcomes in patients with highly drug-resistant tuberculosis. Finally, research is also being conducted to repurpose already approved drugs for the treatment of tuberculosis. For instance, moxifloxacin, linezolid and clofazimine are all repurposed antibiotics that have recently been approved for the treatment of XDR Tuberculosis (Cardoso et al., 2021; Singh et al., 2019).

Vaccines

Currently, the only approved vaccine against *M. tuberculosis* is the Bacille Calmette-Guerin (BCG) vaccine. BCG is an attenuated vaccine derived from M. bovis that protects against childhood tuberculosis but is ineffective against adult pulmonary tuberculosis (Fatima et al., 2020). Different strategies are studied to develop a more efficacious vaccine against tuberculosis. One such strategy involves modifying the current route of administration of BCG vaccination. Murine infection studies have demonstrated that delivering the BCG vaccine via aerosols, rather than intradermally, leads to a heightened CD4 and CD8 T cell response to *M. tuberculosis* infection and reduces bacterial burdens after vaccination (Derrick et al., 2014; Kaveh et al., 2020). Unfortunately, this administration route has also been associated with increased inflammation and potential lung tissue damage, which warrants further investigation into the safety of this method (Kaveh et al., 2020). Another possibility would be to administer BCG intravenously, as it has been shown to elicit a robust immune response in macaque monkeys (Darrah et al., 2020). Nonetheless, this approach is unlikely to have broad applicability due to the limited availability of the infrastructure and medical expertise required for intravenous administration, especially in countries with a high tuberculosis burden (Cho et al., 2021).

Another potential strategy to develop a more effective tuberculosis vaccine is the creation of a novel recombinant vaccine derived from the existing BCG vaccine. Presently, there are four novel vaccines utilizing recombinant BCG strains under investigation, with three in pre-clinical studies and one in a phase III clinical trial (Cho et al., 2021). Two other vaccines based on other mycobacterial species are also in phase III clinical trials: one derived from *M. indicus pranii*, and another from heat-killed *M. vaccae*. The *M. indicus pranii* vaccine has demonstrated safety in mice and guinea pigs, and has been shown to enhance bacterial clearance in guinea pig models of infection and in advanced pulmonary tuberculosis patients (Gupta et al., 2012; Sharma et al., 2017). The *M. vaccae* vaccine has proven protective against *M. tuberculosis* infections in animal models, and is immunogenic in humans when administered in a three-dose regimen (Mayo & Stanford, 2000; Onyebujoh et al., 1999; Von Reyn et al., 1997). Additionally, several antigenic vaccines based on the insertion of an antigenic *M. tuberculosis* protein into a viral vector are also being studied, some of which are currently in phase II clinical trials (Cho et al., 2021). Finally, several vaccines are under consideration for use as boosters in BCG-vaccinated populations. One such vaccine, MTBVAC, a live-attenuated *M. tuberculosis* vaccine with deletions of the virulence factors fadD26 and phoP, has been deemed safe in humans in a phase I clinical trial (Spertini et al., 2015). In animal models of infection, this vaccine confers greater immunity than BCG when administered alone, and its efficacy further

increases when used as a booster in BCG-primed animals (Aguilo et al., 2020; Clark et al., 2017).

1.2 Interplay between the innate immune system and *Mycobacterium tuberculosis*

Innate immunity is an important determinant not only for early control of *M. tuberculosis* dissemination but also for long-term control of tuberculosis disease by priming adaptive immune responses and regulating inflammation. In the following paragraphs, I will present the innate immune cell types important in tuberculosis pathophysiology, how the bacteria are recognized by these cells, the cellular mechanisms associated with defense against *M. tuberculosis* and the bacterial components that can modulate the innate immune response.

1.2.1 Innate immune cell types in Tuberculosis

Macrophages

Macrophages play a crucial role at different stages of tuberculosis disease progression and are the primary cellular niche for *M. tuberculosis* during early and chronic infection. During the early phases of the disease, macrophages can have a direct anti-bacterial activity against *M. tuberculosis*, be used as a reservoir for *M. tuberculosis* growth, or help regulate immune responses by secreting chemokines and cytokines (Ahmad et al., 2022). They also play a role at later stages of the disease, as they are the main cell type present in granulomas (Cadena et al., 2017; Flynn et al., 2011). At least two populations of macrophages of different ontologies co-exist in the lungs of infected individuals: alveolar macrophages and interstitial macrophages.

Alveolar macrophages

Alveolar macrophages reside in the lung alveoli, at the interface of lung tissue and environment, and present a first line of defense against airborne pathogens (Corleis & Dorhoi, 2020). They are professional phagocytes that play a role not only in innate immunity but also in the metabolism of lung surfactant and clearance of debris (Aegerter et al., 2022). Studies using RNA sequencing or metabolic profiling on cells extracted from the lungs of mice infected with *M. tuberculosis* showed that, in general, infected alveolar macrophages display an M2/anti-inflammatory phenotype and present a permissive environment for *M. tuberculosis* growth (Huang et al., 2018; Pisu et al., 2020; Rothchild et al., 2019). A recent study however showed that a sub-population of pro-inflammatory alveolar macrophage that upregulate iNOS also exists in the lungs of infected mice and is associated with intracellular bacteria exhibiting higher levels of stress (Pisu et al., 2021). These pro- and anti-inflammatory sub-populations of alveolar macrophages seem to be conserved in humans, though

it has not been characterized whether they play a different role in the control of early *M. tuberculosis* infection (Pisu et al., 2021).

During the first few days of infection, alveolar macrophages containing *M. tuberculosis* secrete chemokines that leads to the recruitment of other innate immune cells to the site of infection. Infected alveolar macrophages have also been shown to leave the alveoli and migrate towards the lung interstitium, contributing to bacterial dissemination and infection of other cells, such as interstitial macrophages (Cohen et al., 2018).

Interstitial macrophages

In contrast to alveolar macrophages, which are thought to be of embryonic origin, interstitial macrophages are derived from monocyte progenitors in the bone marrow (Tan & Krasnow, 2016). They are found with a low frequency in the lungs of uninfected mice, but their number increases approximately 40-fold in response to infection with *M. tuberculosis* (Pisu et al., 2021; Wolf et al., 2007). Interstitial macrophages are recruited from the blood to the site of the infection by cytokines released by alveolar macrophages and epithelial cells. In comparison to alveolar macrophages, these macrophages display a more pro-inflammatory phenotype with higher expression of genes such as IFNγ, NOS2, or TNFα (Gibbings et al., 2017; L. Huang et al., 2018; Pisu et al., 2020, 2021). *M. tuberculosis* bacteria inside interstitial macrophage are exposed to higher levels of nitric oxide related stresses and display slower growth rates than bacteria inside alveolar macrophages (Huang et al., 2018; Pisu et al., 2021). As for alveolar macrophages, different sub-populations of interstitial macrophages with different anti-bacterial capacities co-exist in the lungs of infected mice (Chakarov et al., 2019; Pisu et al., 2021).

Macrophages in granulomas

As the disease progresses, organized multi-cellular structure called granulomas form in the lungs in an effort to contain *M. tuberculosis* growth (Delogu et al., 2013). Within these structures, infected macrophages can form multinucleated giant cells, transform into lipid-rich foamy macrophages or transition to epithelioid cells (McClean & Tobin, 2016). Multinucleated giant cells are thought to be formed either via cell-cell fusion or via modified cell divisions, and arise in response to persistent inflammatory stimuli (Herrtwich et al., 2016, Helming et al., 2009). Foamy macrophages are a hallmark of tuberculosis granulomas and are a major niche for *M. tuberculosis* survival (Agarwal et al., 2021; Shim et al., 2020). They contain bubble-like droplets in their cytoplasm which are filled with fatty acids, cholesterols, and triglycerides. *M. tuberculosis* has been found to associate with these droplets and

can utilize the lipids they contain as carbon sources for survival (Shim et al., 2020). Finally, even though they resemble epithelial cells, the epithelioid cells present in tuberculosis granulomas are thought to be derived from macrophages. The molecular mechanisms underlying this transition are poorly understood, but it is hypothesized that it relies on the increased production of E-cadherin by infected macrophages (Cronan et al., 2016).

Neutrophils

Neutrophils are a sub-type of granulocytes and are highly abundant, making up 50-70% of all leukocytes in the blood (Nathan, 2006). Their role in tuberculosis is controversial, as they have been shown to have both a protective and deleterious effect on the host (Gaffney et al., 2022). They are the first immune cell to be recruited to the site of infection, and are thought to serve as an early line of defense through the secretion of pro-inflammatory mediators and antimicrobial molecules (Martineau et al., 2007; Sia & Rengarajan, 2019). Neutrophils can however also serve as a replicative niche for *M. tuberculosis* and elevated neutrophil counts have been associated with poor prognosis (Barnes et al., 1988).

Neutrophils display a range of mechanisms to defend against invading pathogens, such as phagocytosis, neutrophil extracellular trap (NET) formation, the generation of reactive oxygen species, and the secretion of pro-inflammatory cytokines and antimicrobial peptides (Gaffney et al., 2022). In the context of tuberculosis, studies have demonstrated that neutrophils can effectively phagocytose *M. tuberculosis*, however uncertainty remains regarding their capacity to directly kill the engulfed bacteria (Corleis et al., 2012; Kisich et al., 2002). Human neutrophils infected with *M. tuberculosis* can also release NETs, consisting of extracellular chromatin scaffolds, yet this mechanism appears inefficient in inducing killing of the bacterium (Ramos-Kichik et al., 2009). Consequently, it is suggested that, rather than exerting a direct antimicrobial activity, neutrophils play a more immunomodulatory role in the context of human tuberculosis through the secretion of pro-inflammatory cytokines (Sia & Rengarajan, 2019).

Monocytes

Monocytes are a type of leukocytes that can differentiate into macrophages or dendritic cells. Monocytes are recruited along with neutrophils to the site of an *M. tuberculosis* infection in a CCL2/CCR2-dependent manner (Serbina & Pamer, 2006; Sia & Rengarajan, 2019). CCR2 knock-out mice unable to recruit monocytes are more susceptible to high-dose, but not low-dose infection with *M. tuberculosis* (Peters et al., 2001; Scott & Flynn, 2002). Monocytes have been shown to be one of the major

cell types producing nitric oxide in mice, a molecule toxic for *M. tuberculosis* (Sköld & Behar, 2008). Additionally, infected monocytes can act as antigen-presenting cells and deliver *M. tuberculosis* antigens to the lymph nodes to prime T cells (Samstein et al., 2013). On the other hand, monocyte recruitment to the site of infection has also been suggested to be detrimental to the host, as monocytes are a permissive environment for intracellular *M. tuberculosis* growth (Antonelli et al., 2010). It is thus likely that recruitment of monocytes to the site of infection represents a host strategy to combat bacterial replication that has been subverted by *M. tuberculosis* to facilitate its dissemination.

Natural Killer (NK) cells

NK cells are innate lymphocytes that can secrete pro-inflammatory cytokines such as Interferon gamma (IFNγ) and that have a cytotoxic function during infections with various pathogens, including *M. tuberculosis* (Sia & Rengarajan, 2019). NK cells can effectively kill monocytes and macrophages infected with *M. tuberculosis* (Vankayalapati et al., 2005) and have been shown to restrict intracellular *M. tuberculosis* growth in macrophages through the secretion of Il-22 and IFNγ (Dhiman et al., 2009). In humans, a reduced number of NK cells in the blood as well as reduction in their expression of different activation markers was shown to correlate with transition from latent tuberculosis infection to active disease (Bozzano et al., 2009; Chowdhury et al., 2018).

Dendritic cells

Dendritic cells are antigen-presenting cells that initiate the adaptive immune response during *M. tuberculosis* infection. They do so by presenting bacterial antigens on their surface along with co-stimulatory molecules to T lymphocytes (Ravesloot-Chavez et al., 2021). They are crucial for the control of the infection as depletion of these cells in mice models of infection delays T cell response to *M. tuberculosis* and exacerbate disease progression (Tian et al., 2010). Priming of T cells requires the migration of infected dendritic cells to the lung-draining lymph nodes, but interestingly, some T cells can be primed not only by the migrating infected dendritic cells but also by uninfected lymph node resident dendritic cells (Srivastava & Ernst, 2014). It has been suggested that *M. tuberculosis* can impair dendritic cell function and T cell priming (Madan-Lala et al., 2014). Indeed, in comparison to other diseases, the priming of T cells and the initiation of the adaptive immune response is delayed in tuberculosis (Marino et al., 2004).

1.2.2 Pathogen recognition by the innate immune system

Innate immune system activation during an infection with *M. tuberculosis* begins with pathogen recognition. Many different pattern recognition receptors (PRRs) have been shown to recognize *M. tuberculosis*, amongst which the toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the nucleotide-binding domain and leucine-rich repeat–containing receptors (NLRs), the Cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway, and the NLRP3 and Aim2 inflammasomes (Liu et al., 2017) (Figure 3).

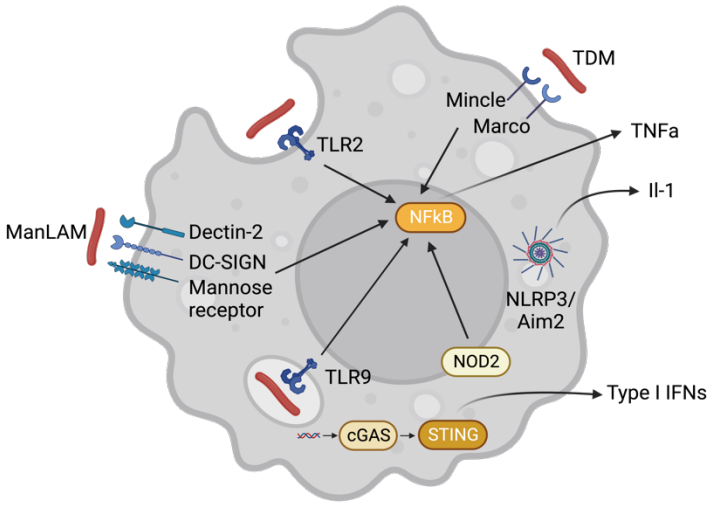


Figure 3: Pattern recognition receptors implicated in detecting *M. tuberculosis*
The C-type lectin receptors Dectin-2, DC-SIGN and the Mannose receptor recognize the glycolipid mannose-capped lipoarabinomannan (ManLAM), while Mincle and Marco recognize Trehalose dimycolate. The NLR NOD2 is suggested to recognize muramyl dipeptide released from bacterial peptidoglycan. TLR9 and Aim2 recognize DNA released by intracellular bacteria, while TLR2 is suggested to recognize lipoproteins and lipoglycans on the surface of *M. tuberculosis*. The cGAS/STING pathway is also activated upon sensing of intracellular DNA, and the NLRP3 inflammasome responds to membrane damage induced by *M. tuberculosis* infection.

Toll-like receptors (TLRs)

TLRs are transmembrane proteins that recognize a wide range of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). TLRs can either be associated with the plasma membrane or be located on the membrane of endosomal compartments (Liu et al., 2017). Upon activation, TLRs can recruit adapter proteins, including MyD88, TRIF, TIRAP and TRAM, subsequently initiating various signaling pathways, such as the NF-kB or MAPK pathways. This activation leads to the secretion of several anti-microbial molecules, cytokines, and

chemokines, which are thought to be beneficial for the host (J. Basu et al., 2012). Additionally, several cellular defense mechanisms, such as autophagy, have also been shown to be associated with TLR signaling in tuberculosis (Shin, Yuk, et al., 2010). Interestingly, it should be noted that *M. tuberculosis*-induced TLR signaling has also been suggested to be detrimental to the host by promoting bacterial survival inside macrophages (Yoshida et al., 2009).

Several TLRs have been implicated in *M. tuberculosis* pathogenesis. MyD88$^{-/-}$ mice, TLR2$^{-/-}$ mice and TLR9$^{-/-}$ mice all exhibit increased susceptibility to infection with *M. tuberculosis* in comparison to wild-type mice (Bafica et al., 2005; Drennan et al., 2004; Scanga et al., 2004). Additionally, genetic polymorphisms in TLR2, TLR4, TLR7, TLR8 and TLR9 have been associated with increased susceptibility to *M. tuberculosis* infection in humans (Z. Chen et al., 2015; Lai et al., 2016; Najmi et al., 2010; Wu et al., 2020). *M. tuberculosis* expresses various molecules that can be recognized by TLRs. For instance, TLR2 has been shown to recognize different lipoproteins, such as LprG, LprA and LpqH, as well as the glycolipids phosphatidylinositol mannoside 6 and 2 and lipoarabinomanna (LAM) (Drage et al., 2010; Pecora et al., 2012; Shukla et al., 2018). Additionally, TLR9, located on the membrane of endosomal compartments, recognizes unmethylated CpG motifs on *M. tuberculosis* DNA, while TLR4 recognizes the heat shock proteins HSP65 and HSP70 (Bafica et al., 2005; Bulut et al., 2005).

Nucleotide-binding domain and leucine-rich repeat–containing receptors (NLRs)

Members of the NLR family are cytoplasmic proteins that recognize microbial components inside the cytosol of the cell. In the context of tuberculosis, a noteworthy NLR is NOD2, which acts upstream of the NF-kB signaling pathway. NOD2 plays a crucial role in the release of pro-inflammatory cytokines and has been associated with the initiation of autophagy and the production of nitric oxide by macrophages (Juárez et al., 2012). NOD2 has been shown to be important for the control of *M. tuberculosis* infections in mice models (Divangahi et al., 2008; Gandotra et al., 2007). Moreover, genetic polymorphisms in NOD2 have been linked to an increased susceptibility to *M. tuberculosis* (Austin et al., 2008; Pan et al., 2012; Wang et al., 2013). NOD2 can recognize muramyl dipeptide (MDP), a constituent of bacterial peptidoglycan (Girardin et al., 2003). Consequently, defects in NOD2 signaling lead to impaired recognition of *M. tuberculosis* and a reduced inflammatory response in both human and murine macrophages (Ferwerda et al., 2005; Juárez et al., 2012; Yang et al., 2007).

C-type leptin receptors (CLRs)

CLRs are a class of receptors characterized by the presence of at least one carbohydrate recognition domain. They play a pivotal role for the recognition of many different bacteria and viruses. Numerous CLRs have been associated with recognition of *M. tuberculosis*, amongst which the mannose receptor (MR), DC-SIGN, Mincle, and Dectin-2 (Liu et al., 2017).

The mannose receptor is a type 1 transmembrane CLR present on macrophages and dendritic cells that can recognize mannose-capped lipoarabinomannan (ManLam) on the surface of *M. tuberculosis* (Kang et al., 2005). This triggers phagocytosis of the bacteria and the secretion of the pro-inflammatory cytokine Il-8 (Naqvi & Endsley, 2020). Interestingly, recognition of *M. tuberculosis* by MR has also been suggested to promote intracellular bacterial survival through the inhibition of phagosome maturation and phagosome-lysosome fusion (Rajaram et al., 2017). DC-SIGN is a mannose-binding type II CLR that is present on dendritic cells and macrophages. It can recognize the surface-exposed lipoglycan lipoarabinomannan (LAM) (Tallieux et al., 2003). Knocking-out the murine homolog of DC-SIGN, SIGNR1, results in only partial impairment of host resistance to *M. tuberculosis* (Tanne & Neyrolles, 2010). However, knocking out both DC-SIGN and MR leads to a more pronounced phenotype characterized by elevated lung inflammation and the presence of large bacterial foci in the lungs (Court et al., 2010). Mincle is a type II transmembrane CLR that is mainly expressed by macrophages and neutrophils. Mincle can recognize trehalose-dimycolate (TDM, also called cord factor) present in the *M. tuberculosis* cell wall, which triggers the production of pro-inflammatory cytokines and of nitric oxide by the cells (Ishikawa et al., 2009). Mincle plays a crucial role in granuloma formation *in vivo*, and Mincle$^{-/-}$ mice are impaired in granuloma formation upon TDM injection (Ishikawa et al., 2009). Similarly to other CLRs, Mincle deficiency only has a modest impact on disease outcome in murine models of infection, suggesting potential overlap with other receptors (Behler et al., 2012; Heitmann et al., 2013). Dectin-2 is present on macrophages and some dendritic cell subsets, and recognizes ManLam on the surface of *M. tuberculosis* (Decout et al., 2018). This recognition leads to the secretion of pro- and anti-inflammatory cytokines such as IL-6, TNFα, IL-10 and TGF-β (Yonekawa et al., 2014). Dectin-2$^{-/-}$ mice display higher lung bacterial counts following infection with mycobacteria than wild-type mice, along with increased lung volume and inflammation, suggesting a role for Dectin-2 *in vivo* (Yonekawa et al., 2014). Additionally, other CLRs, such as DCIR or Dectin-1, have also been implicated in immunity against tuberculosis, although further investigation is needed to elucidate their precise roles (Liu et al., 2017).

Cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING)

cGAS is a DNA sensor that is activated upon binding with foreign cytosolic DNA leading to the catalysis of cyclic GMP-AMP (cGAMP). Subsequently, this activation triggers the activation of the sensor STING, which recruits the kinase TBK-1. TBK-1 then phosphorylates the transcription factor IRF-3, promoting the transcription of IFN-β and interferon-stimulated genes (Chai et al., 2020). cGAS has been shown to be essential for mounting type I IFN responses during *M. tuberculosis* infection, and plays a pivotal role in the induction of autophagy by infected macrophages (Wassermann et al., 2015; Watson et al., 2015). Additionally, it has been suggested that cGAS is important for the activation of dendritic cells (Li et al., 2019; Marinho et al., 2018). Interestingly, despite being important for mycobacterial control in macrophages, the cGAS/STING pathway does not seem to be protective against *M. tuberculosis in vivo*, suggesting that other immune receptors could compensate for its role (Collins et al., 2015; Marinho et al., 2018; Watson et al., 2015).

Scavenger receptors

Scavenger receptors are found on the surface of the cell and can bind many bacterial ligands. Both class A and class B scavenger receptors have been shown to be able to recognize mycobacterial lipoarabinomannans and lipopeptides (Drage et al., 2009; Józefowski et al., 2011). One class A scavenger receptor that is important for the immune response against tuberculosis is the macrophage receptor with collagenous structure (MARCO). MARCO participates in phagocytosis of *M. tuberculosis*, via recognition of TDM on the mycobacterial surface (Bowdish et al., 2009). In humans, genetic polymorphism in MARCO have been associated with impaired phagocytosis of TDM-coated beads by macrophages and with increased risk of developing active tuberculosis (Thuong et al., 2016).

Inflammasome

Inflammasomes are molecular platforms that are assembled in the cytosol upon sensing of danger signals by the cell. This process results in the secretion of the cytokines IL-1β and IL-18 and ultimately triggers a programmed cell death known as pyroptosis. Since IL-1β is a potent pro-inflammatory cytokine, its production is tightly regulated and a second signaling pathway in addition to inflammasome activation must be engaged for its release from the cell. This second pathway involves other pattern recognition receptors, such as TLRs, or cytokine receptors and its engagement is required for the transcription of key inflammasome-related genes (Rastogi & Briken, 2022).

Two types of inflammasomes are relevant in *M. tuberculosis* infections: the AIM2 inflammasome and the NLRP3 inflammasome (Rastogi & Briken, 2022). AIM2, an interferon-inducible member of the HIN-200 family, can bind intracellular *M. tuberculosis* DNA (Fernandes-Alnemri et al., 2009; Saiga et al., 2012). NLRP3, on the other hand, can sense various *M. tuberculosis* components and also responds to cellular perturbations caused by the infection (reviewed in Rastogi & Briken, 2022). Upon sensing of these signals, NLRP3 associates with the protein ASC, and recruit the cysteine protease pro-Caspase-1. This results in the formation of the inflammasome complex, which facilitates the cleavage and activation of Caspase-1. Activated Caspase-1, in turn, cleaves pro-IL-1β into its active form and GasderminD into a pore-forming N-terminus fragment. These GasderminD pores allow active IL-1β to be released from the cell in a process known as pyroptosis, ultimately leading to death of the infected cell (Figure 4).

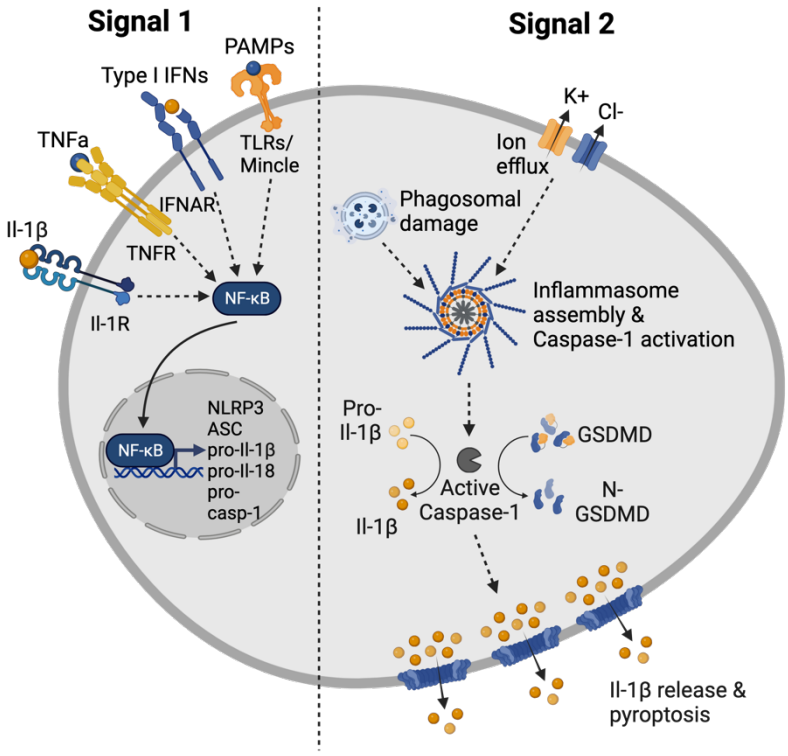


Figure 4: Inflammasome signaling
Inflammasome activation requires two signals. The first signal (a PAMP or a pro-inflammatory cytokine) leads to the upregulation of inflammasome-related genes in an NF-kB-dependent manner. The second signal leads to the assembly of the inflammasome, activation of caspase-1, cleavage of pro-IL-1β and GSDMD, secretion of IL-1β through GSDMD pores, and ultimately death of the cell by pyroptosis.

Different *M. tuberculosis* proteins or lipids have been shown to promote NLRP3 inflammasome assembly. PPE13, a Proline-Proline-Glutamate family protein, can induce assembly of the NLRP3 inflammasome complex by interacting directly with the NACHT and the LRR domain of NLRP3 (Yang et al., 2020). EST12, a pyroptosis-inducing protein, can trigger inflammasome assembly through the deubiquitination of NLRP3 (Qu et al., 2020). The lipoprotein LpqH induces the upregulation of NLRP3, ASC and Caspase-1 gene expression in a TLR2-dependent manner, and triggers NLRP3 assembly by promoting potassium efflux from the cell (Liu et al., 2021). The ESX-1 secretion system also seems to play a primordial role in inducing the assembly of the NLRP3 inflammasome. Indeed, several studies performed in human and mouse macrophages have shown that this secretion system is required for NLRP3 inflammasome assembly (Abdallah et al., 2011; Basu et al., 2018; Carlsson et al., 2010; Kurenuma et al., 2009; Mishra, Moura-Alves, et al., 2010; Wassermann et al., 2015). Additionally, the mycobacterial cell wall lipid trehalose dimycolate (TDM) can also induce NLRP3 assembly and release of IL-1β from infected cells (Schweneker et al., 2013). It is however important to note that the NLRP3 inflammasome acts as a cell stress sensor, and bacterial activation of NLRP3 inflammasome might not be mediated by a direct interaction between a bacterial molecule and NLRP3.

On the other hand, *M. tuberculosis* has also developed mechanisms to inhibit inflammasome activation. For instance, the *M. tuberculosis* serine threonine kinase PknF can inhibit NLRP3 inflammasome formation and IL-1β secretion from human and murine macrophages (Rastogi et al., 2021). Additionally, the mycobacterial serine hydrolase Hip1 has also been suggested to inhibit NLRP3 inflammasome activation by dampening TLR2 signaling in macrophages (Madan-Lala et al., 2011; Rastogi & Briken, 2022). Finally, it has been shown that the *M. tuberculosis* inhibits AIM2 inflammasome activation in an ESX-1 dependent manner. Indeed, AIM2 is activated in macrophages infected with non-virulent mycobacteria and can even recognize and bind *M. tuberculosis* DNA, but AIM2 is not activated in murine macrophages infected with *M. tuberculosis* (Saiga et al., 2012; Shah et al., 2013). It is thus hypothesized that a potential AIM2 inhibitor could be released into the cytosol in an ESX-1-dependent manner along with *M. tuberculosis* DNA. However, further research needs to be conducted to identify this potential inhibitor and assess the importance of AIM2 inflammasome evasion for *M. tuberculosis* virulence.

1.2.3 Cellular mechanisms associated with defense against *Mycobacterium tuberculosis*

Upon recognition of *M. tuberculosis* by the cells of the innate immune system, a variety of cellular events are triggered to control and kill the pathogen. Amongst these events are the release of pro- and anti-inflammatory cytokines, the phagocytosis of the pathogenic bacteria, the initiation of autophagy, and the production of reactive oxygen and nitrogen species.

Cytokine secretion

Cytokines released by infected immune cells are crucial to regulate the inflammatory process and act in a complex network of interactions. They can have local effects, such as increasing vascular permeabilization and recruiting immune cells as well as systemic effects, such as inducing fever or promoting tissue homeostasis (Etna et al., 2014). Several cytokines are important in tuberculosis pathophysiology:

- **TNFα** is produced by macrophages and is implicated in immune cell proliferation, recruitment of lymphocytes and monocytes to the site of infection and macrophage activation (Etna et al., 2014). In tuberculosis patients, TNFα seems to be a double-edged sword. Indeed, systemic TNFα production is linked to fever and wasting (Bekker et al., 1998). However, blocking TNFα with neutralizing antibodies has been linked to reactivation of latent tuberculosis (Keane et al., 2001). TNFα also plays a crucial role in granuloma formation, as blocking it with neutralizing antibodies inhibits early lesion formation in an in vitro model of infection (Alves da Silva et al., 2018). Finally, TNFα receptor knockout mice infected with *M. tuberculosis* die earlier and exhibit higher bacterial burdens than wild-type mice (Flynn et al., 1995).
- **IFNγ** is a cytokine secreted mainly by natural killer cells and T cells during an *M. tuberculosis* infection, but it can also be secreted in smaller quantities by infected macrophages (Etna et al., 2014). In humans, release of IFNγ by T cells upon exposure to *M. tuberculosis* antigens is commonly used to diagnose tuberculosis (Lalvani & Pareek, 2010). IFNγ secretion by T cells and NK cells increases antigen presentation in macrophages and results in the upregulation of proteins important to fight against infections, such as inducible nitric oxide synthase (iNOS) and IRGM-1 (Herbst et al., 2011). It is a crucial cytokine for protection against the disease, and IFNγ knockout mice are more susceptible to the disease than WT mice (Flynn et al., 1993).

- **IL-1β** is mainly produced by macrophages and monocytes during M. tuberculosis infection and is implicated in the recruitment of lymphocytes to the site of infection. It can also have systemic effects, such as the induction of fever and prostaglandin secretion (Silvério et al., 2021). *M. tuberculosis* induces IL-1β secretion from both human and murine macrophages through activation of the NLRP3 inflammasome (reviewed in Rastogi & Briken, 2022). IL-1β sensing is mediated through the IL-1 receptor and is dependent on the presence of MyD88, an adaptor protein also involved in TLR signaling (Fremond, Togbe, et al., 2007). In vitro, IL-1β has been shown to participate in defense against *M. tuberculosis* by inducing autophagy in infected macrophages (Pilli, Arko-Mensah, et al., 2012). IL-1β also seems play a protective role *in vivo*, as mice lacking the receptor to IL-1β and mice unable to secrete IL-1β and Il-1α show a high susceptibility to *M. tuberculosis* with a high mortality and an elevated bacterial burden (Fremond, Togbe, et al., 2007; Juffermans et al., 2000; Mayer-Barber et al., 2010; Yamada et al., 2000). Interestingly, there seems to be a certain level of redundancy between IL-1β and Il-1a, as the presence of either one of these cytokines allows better control *M. tuberculosis* infection in mice in comparison to double knock-out mice (Bourigault et al., 2013). In humans, genetic variants in the IL-1β gene have been linked to an increased susceptibility to *M. tuberculosis* infection (Kusuhara et al., 2007; J. Wang et al., 2018). Furthermore, blocking IL-1β signaling with IL-1 receptor antagonists has been associated with an elevated risk of developing active tuberculosis in humans, supporting a protective role for IL-1β (He et al., 2013). On the other hand, there is also evidence pointing towards IL-1β having a deleterious role in tuberculosis infections, and increased levels of IL-1β in the BAL have been linked with tissue necrosis and cavity formation in tuberculosis patients (Tsao et al., 2000).
- **IL-12** is a heterodimeric cytokine that is composed of two subunits and is secreted by macrophages and dendritic cells upon infection with *M. tuberculosis* (Ladel, Szalay, et al., 1997). IL-12 participates in polarizing naïve T cells to a Th1 phenotype that can migrate to the site of infection and that secrete IFNγ (Cooper et al., 2007). IL-12 is important for protection against *M. tuberculosis* as infected IL-12 KO mice die earlier and with higher bacterial loads than WT mice (Cooper et al., 1997). Furthermore, administrating IL-12 to BALB/c mice increased their resistance to infection with *M. tuberculosis* (Flynn et al., 1995). In humans, genetic mutations resulting in defects in IL-12 signaling confers high susceptibility to tuberculosis and infection with other mycobacteria (Altare et al., 1998).
- **IL-6** is a cytokine that has both pro- and anti-inflammatory properties. It has been reported to promote tissue homeostasis, activate T cells, induce the differentiation of B cells to plasma cells, recruit monocytes to the site of infection and induce fever (Etna et al., 2014). In tuberculosis, IL-6 seems to have a primarily beneficial role as

IL-6 KO mice show increased susceptibility to *M. tuberculosis* with increased bacterial load and mortality (Ladel et al., 1997). However, IL-6 secretion can also have some harmful effects since it can inhibit the secretion of pro-inflammatory cytokines such as TNFα, IL-1β and IFNγ (Schindler et al., 1990; VanHeyningen et al., 1997).

- **GM-CSF** is a glycoprotein that acts as a cytokine and is secreted by macrophages, T cells, NK cells and fibroblasts (Mishra et al., 2020). It is implicated in the activation of macrophages and the recruitment of T cells to the site of infection. GM-CSF KO mice control infection by *M. tuberculosis* less well than WT mice and are deficient in the secretion of several cytokines and chemokines, such as TNFα and RANTES (Gonzalez-Juarrero et al., 2005). Heterogeneity in GM-CSF signaling in macrophages has also been linked to differential control of *M. tuberculosis* growth (Bryson et al., 2019).
- **The type I interferon family** is a multigene cytokine family that encodes for several IFN-α subtypes and for IFN-β. Type I interferons signal through STAT1 and STAT2 and activate many different interferon-stimulated genes (Lúcia Moreira-Teixeira et al., 2018). These genes have been shown to have both host-protective and detrimental effects in the context of tuberculosis. Both human and murine cells infected with *M. tuberculosis* can secrete type I IFNs, and this effects seems to be dependent on the ESX-1 secretion system (Manzanillo et al., 2012; Pandey et al., 2009; Wassermann et al., 2015). There are many studies that point towards a deleterious effect of type I interferons in tuberculosis (reviewed in Moreira-Teixeira et al., 2018). High levels of type I IFNs in the blood are associated with increased susceptibility to tuberculosis in humans, mice, and non-human primates (Berry et al., 2010; Domaszewska et al., 2017; Gideon et al., 2016). Furthermore, more virulent *M. tuberculosis* strains are associated with a higher production of type I IFNs in murine models of infection (Carmona et al., 2013; Manca et al., 2001). Several studies have also shown that mice deficient in the receptor for type I interferons (IFNAR) show reduced bacterial loads and improved survival, once again suggesting a detrimental role for type I interferons in tuberculosis (reviewed in Moreira-Teixeira et al., 2018). Interestingly, there is also evidence point towards a beneficial role of type I interferons under specific conditions. Clinical cases reports have suggested that co-administrating IFN-α along with the usual treatment improved symptoms in patients with active tuberculosis (Giosuè et al., 1998; Mansoori et al., 2002). It is thus likely that a balanced production of type I interferons is required for optimal protection against *M. tuberculosis*.
- **IL-10** is one of the two main anti-inflammatory cytokines involved in tuberculosis disease pathophysiology. It is a cytokine secreted by macrophages, T and B cells that can suppress macrophage activation and secretion of pro-inflammatory

cytokines such as TNFα, IL-12, IL-6 and GM-CSF (Etna et al., 2014). In mice, IL-10 negatively influences disease progression, and IL-10 KO mice show a decreased bacterial burden in comparison to WT mice (Redford et al., 2010). Furthermore, it seems that IL-10 specifically produced by T cells and not by macrophages has a deleterious role in disease progression in murine models of infection (Moreira-Teixeira et al., 2017). IL-10 also seems to prevent phagosome maturation in infected macrophages and impair immune response in early granulomas (O'Leary et al., 2011; E. A. Wong et al., 2020).

- **TGF-β** is the other main anti-inflammatory cytokine that is secreted during a tuberculosis infection. It secreted mainly by macrophages and has anti-inflammatory effects on B cells, T cells and macrophages (Etna et al., 2014). In patients infected with *M. tuberculosis*, circulating blood monocytes were found to express higher levels of TGF- β than those from uninfected patients (Toossi et al., 1995). TGF-β was also found to be expressed in granulomas from the same infected patients (Toossi et al., 1995). In guinea pigs, daily injections of recombinant TGF-β lead to increase bacterial loads and decreased proliferation of lymphocytes as well as secretion TNFα and IFNγ (Dai & McMurray, 1999). Conversely, blocking TGF-β with neutralizing antibodies lead to opposite effects (Allen et al., 2004).

Phagocytosis

M. tuberculosis can be phagocytosed by macrophages through interaction with a wide range of host-cell receptors. For instance, the bacteria can be taken up after opsonization with complement factor C3 followed by uptake by complement receptors C1, C2 or C4 (Hirsch et al., 1994), or they can be recognized and phagocytosed through the macrophage mannose receptor and through the scavenger receptor MARCO that both sense lipoarabinomannan on the surface of the bacteria (Sia & Rengarajan, 2019). After phagocytosis, the bacteria are engulfed in phagosomes that can be acidified and fuse with lysosomes (Westman & Grinstein, 2021). This exposes the bacteria to different stressors, such as a low pH, reactive nitrogen and oxygen species, proteases, and anti-microbial peptides. *M. tuberculosis* is however able to circumvent this process by preventing phagosome-lysosome fusion and escaping from the phagosome to the cytosol (Welin & Lerm, 2012). Several *M. tuberculosis* lipids have been shown to be able to inhibit phagosome maturation. Phosphatidylinositol mannosides (PIMs) can for instance inhibit phagosome acidification by promoting the fusion between phagosomes and early endosomes (Kyei et al., 2006; Vergne et al., 2004). ManLAM can also limit phagosome maturation by interacting with the mannose receptor (Kang et al., 2005). Several *M. tuberculosis* proteins are also linked to the inhibition of phagosome maturation, such as the

serine/threonine kinase PknG, the secreted phosphatase PtpA, and the PPE family protein PPE57 (Walburger et al., 2004; D. Wong et al., 2011; Xu et al., 2015). In addition to inhibiting phagosome maturation and acidification, *M. tuberculosis* can also escape from the phagosome into the cytosol, an environment more permissive to its growth. The ESX-1 secretion study has been demonstrated in many different studies to be essential for this process (Houben et al., 2012; Simeone et al., 2012; van der Wel et al., 2007; K. W. Wong & Jacobs, 2011). The mycobacterial lipid phthiocerol dimycocerosate (PDIM) is also required for bacterial escape from the phagosome and seems to act in conjunction with the ESX-1 secretion system (Augenstreich et al., 2017; Lerner et al., 2017; Quigley et al., 2017). Once the bacteria are inside the cytosol, macrophages can detect them and resort to other defense mechanisms, such as autophagy, which targets cytosol content to lysosomes for destruction.

Autophagy

Autophagy is a process that involves breaking down and removing cellular debris from the cytosol, and is also involved in killing intracellular pathogens (Deretic & Wang, 2023; Strong & Lee, 2021) **(Figure 5).** This mechanism has been shown to be crucial for the control of tuberculosis as mice lacking genes necessary for autophagy are more susceptible to *M. tuberculosis* than WT mice (Castillo et al., 2012; Watson et al., 2012). Furthermore, inducing autophagy in murine macrophages infected with *M. tuberculosis* increases phagosome maturation and suppresses intracellular bacterial survival (Gutierrez et al., 2004; Ponpuak et al., 2010). In humans, there is also evidence that autophagy is an important contributor to the immune response against *M. tuberculosis*. Indeed, genetic polymorphisms in several autophagy-related genes have been associated with changes in the susceptibility to tuberculosis (Cheng et al., 2019; Intemann et al., 2009; King et al., 2011; Songane et al., 2012). Additionally, autophagy was found to be elevated in PBMCs from patients with active tuberculosis, with the level of autophagy correlating with the concentration of IFNγ released by the same PBMCs in response to *M. tuberculosis* antigens (Rovetta et al., 2014). Beclin-1, another marker for autophagy, is also increased in alveolar macrophages from patients ith active tuberculosis (Yu et al., 2016). Recently, a study performed with iPSCs-derived macrophages highlighted the role of ATG7 and ATG14, two autophagy-related proteins, in the control of intracellular *M. tuberculosis* growth in human cells (Aylan et al., 2023). Interestingly, autophagy also seems to play a role in mediating the antimycobacterial effect of the drugs isoniazid and pyrazinamide. Indeed, treatment of *M. tuberculosis*–infected macrophages with these drugs causes an increase in autophagy, which is crucial for effective defense against the intracellular bacteria (Kim et al., 2012).

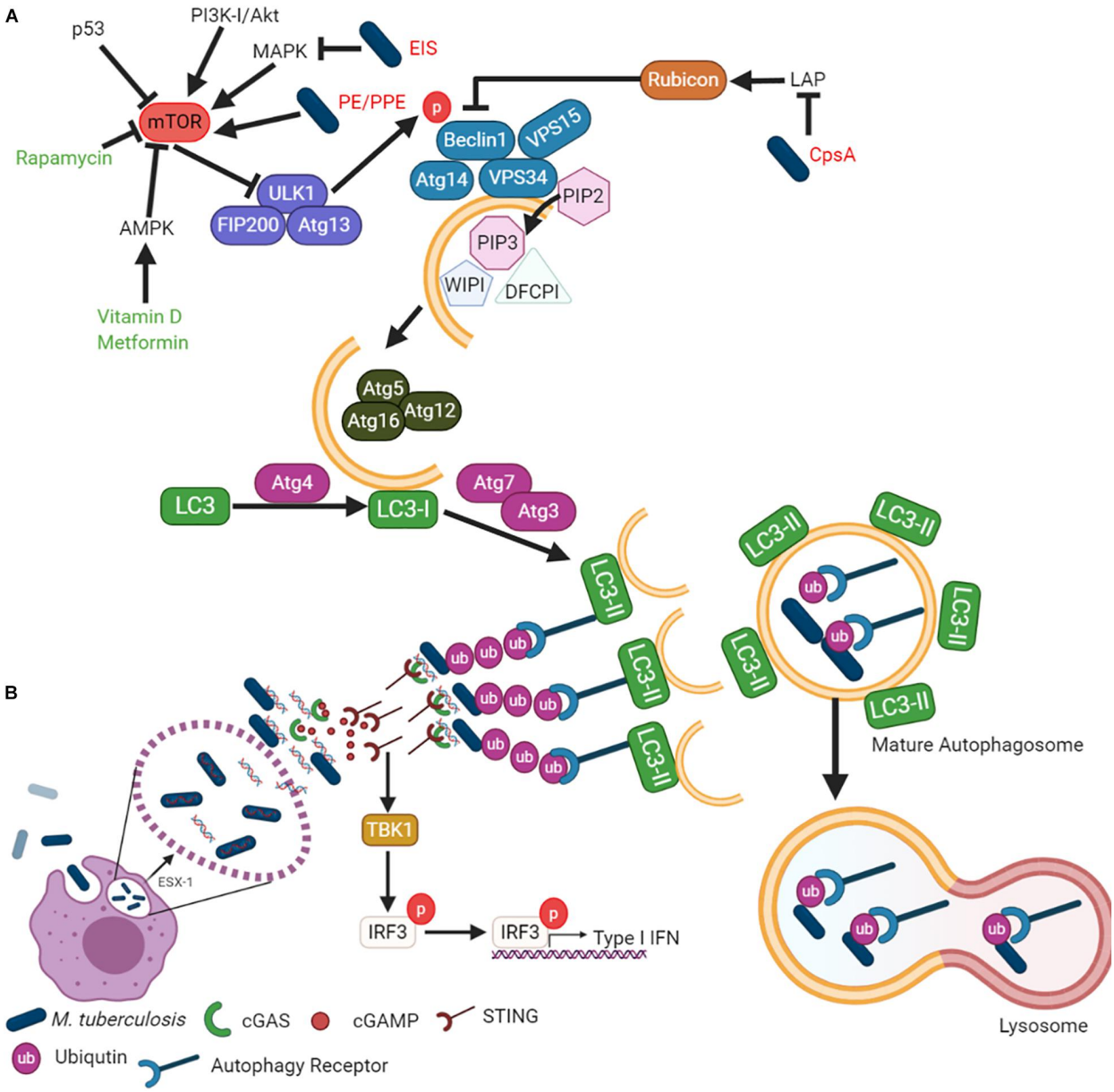


Figure 5: Autophagy in *M. tuberculosis* infections
Autophagy begins with the activation of the ULK1 complex, which is regulated by mTOR. The Beclin-1 complex then generates phosphatidylinositol 3-phosphate (PI3P), which is allows the recruitment of other autophagy-related proteins to the site of autophagosome formation. The ATG proteins, including ATG5, ATG12, and ATG16L1, subsequently form complex, which assists in the formation of the autophagosome. LC3, a protein associated with autophagosomal membranes, exists in two forms: LC3-I and LC3-II. LC3-I is cytosolic, while LC3-II is lipidated and incorporated into the autophagosomal membrane. LC3-II serves as a marker for autophagosome formation and is involved in cargo recognition and autophagosome-lysosome fusion. Autophagy receptors, recognize specific cargo marked for degradation and link them to LC3-II on the autophagosomal membrane. M. tuberculosis is targeted for degradation through its ubiquitination. Autophagosomes, then undergo fusion with lysosomes to form autolysosomes. This fusion requires Rab proteins, such as Rab7, which help in the maturation of autophagosomes into autolysosomes by facilitating the fusion process. Figure adapted from Strong & Lee (2021)

Production of reactive nitrogen species

In response to pro-inflammatory cytokines, macrophages can express defense mechanisms such as inducible nitric oxide synthase. Nitric oxide synthases (NOS) are enzymes that catalyze the production of nitric oxide, a molecule implicated in cellular signaling and host defense against bacteria, viruses, and parasites (Cinelli et al., 2020). Three main nitric oxide synthases exist in mammals: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). The first two are involved in tissue homeostasis while the latter plays an important role in the innate immune response (Cinelli et al., 2020). iNOS is expressed in different cells of the immune system such as macrophages and neutrophils. As its name suggests, iNOS has a low baseline expression and can be induced through signaling events implicating toll-like receptors and the NF-kB transcription factor or the IFNγ pathway (Figure 6). Upon induction, iNOS will convert l-arginine to l-citruline, producing nitric oxide in the process. Since this usually occurs in an oxidative environment, the nitric oxide produced can form reactive nitrogen species, which are toxic to many bacteria, including *M. tuberculosis* (Firmani & Riley, 2002). Furthermore, since nitric oxide is a highly diffusible molecule, it can also act as a signaling molecule and has been shown to impact macrophage transcriptome during an *M. tuberculosis* infection and have immunomodulatory properties (Braverman & Stanley, 2017; Mishra et al., 2013).

Reactive nitrogen species and iNOS play a crucial role in host defense against *M. tuberculosis* in murine models of infection. iNOS knockout mice or mice treated with iNOS inhibitors infected with *M. tuberculosis* fail to control bacterial growth and have a higher mortality rate than wild-type mice (Chan et al., 1995; MacMicking et al., 1997). Studies have highlighted the role of macrophages in this process by showing that *M. tuberculosis* and IFNγ have a synergistic effect on iNOS expression in primary murine macrophages, and that the reactive nitrogen species produced by these cells are toxic to *M. tuberculosis* (Chan et al., 1992; Ehrt et al., 2001). Furthermore, macrophages expressing low and high levels of iNOS co-exist in the lungs of infected mice, and bacteria in high iNOS-expressing macrophages exhibit higher levels of stress than those in low iNOS-expressing cells (Pisu et al., 2021). In humans, iNOS also seems to act as a defense mechanism against *M. tuberculosis*, even though the evidence is not as clear as for murine models of infection. Immunological stainings of resected lung sections from infected patients show increased iNOS expression in the inflammatory zone surrounding granulomas (Choi et al., 2002; Schön et al., 2004; Ufimtseva et al., 2021). Furthermore, macrophages extracted from the lungs of infected patients also show increased expression of iNOS in comparison to those from healthy patients (Cho et al., 2020; Nicholson et al., 1996; Wang & Kuo, 2001). Interestingly, blocking nitric oxide production in alveolar macrophages extracted from

the lungs of infected patients resulted in decreased production of IL-1β and TNFα, suggesting that nitric oxide may act as an immunomodulatory signaling molecule in humans as well (Wang & Kuo, 2001). Multiple studies using either human macrophage cell lines or primary macrophages have also shown upregulation of iNOS by *M. tuberculosis* in vitro, but it is not always clear if the resulting concentration of nitric oxide secreted is high enough to be toxic to *M. tuberculosis* (Jagannath et al., 1998; Jung et al., 2013; Landes et al., 2015).

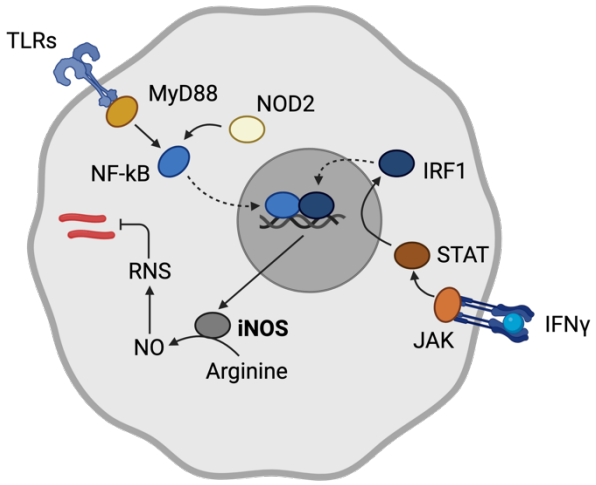


Figure 6: Regulation of inducible nitric oxide synthase (iNOS) expression
iNOS expression is regulated through two main pathways. Its expression can be induced by binding of molecules such as LPS to Toll-like receptors (TLRs), which causes NF-κB to translocate to the nucleus, where it binds to the iNOS promoter and induces its expression. iNOS can also be induced by binding of IFNγ to its receptors (IFR1/2). In the case of an M. tuberculosis infection, it is the IFNγ pathway that is most relevant.

Reactive nitrogen species are toxic to mycobacteria by interacting with many different molecules, such as nucleic acids, lipids, carbohydrates and proteins (Ehrt & Schnappinger, 2009). *M. tuberculosis* has evolved mechanisms both to detoxify reactive nitrogen species and to repair the damage they cause. For instance, the co-enzyme F_{420} has been suggested to protect *M. tuberculosis* from damage by converting NO_2 back to nitric oxide, which is less toxic to the bacteria (Purwantini & Mukhopadhyay, 2009). *M. tuberculosis* also expresses an NADH-dependent peroxidase and peroxynitrite reductase made of four proteins. Bacterial mutants in one of these proteins, dlaT, show increased susceptibility to reactive nitrogen species *in vitro* and was attenuated in wild-type macrophages (Shi & Ehrt, 2006). When exposed to reactive nitrogen species, methionine residues in bacterial proteins can be converted to methionine sulfoxide, which interferes with the function of proteins. To counteract this, *M. tuberculosis* expresses two methionine sulfoxide reductases,

msrA and msrB that can convert methionine sulfoxide back to methionine (Lee et al., 2009). Furthermore, several genes associated with the proteasome and protein degradation are also thought to be important in resisting stress induced by reactive nitrogen species (Darwin et al., 2003). Interestingly, *M. bovis* and *M. tuberculosis* can also inhibit colocalization of iNOS with the phagosome (Miller et al., 2004). Finally, a more recent study has shown that PPE2, an *M. tuberculosis* protein containing proline and glutamate residues and part of the PPE protein family, can bind to the iNOS promoter and inhibit its expression (Bhat et al., 2017).

Production of reactive oxygen species

The production of reactive oxygen species (ROS) is another mechanism used by macrophages against intracellular pathogens. ROS are produced after phagocytosis of mycobacteria via the enzymatic activity of the phagocyte NADPH oxidase (NOX2) (Awuh & Flo, 2017). Similarly to the production of RNS, the production of ROS is increased in macrophages activated with IFNγ. In humans, polymorphisms in the catalytic subunit of the phagocyte oxidase are associated with increased susceptibility to *M. tuberculosis* infection (Liu et al., 2015). In murine models of infection however, mice deficient in subunits of the phagocyte oxidase show only a slight increase in their susceptibility to *M. tuberculosis*, suggesting that the bacteria have evolved mechanisms to resist to ROS-induced stress (Adams et al., 1997; Cooper et al., 2000; Thomas & Olive, 2023). Once such mechanism is the degradation of ROS to water and oxygen by the sequential action of a superoxide dismutase and a catalase. *M. tuberculosis* possess two superoxide dismutases (SODA and SODC) and one catalase-peroxidase-peroxynitritase (KatG), all of which have been shown to be important for *M. tuberculosis* virulence (Dussurget et al., 2001; Ng et al., 2004; Piddington et al., 2001). Furthermore, *M. tuberculosis* can produce anti-oxidant molecules, such as mycothiol, which helps mitigate ROS-related stress (Buchmeier & Fahey, 2006). Finally, *M. tuberculosis* can also directly inhibit the production of ROS via the secreted EIS protein (Shin, Jeon, et al., 2010).

Interferon-γ-inducible regulatory immunity-related GTPase (IRMG1)

The Interferon-γ-inducible regulatory immunity-related GTPase (IRMG1), also known as LRG47, is an IFNγ-inducible macrophage protein important for defense against intracellular pathogens such as *M. tuberculosis*. It has been shown to protect against *M. tuberculosis* infections in murine models of infection independently from iNOS (MacMicking, 2003). IRGM1 seems to play a role in promoting phagosome maturation and autophagy in both human and murine cells infected with *M. tuberculosis* (Singh, 2006). Furthermore, IRGM1 has been shown to mediate adhesion and motility of IFNγ-activated macrophages (Henry, 2007). When expressed in CD4+

T cells, IRGM1 promotes expansion and protects against IFNγ-induced cell death (Feng, 2008). In humans, IRGM is not induced by IFNγ but is constitutively expressed, suggesting that its role in the control of Mycobacterial infections might be slightly different than in mice (Singh, 2006). Several studies have however showed that genetic polymorphisms in IRGM are associated with susceptibility to tuberculosis, likely through modulation of autophagy in infected phagocytes (Intemann et al., 2009; King et al., 2011).

1.2.4 *Mycobacterium tuberculosis* fights back – mycobacterial lipids and the ESX-1 secretion system

M. tuberculosis has evolved different mechanisms to survive inside cells of the immune system and can persist inside its host for many years. It grows extremely slowly, with doubling times of around 20 hours, and has a thick, waxy cell wall that consists mainly of peptidoglycan, arabinogalactan, mycolic acids, lipoarabinomannan and free lipids surrounded by a capsule (Abrahams & Besra, 2021). Several lipids present in the cell wall, such as lipoarabinomannan (LAM), trehalose dimycolate (TDM) and phthiocerol dimycocerosates (PDIM) act as important virulence factors (Guenin-Macé et al., 2009; Ly & Liu, 2020). LAM is a glycolipid that is present in the cell wall of all mycobacterial species (Correia-Neves et al., 2019). It can interact with host cell membranes, inhibit phagosomal maturation and induce a pro-inflammatory response in infected and bystander macrophages (Turner & Torrelles, 2018; Welin et al., 2008; Wojtas et al., 2011). TDM, also known as mycobacterial cord factor, is the most abundant glycolipid in the mycobacterial cell wall. It is recognized by the Mincle receptor on macrophages, has strong immunomodulatory properties and can inhibit phagosome maturation (Huber et al., 2020; Nguyen et al., 2020; Patin et al., 2017). PDIM is present in the cell wall of all pathogenic mycobacteria but absent in non-pathogenic mycobacteria. *M. tuberculosis* mutants lacking PDIM are attenuated in mouse and guinea pig models of infection (Camacho et al., 1999; Cox et al., 1999; Goren et al., 1974). PDIM is able to spread into host membranes and act together with other bacteria virulence factors, such as ESX-1, to disrupt phagosomal membranes (Augenstreich et al., 2020; Cambier et al., 2020; Osman et al., 2020; Rens et al., 2021). It has also been suggested to play a role in masking PAMPs and avoiding bacterial detection by toll-like receptors on immune cells (Cambier, Takaki, et al., 2014).

Another important virulence factor for *M. tuberculosis* is the type VII secretion system ESX-1, which allows secretion of small molecules across the mycobacterial cell wall. The esx-1 locus contains genes encoding for the secreted effector proteins EsxA and EsxB (also known as ESAT-6 and CFP-10), ecc genes involved in forming the core ESX complex and esp genes such as espA, espB, espC, espD, and espK that also encode for ESX-secreted proteins and are necessary for virulence (Gröschel et al., 2016). This locus is absent from the vaccine strain *M. bovis* BCG and its deletion in *M. tuberculosis* leads to decreased pathogenicity and bacterial burden in mice (Lewis et al., 2003). EsxA and EsxB are co-secreted as a heterodimer and are both potent T-cells antigens (Renshaw et al., 2002). EsxA is implicated in inhibiting phagosome-lysosome fusion and disrupting phagosomal membranes to allow access of the bacteria to the cytosol (Houben et al., 2012; Simeone et al., 2012). EsxA and

EsxB have also been linked to inhibition of MyD88-dependent TLR signaling and NF-kB (Pathak et al., 2007), inhibition of autophagy (Romagnoli et al., 2012), and induction of apoptosis in macrophages (Aguilo et al., 2013). Of the other ESX-1 proteins, **EspA** is required for secretion of EsxA and EsxB and *M. tuberculosis* virulence (Chen et al., 2013). In addition, mutants lacking EspA are hypersusceptible to detergents, suggesting that it could be implicated in maintaining cell surface integrity (Garces et al., 2010). **EspB** is expressed as a 60 kDa protein but is cleaved into a mature 50 kDa isoform by the protease MycP1 during translocation across the cell wall (Ohol et al., 2010). It is necessary for virulence and has been shown to induce host-cell death and inhibit autophagy (Chen et al., 2013; Huang & Bao, 2016; Toniolo et al., 2023). **EspC** is important for *M. tuberculosis* survival *in vivo* and has been shown to induce ER-related stress and apoptosis in infected cells (Guo et al., 2021). **EspD** is secreted by *M. tuberculosis,* but interestingly, its secretion does not depend exclusively on the ESX-1 secretion system. Similarly to EspA, EspD expression is required for secretion of EsxA (Chen et al., 2012). Interestingly, not all of the Esp proteins are secreted, and several of these proteins, such as **EspK** and **EspL** have been suggested to act as chaperones to promote proper folding of other Esp proteins (Gijsbers et al., 2023; Lim et al., 2022; Sala et al., 2018).

1.3 Phenotypic heterogeneity in *Mycobacterium tuberculosis* infections

Tuberculosis disease progression is extremely variable with outcomes ranging from sterilization of the infection to progression to active tuberculosis (Behr et al., 2019; Cadena et al., 2017) **(Figure 7)**. This heterogeneity is often attributed to diversity in granuloma evolution at later stages of the disease (Lin et al., 2014; Marakalala et al., 2016; Martin et al., 2017). However, some individuals highly exposed to *M. tuberculosis* never display any symptoms nor develop granulomatous lesions, suggesting that early-stage sterilization of the bacteria is also possible (Houk et al., 1968; Simmons et al., 2018). In these cases, whether the infection is sterilized or not may be linked to heterogeneity in the host cells first encountered by the bacteria (as discussed in Toniolo, Rutschmann et al., 2021). In the following paragraphs, I will discuss how heterogeneity in the environments *M. tuberculosis* is exposed during an infection can influence disease outcome and highlight why it is important to study the interactions between *M. tuberculosis* and host cells at the single-cell level **(Figure 8)**. Even though *M. tuberculosis* can be found in different organs, I will only discuss heterogeneity in pulmonary tuberculosis as it is the focus of this thesis. Most of these ideas were summarized in a review I wrote in collaboration with Dr. Chiara Toniolo published in Current Opinion in Microbiology (Toniolo, Rutschmann et al., 2021). The following paragraphs are an adaptation of this review.

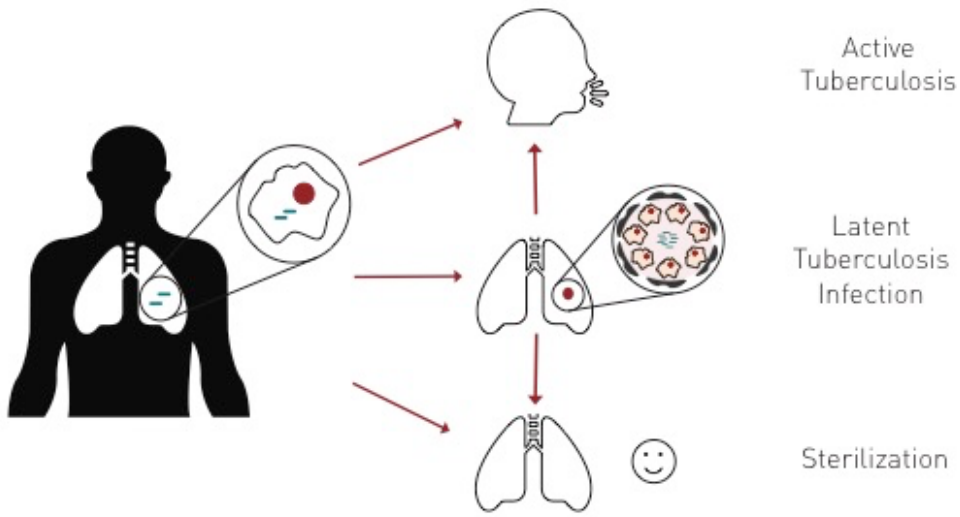


Figure 7: Tuberculosis disease pathophysiology
The outcome of an *M. tuberculosis* infection is very variable. Some individuals sterilize the infection, some contain the bacteria inside granulomas for years and others quickly fall ill and develop symptoms.
An edited version of this figure has been published in Toniolo, Rutschmann et al.,, 2021.

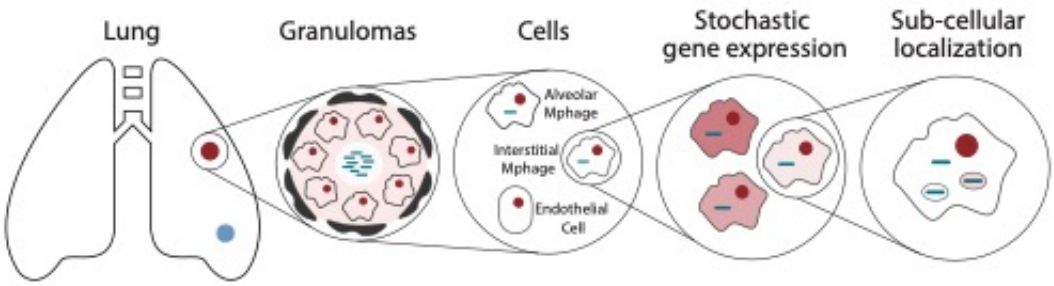


Figure 8: *M. tuberculosis* is exposed to diverse host environments
An *M. tuberculosis* infection initiates an immune response leading to the formation of primary granulomas, which can occasionally achieve bacterial sterilization (depicted in blue). With disease progression, *M. tuberculosis* can escape these granulomas and enter the vascular system, allowing reseeding in the lungs and containment within secondary granulomas (illustrated in red). Notably, both primary and secondary granulomas may exhibit solid or caseating centers, with the latter associated with more extensive disease progression and bacterial dissemination. Heterogeneity is not limited to differences between granulomas but also extends to the cellular level within a single granuloma. *M. tuberculosis* can infect various cell types, with some, such as epithelial cells, more supportive to bacterial growth than others, such as interstitial macrophages. Differential gene expression within the host-cell population further contributes to the diverse microenvironments *M. tuberculosis* encounters, affecting bacterial dissemination. Moreover, even within a single host cell, *M. tuberculosis* faces varying stressors within different subcellular compartments, adding to the complexity of the infection process.
An edited version of this figure has been published in Toniolo, Rutschmann et al., 2021).

1.3.1 Heterogeneity in granulomas

Granulomas are made of several different cell types, such as macrophages, dendritic cells, fibroblasts, neutrophils, natural killer cells and lymphocytes. Individual granulomas are each initiated by a single bacterium, and can progress independently from others in the lungs (Martin et al., 2017). Primary granulomas found in the lower lungs often co-exist with secondary granulomas in the upper lungs originating from bacteria re-seeded from the lymphatic or vascular systems (Converse et al., 1996). For both types of granuloma, evolution is heterogeneous over time, with outcomes ranging from sterilization of the bacteria to cavitation of the lesion and further propagation of the pathogen (Lin et al., 2014; Martin et al., 2017; Subbian et al., 2015).

Interestingly, there is significant heterogeneity in the cell composition of granulomas even within a single host. Lesions extracted from the lungs of infected individual can exhibit different levels of immune cell infiltration and inflammatory markers (Abengozar-Muela et al., 2020; Gideon et al., 2022). Thus, bacteria residing in different granulomas can be exposed to very different immune environments within a single host. There is also significant heterogeneity in the environment *M. tuberculosis* is exposed to within single lesions, as cell types and protein expression vary in different regions of individual granulomas. For instance, pro-inflammatory

proteins are expressed more in the center of certain lesions, while anti-inflammatory proteins are found more in the periphery (Carow et al., 2019; Marakalala et al., 2016; Marino et al., 2015; McCaffrey et al., 2022; Seto et al., 2020).

Inter and intra-granuloma heterogeneity can also impact the efficacy of anti-tuberculosis drugs. Several studies have shown that drug penetration varies from granuloma to granuloma, and even differs depending on the area within a granuloma. For example, fluoroquinolones accumulate preferentially in regions with a high density of macrophages, while pyrazinamide and isoniazid concentrations are higher in the caseum of granulomas (Blanc et al., 2018; Prideaux et al., 2015). Furthermore, rifampicin penetration and temporal evolution has been shown to vary from lesion to lesion, even within the same patient (Ordonez et al., 2020).Thus, depending on where they are, bacteria can be differently exposed to drugs which could influence their chance of survival.

1.3.2 Heterogeneous host cells

Over the course of an infection, *M. tuberculosis* interacts with many different cell types. For instance, within a granuloma, different cells such as fibroblasts, neutrophils, macrophages, lymphocytes and dendritic cells co-exist and can all be infected by *M. tuberculosis* (Cadena et al., 2017). All of these cells have different anti-microbial properties and can control intracellular bacteria differently (Bussi & Gutierrez, 2019). Thus, heterogeneity in their distribution and their infection by *M. tuberculosis* could impact how a granuloma progresses over time, which could potentially influence disease outcome. Outside of granulomas, *M. tuberculosis* can be internalized by both immune and non-immune cells, such as endothelial or epithelial cells (Barrios-Payán et al., 2012; Lerner et al., 2020; Thacker et al., 2020). Since non-immune cells are more permissive to intracellular bacterial growth than immune cells, whether *M. tuberculosis* is internalized by them or not could also influence the outcome of the disease.

Interestingly, there can also be significant heterogeneity even within a single class of cells. Over the course of an infection, bacteria for instance interact with different types of macrophages, such as interstitial and alveolar macrophages, that have been shown to control *M. tuberculosis* growth differently (Huang et al., 2018; Pisu et al., 2020, 2021). Furthermore, there can also be differences even within a population of cells, and at least two different populations of alveolar macrophages have been for instance shown to co-exist in the lungs of infected mice (Lafuse et al., 2019). Interestingly, these two subpopulations not only express different levels of pro- and anti-inflammatory cytokines, but also differ in the number of bacteria they internalize, suggesting differences in their phagocytic and anti-microbial capacities.

Recent studies further showed that in the lung of infected mice, both alveolar and interstitial macrophages can be segregated into multiple sub-categories expressing different levels of immune-related genes and exerting different levels of stress on internalized bacteria (Pisu et al., 2020, 2021). Thus, the characteristics of the host cells that interact with *M. tuberculosis* could potentially influence disease progression.

1.3.3 Heterogeneous intracellular localization

Intracellular bacteria can also be exposed to diverse environments within a single host cell. Already in 1971, Armstrong et al observed that *M. tuberculosis* could be found within permissive phagosomes or less permissive phagolysosomes inside macrophages (Armstrong & D'Arcy Hart, 1971). More recently, fluorescence microscopy in combination with *M. tuberculosis* reporter strains for stress response and bacterial growth revealed that bacteria sensing different stresses, and presumably localized in different sub-cellular compartments, can co-exist within a single infected cell (MacGilvary & Tan, 2018). Bacteria with a more oxidized signature were for instance preferentially found in autophagosomes, while endosomes were enriched for bacteria with a reduced signature (Bhaskar et al., 2014). This could be particularly relevant for the treatment of tuberculosis, as bacteria with a more oxidized or reduced signature have a different sensitivity to antibiotics. Differences in antibiotic efficacy could also depend on differences in local drug accumulation. Bedaquiline, for instance, accumulates in cell lipid droplets and thus is more effective against bacteria localized in proximity to these droplets (Greenwood et al., 2019).

Interestingly, subcellular localization of *M. tuberculosis* is not only heterogeneous in space, but also changes over time (Sachdeva et al., 2020; Schnettger et al., 2017). Indeed, phagosomes are dynamic and can fuse with other sub-cellular compartments, such as lysosomes. Furthermore, *M. tuberculosis* has evolved mechanisms, such as the ESX-1 secretion system, to halt the fusion of phagosomes to lysosomes, to damage the membranes of these subcellular compartments, and to escape into the cytosol, an environment more permissive to bacterial growth (Lienard et al., 2020; Simeone et al., 2012). Thus, the subcellular localization of *M. tuberculosis* likely depends on a balance between host defense mechanisms and bacterial virulence factors.

1.3.4 Phenotypic heterogeneity of host cells and bacteria

As discussed above, differences between host cells can be programmed (e.g. macrophages of different ontologies) or can come from differences in the external signals sensed by the cells. However, even cells grown under identical conditions can have different phenotypes and gene expression profiles (Gierahn et al., 2017).

Human monocyte-derived macrophages cultured in vitro can for instance be clustered into different subpopulations according to sc-RNAseq. Furthermore, when infected with *M. tuberculosis*, each subpopulation displays different shifts in gene expression (Gierahn et al., 2017). Similarly, subpopulations of human macrophages displaying different antibacterial activity can be discriminated according to their GM-CSF signaling. In this case, heterogeneity in GM-CSF signaling is not pre-existent and is triggered by *M. tuberculosis* infection (Bryson et al., 2019). In this example, it is not possible to know if the observed heterogeneity is the consequence or the cause of fast and slow bacterial growth, and to my knowledge, no study has been able to address this point in cells infected with *M. tuberculosis*. However, in studies using *Salmonella typhimurium*, stochastic differences in the expression of bacterial virulence factors were shown to drive different responses in infected macrophages (Avraham et al., 2015). It is likely that such results could also be obtained with *M. tuberculosis*, as studies performed with mutant strains show that differences in the expression of virulence factors can have an influence on the intracellular localization of the bacteria and the host response to the infection (Augenstreich et al., 2017; Ohol et al., 2010; Simeone et al., 2012).

Finally, phenotypic heterogeneity in the expression of bacterial genes could also influence the way they respond to host stresses. This phenomenon is thought to confer an evolutionary advantage and increase chances of survival in response to environmental changes. Phenotypic heterogeneity in bacteria such as *Escherichia. coli* has been known to occur for decades and can originate from noise in gene expression (Elowitz et al., 2002; Novick & Weiner, 1957). In *M. tuberculosis*, phenotypic heterogeneity in ribosomal RNA expression has been observed in vitro and is further amplified by host stresses (Manina et al., 2015). Heterogeneous expression of bacterial genes could also influence the way *M. tuberculosis* respond to antibiotics. Indeed, the expression of the catalase-peroxidase KatG has been shown to occur in stochastics bursts in *M. smegmatis* and influence the susceptibility of the bacteria to isoniazid (Wakamoto et al., 2013). Similar observations have also been made concerning the expression of recA, involved in DNA damage repair, and susceptibility of *M. smegmatis* to fluoroquinolones (Manina et al., 2019). Thus, control of the pathogen by the host is likely a dynamic and heterogeneous process that continuously changes in function of bacterial virulence, differential exposure to drugs and host defense mechanisms.

1.3.5 The importance of single-cell studies

To be able to accurately observe heterogeneity in host-pathogen interactions, single-cell techniques such as single-cell RNA sequencing or single-cell proteomics are necessary. One interesting technique that has recently been applied to the study

of host cells infected by *M. tuberculosis* is dual single-cell RNA sequencing, which allows quantifying the expression of genes expressed by both the host and the pathogen in parallel (Pisu et al., 2020). Though not yet applied to the study of *M. tuberculosis* infections, other single-cell omics techniques, such as single-cell proteomics or epigenomics could also be used to decipher heterogeneity in host-pathogen interactions. However, single-cell 'omics techniques only allow assessing gene expression and cell-to-cell heterogeneity at defined timepoints, whereas host-pathogen interactions are highly dynamic processes. On the other hand, time-lapse microscopy of single infected cells can reveal dynamic changes in the interactions between host and bacterial cells. The throughput of these experiments is however limited, as one can only follow the expression of a small number of genes over time using fluorescent reporters. Nonetheless, these dynamic studies are necessary to define causality in observed phenomena and assess the role of chance and random events in host-pathogen interactions. This is especially important when studying infections initiated by few bacteria, such as Tuberculosis, as chance encounters with "stronger" or "weaker" host cells could influence whether a bacterium grows or is killed by the host, which could impact disease progression.

1.4 Aim of the thesis

During my thesis, I studied the interactions between *M. tuberculosis* and macrophages at the single-cell level. The thesis was subdivided into two main goals:

Aim 1: Characterize phenotypic heterogeneity in the single-cell interactions between *M. tuberculosis* and macrophages.

In this project, we used a combination of time-lapse microscopy and fluorescent reporters to study heterogeneity in the interactions between macrophages and *M. tuberculosis*. We observed that infection of single macrophages with *M. tuberculosis* can lead to diverse outcomes, ranging from the death of the pathogen to its replication and subsequent killing of the host cell. By using two *M. tuberculosis* strains expressing different fluorescent proteins, we showed that bacteria inside the same macrophage behave more similarly than bacteria in different host cells, suggesting that some macrophages are more permissive to bacterial growth than others. Furthermore, by using a macrophage reporter cell line for inducible nitric oxide synthase (iNOS) expression, we showed that populations of macrophages expressing high and low levels of iNOS co-exist and that this pre-existing heterogeneity in iNOS expression is linked to the control of intracellular *M. tuberculosis*. As alveolar macrophages are considered to be the initial cells in contact with *M. tuberculosis*, studying how they interact with this pathogen is essential to understand the early stages of the infection. Pre-existing heterogeneity in these macrophages could explain part of the variability observed in the outcome of tuberculosis infections, especially since in the case of *M. tuberculosis*, an infection may initiate from a single bacterium coming in contact with a single host macrophage. Thus, the level of iNOS this macrophage expresses could influence how well it controls the initial infection and how the disease progresses.

The results of this study were published in mBio (Rutschmann et al., 2022).

Aim 2: Characterize non-canonical inflammasome activation in macrophages infected with *M. tuberculosis*.

In this project, we dissected the mechanisms leading to IL-1β secretion by macrophages infected with *M. tuberculosis*. IL-1β plays a key role in host defense against *M. tuberculosis* by promoting inflammation and recruiting immune cells to the site of infection. Its secretion however needs to be tightly regulated, as overproduction of IL-1β can lead to disease exacerbation. Previous studies have demonstrated that IL-1β secretion can be mediated by the NLRP3 and AIM2 inflammasomes upon *M. tuberculosis* infection. In this study, we investigated if an alternative pathway known as the non-canonical inflammasome and involving Caspase-11 (in mice) or Caspase-

4 and Caspase-5 (in humans) could also be involved in regulating IL-1β secretion from infected macrophages. We demonstrated that Caspase-11, Caspase-4 and Caspase-5 undergo auto-proteolysis and are activated in macrophages infected with *M. tuberculosis*. Caspase-11/-4/-5 activation results in IL-1β secretion from infected macrophages, followed by pyroptotic death of the cell. We further showed that IL-1β secreted from infected macrophages can enhance the anti-mycobacterial properties of neighboring cells in a paracrine manner. Interestingly, we observed that Caspase-11 expression is induced in myeloid cells providing from the lungs of infected mice, suggesting a role for the control of *M. tuberculosis* infection *in vivo*. Our findings thus identify a novel mechanism leading to IL-1β secretion in *M. tuberculosis*-infected cells and suggest that the non-canonical inflammasome could potentially be involved in defense against mycobacterial infections.

The results of this study are the object of a manuscript currently in preparation.

Chapter 2. Preexisting Heterogeneity of Inducible Nitric Oxide Synthase Expression Drives Differential Growth of *Mycobacterium tuberculosis* in Macrophages

Ophélie Rutschmann, Chiara Toniolo & John D. McKinney

School of Life Sciences, EPFL, Lausanne, Switzerland

Manuscript published at mBio (2022)

Abstract

Mycobacterium tuberculosis infection is initiated by the inhalation and implantation of bacteria in the lung alveoli, where they are phagocytosed by macrophages. Even a single bacterium may be sufficient to initiate infection. Thereafter, the clinical outcome is highly variable between individuals, ranging from sterilization to active disease, for reasons that are not well understood. Here, we show that the rate of intracellular bacterial growth varies markedly between individual macrophages, and this heterogeneity is driven by cell-to-cell variation of inducible nitric oxide synthase (iNOS) activity. At the single-cell level, iNOS expression fluctuates over time, independent of infection or activation with gamma interferon. We conclude that chance encounters between individual bacteria and host cells randomly expressing different levels of an antibacterial gene can determine the outcome of single-cell infections, which may explain why some exposed individuals clear the bacteria while others develop progressive disease.

Importance

In this report, we demonstrate that fluctuations in the expression of antimicrobial genes can define how single host cells control bacterial infections. We show that preexisting cell-to-cell variation in the expression of a single gene, that for inducible nitric oxide synthase, is sufficient to explain why some macrophages kill intracellular *M. tuberculosis* while others fail to control bacterial replication, possibly leading to disease progression. We introduce the concept that chance encounters between heterogeneous bacteria and host cells can determine the outcome of a host-pathogen interaction. This concept is particularly relevant for all the infectious diseases in which the number of interacting pathogens and host cells is small at some point during the infection.

Introduction

A *Mycobacterium tuberculosis* infection usually starts when airborne droplets containing one or more bacteria enter an individual's lungs and interact with alveolar macrophages (Cohen et al., 2018). From this initial encounter, outcomes range from sterilization to latent infection or active disease (Cadena et al., 2017). These heterogeneous outcomes may be linked to differences in the evolution of granulomas, the characteristic multicellular structures that form around bacteria in the lungs (Lin et al., 2014; Marakalala et al., 2016; Martin et al., 2017). However, clinical studies have shown that some individuals who are heavily exposed to *M. tuberculosis* remain tuberculin skin test negative and presumably uninfected, suggesting that it is possible for the host to suppress or clear the bacteria during the earliest stages of infection (Houk et al., 1968; Simmons et al., 2018). Thus, different infection outcomes may depend not only on granuloma evolution at later stages of the disease, but also on differences in the outcomes of interactions between *M. tuberculosis* and host cells during the initial phases of the infection.

Several findings support this hypothesis. During the first few weeks of infection, macrophages control *M. tuberculosis* growth differently due to differences in their metabolism or expression of immune-related genes, such as the gene for inducible nitric oxide synthase (iNOS) and genes downstream of NF-κB (Huang et al., 2018; Pisu et al., 2020). Heterogeneous control of intracellular *M. tuberculosis* growth is observed not only in macrophages of different lineages, but also in homogeneous populations of primary macrophages cultivated in vitro (Bryson et al., 2019). However, those previous studies did not distinguish whether cell-to-cell differences in the control of intracellular *M. tuberculosis* depended on different adaptation of infected host cells to the bacteria (Avraham et al., 2015) or on preexisting heterogeneity in the host cells (Gierahn et al., 2017). Answering this fundamental question is particularly relevant in the context of Tuberculosis because an infection can start with a single bacterium coming in contact with a single host macrophage (Saini et al., 2012). Thus, preexisting phenotypic heterogeneity among host cells, or heterogeneous cellular responses to intracellular bacteria, could influence whether an infection progresses or, conversely, the host is able to control and possibly eliminate the pathogen.

In this study, we investigated how preexisting phenotypic diversity in a population of macrophages contributed to the control of intracellular *M. tuberculosis*. To explore the behavior of individual macrophages infected with fluorescently labeled *M. tuberculosis*, we used single-cell time-lapse microscopy, a technique that has been successfully used to assess infection dynamics at the single-cell level and to

investigate the links between host cell death and bacterial growth rate (Lerner et al., 2017; Mahamed et al., 2017). We observed that bacteria within the same macrophage displayed more similar growth rates than bacteria in different macrophages, suggesting that some host cells are better than others at controlling *M. tuberculosis* infection. Using fluorescent reporter macrophages, we found that iNOS expression varied between individual macrophages and fluctuated over time. This preexisting heterogeneity of iNOS expression explains the differential control of intracellular *M. tuberculosis* growth even within a clonal population of macrophages. Our findings highlight the importance of considering preexisting phenotypic heterogeneity in host cells when studying the pathophysiology of an infectious disease, as these differences may determine the outcome of the initial encounter between host and pathogen.

Results

Intracellular growth of *M. tuberculosis* and survival of infected host cells are heterogeneous at the single-cell level.

We used fluorescence time-lapse microscopy to image individual mouse bone marrow-derived macrophages (BMDMs) infected with green fluorescent protein (GFP)-expressing *Mycobacterium tuberculosis* (Figure 1A). The total fluorescent area per macrophage measured at 2-h intervals was used as a proxy for the number of intracellular bacteria and to calculate the growth rate of each intracellular microcolony. Macrophages can partially control *M. tuberculosis* growth, as the median growth rate of intracellular bacteria (0.036 per hour, corresponding to a doubling time of 27.8 h) (Figure 1B) was reduced in comparison to extracellular bacteria growing on the debris of dead cells in the same culture (growth rate of 0.078 per hour, corresponding to a doubling time of 12.8 h) (Figure 1C). Intracellular bacterial growth was heterogeneous: some intracellular bacteria grew with a growth rate above 0.08 per hour, corresponding to a doubling time of 12.5 h (Figure 1A, lower panel, and Figure 1B), while others grew very slowly, with doubling times of more than 1 week (Figure 1A, upper panel, and Figure 1B). Over 168 h of continuous imaging, ~60% of infected host cells died at different time points after infection (Figure 1D). Surprisingly, the fate of individual infected macrophages (death or survival over the 168-h imaging period) did not correlate with the initial bacterial load (Figure 1E), final bacterial load (Figure 1F), or growth rate of intracellular *M. tuberculosis* (Figure 1G; see also Figure S1 in the supplemental material). Indeed, some macrophages survived even with a bacterial load that was higher than the median bacterial load in macrophages that died (Figure 1F). It is worth noting, however, that even though no significant trend appeared, a subpopulation of exceptionally fast-growing intracellular bacteria with growth rates greater than 0.8 per hour, corresponding to a doubling time of less than 12.5 h, eventually did kill their host cells (Figure 1G).

Heterogeneity of intracellular *M. tuberculosis* growth rates is linked to variability in the macrophage population.

The observed single-cell heterogeneity in intracellular *M. tuberculosis* growth rates could reflect heterogeneity in the bacterial population (some bacteria resist macrophage-imposed stresses better than others) or variability in the macrophage population (some cells control *M. tuberculosis* growth better than others). To address this question, we coinfected BMDMs with two fluorescently labeled *M. tuberculosis*

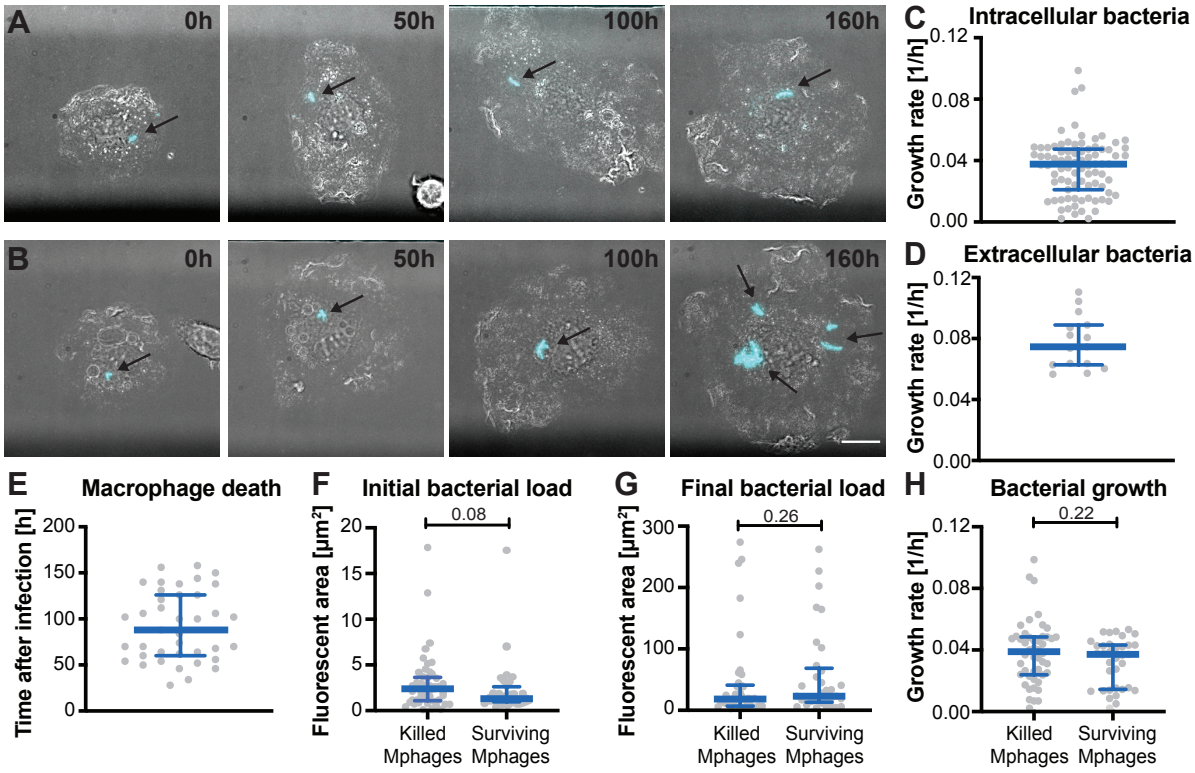


Figure 1: Intracellular growth of *M. tuberculosis* and survival of infected host cells are heterogeneous at the single-cell level

Murine BMDMs were infected with GFP-expressing *M. tuberculosis* and imaged by time-lapse microscopy at 2-h intervals for 168 h. **(A)** Example of an infected macrophage in which bacteria (cyan) grew slowly (doubling time, >168 h). **(B)** Example of an infected macrophage in which bacteria grew quickly (doubling time, <20 h). **(C)** Growth rates of intracellular *M. tuberculosis*. Each symbol represents a bacterial microcolony inside a single infected macrophage. **(D)** Growth rates of extracellular *M. tuberculosis*. Each symbol represents a single extracellular bacterial microcolony. **(E)** Survival time of infected macrophages that died before the end of the experiment. Each symbol represents a single macrophage. Survival time was calculated from the frame when initial infection occurred to the frame when death occurred. **(F to H)** Initial bacterial load **(F)**, final bacterial load **(G)**, and growth rate of intracellular bacteria **(H)** for macrophages that died during the experiment (killed Mphages) or macrophages that survived until the end of the experiment (surviving Mphages). Each symbol represents a single infected macrophage. Blue lines indicate median values and interquartile ranges. Scale bar, 10 µm. P values were calculated using a Mann-Whitney test.

strains expressing either constitutive GFP or tdTomato (Figure 2A), which displayed similar intracellular growth rates (Figure S2). BMDMs infected with one bacterial cell of each color were imaged by time-lapse microscopy, and growth rates were calculated independently for intracellular bacterial microcolonies originating from a GFP+ or tdTomato+ bacterium. This approach permitted the comparison of two microcolonies (one GFP+, one tdTomato+) growing inside the same macrophage, or of two microcolonies growing in different macrophages (Figure 2B). If variability in the macrophage population contributes to heterogeneity in intracellular bacterial growth rates, then two microcolonies in the same macrophage should behave more similarly than two microcolonies in different macrophages. We tested this hypothesis

in unactivated macrophages and in macrophages preactivated with gamma interferon (IFNγ), a cytokine that induces the expression of antibacterial host defense mechanisms (18) (Figure S2C). We found that the difference in growth rates between two intracellular bacterial microcolonies was smaller, on average, if they were in the same host cell than if they were in different cells, in both unactivated and IFNγ-activated macrophages (Figure 2C). These results suggest that some individual host cells control *M. tuberculosis* growth better than others, irrespective of their activation status.

Single-cell variation in nitric oxide production by macrophages drives heterogeneous growth of intracellular *M. tuberculosis.*

Nitric oxide production by iNOS is one of the IFNγ-induced mechanisms that macrophages use to control intracellular growth of *M. tuberculosis* (Chan et al., 1992; MacMicking et al., 1997). We hypothesized that cell-to-cell differences in iNOS activity might explain why some host cells control *M. tuberculosis* growth better than others. We tested this hypothesis by coinfecting BMDMs with single GFP+ and tdTomato+ bacteria while inhibiting iNOS activity with aminoguanidine (Figure 2C; Figure S3A). In both unactivated and IFNγ-activated BMDMs treated with aminoguanidine, inter-macrophage and intramacrophage differences in bacterial growth rates were not significantly different (Figure 2D). This suggests that, when iNOS activity is inhibited, all macrophages control *M. tuberculosis* growth about equally well. Consistent with this hypothesis, we found that inter-macrophage and intramacrophage bacterial growth rates were not significantly different in BMDMs from iNOS$^{-/-}$ mice. This observation held true in both unactivated and IFNγ-activated iNOS$^{-/-}$ BMDMs (Figure 2E). Despite inhibition or lack of iNOS activity, IFNγ activation was still effective in reducing intracellular bacterial growth (Figure S2C), presumably due to other IFNγ-activated defenses, such as IRGM1 (MacMicking et al., 2003). We conclude that cell-to-cell variation in iNOS activity is linked to the control of intracellular *M. tuberculosis* growth in macrophages, irrespective of their activation status.

Single-cell variability of macrophage iNOS expression in a reporter cell line contributes to heterogeneous growth of intracellular *M. tuberculosis*.

To further investigate the link between cell-to-cell heterogeneity of iNOS gene expression and intracellular *M. tuberculosis* growth, we used RAW 264.7 reporter macrophages that expressed yellow fluorescent protein (YFP) from a copy of the iNOS promoter stably integrated in the genome (Beaulieu et al., 2010). In these macrophages, iNOS-YFP is expressed at low basal levels in unactivated cells and is strongly induced upon activation with IFNγ (Figure 3A; Figure S4). To verify that

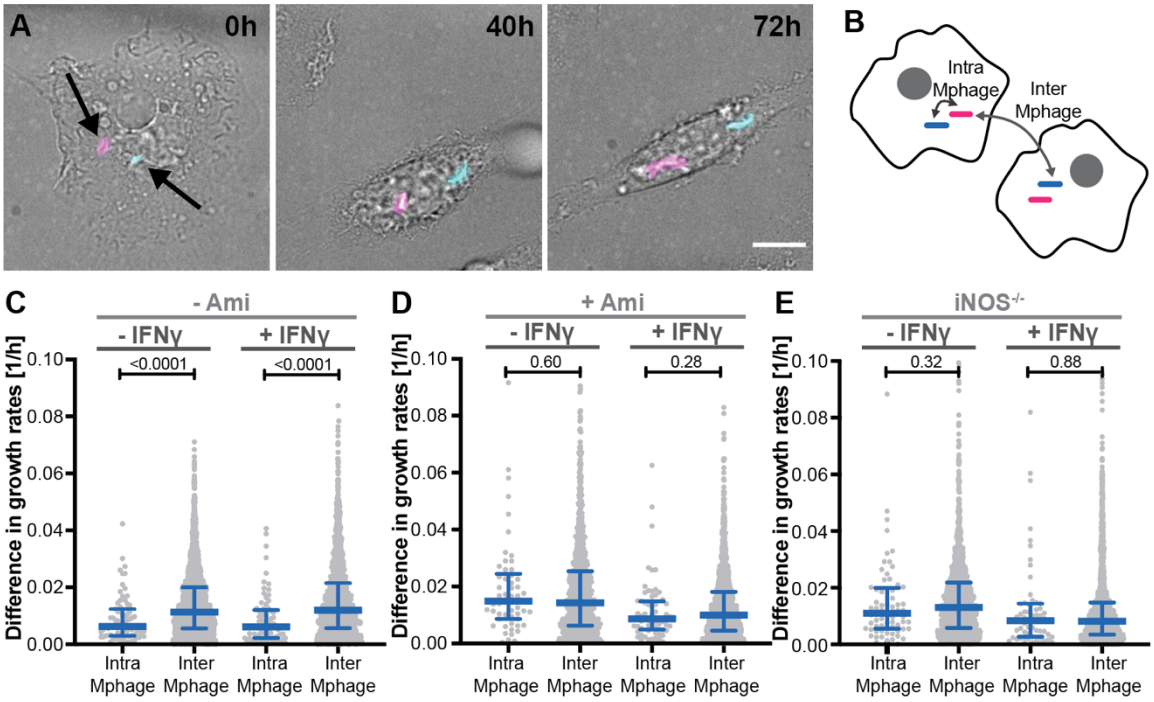


Figure 2: Single-cell variability of macrophage nitric oxide production drives heterogeneous growth of intracellular *M. tuberculosis*
Murine bone marrow-derived macrophages were simultaneously infected with *M. tuberculosis* strains expressing GFP or tdTomato and imaged by time-lapse microscopy at 1-h or 2-h intervals for 72 h. (A) Representative time-lapse images of a macrophage coinfected with GFP-expressing (cyan) and tdTomato-expressing (magenta) *M. tuberculosis*. (B) Schematic representation of the experimental design. The growth rates of two bacterial microcolonies (one green, one red) inside the same macrophage (intra-Mphage) or in two different macrophages (inter-Mphage) were compared. For calculation of intermacrophage differences in growth rate, each GFP-expressing bacterium was compared to all tdTomato-expressing bacteria not in the same macrophage. (C to E) Differences in growth rates between two bacterial microcolonies inside the same macrophage or in two different macrophages. Each symbol represents the difference in growth rates between one green and one red bacterium in unactivated or preactivated wild-type macrophages that were untreated (C), treated with aminoguanidine to inhibit nitric oxide production (D), or in untreated iNOS$^{-/-}$ macrophages (E). Blue lines indicate median values and interquartile ranges. Scale bar, 10 µm. P values were calculated using a Mann-Whitney test.

iNOS-YFP expression was indeed linked to nitric oxide production, we flow-sorted the macrophages into low- and high-fluorescence subpopulations (Figure 3A) and performed a Griess assay to measure the concentration of reactive nitrogen species (RNS) in the culture supernatants 24 h after sorting. iNOS-YFP^{low} cells produced less RNS than iNOS-YFP^{high} cells in both unactivated and IFNγ-activated samples, confirming that iNOS-YFP expression is linked to RNS production (Figure 3B).

We infected four flow-sorted subpopulations of macrophages (± IFNγ iNOS-YFP^{low} and ± IFNγ iNOS-YFP^{high}) with tdTomato-expressing *M. tuberculosis* and used time-lapse microscopy to measure the growth rates of bacterial microcolonies within

individual macrophages. In both unactivated and IFNγ-activated macrophage subpopulations, iNOS-YFP^{high} cells controlled bacterial growth significantly better than iNOS-YFP^{low} cells (Figure 3C). This difference was abolished when iNOS activity was inhibited with aminoguanidine, confirming that it is dependent on nitric oxide production (Figure 3D; Figure S3B). As we observed in infected BMDMs (Figure S2C), when iNOS activity was inhibited, IFNγ-activated RAW 264.7 macrophages still controlled M. tuberculosis growth better than unactivated macrophages (Figure 3D).

For all conditions tested, the survival rate of infected macrophages was similar over the course of the experiments, indicating that the observed differences in bacterial growth rates were not due to differences in host cell viability (Figure S5).

iNOS expression is not linked to differences in macrophage polarization or expression of other IFNγ-regulated genes.

We investigated whether cell-to-cell differences in iNOS expression are linked to macrophage polarization or single-cell variability in IFNγ-responsive gene expression by quantitative real-time PCR (qRT-PCR) analysis of unactivated or IFNγ-activated iNOS-YFPlow and iNOS-YFPhigh cells. These experiments confirmed that iNOS mRNA expression correlates with iNOS-YFP fluorescence levels (Figure 3E). However, we did not observe any differences between iNOS-YFPhigh and iNOS-YFPlow macrophages in the expression of other genes associated with macrophage polarization or the IFNγ response (Figure 3E).

iNOS expression and RNS production fluctuate over time.

The observation that individual macrophages express iNOS-YFP at different levels prompted us to ask whether these cell-to-cell differences are stable or unstable over time. We used flow cytometry to measure fluorescence levels in flow-sorted macrophage subpopulations 0, 24, 48, 72, and 96 h after sorting. This analysis revealed that the iNOS-YFP^{low} and iNOS-YFP^{high} subpopulations were not stable over time and slowly converged toward each other in both unactivated and IFNγ-activated samples (Figure 4A). Convergence of the iNOS-YFP^{low} and iNOS-YFP^{high} subpopulations was faster in the unactivated samples than in the IFNγ-activated samples. To confirm that the fluctuations in iNOS-YFP expression reflected changes in RNS production, we performed a Griess assay to measure levels of RNS secreted by iNOS-YFP^{low} and iNOS-YFP^{high} subpopulations 24, 48, 72, and 96 h after sorting. The amount of RNS secreted by the different subpopulations also fluctuated over time and converged within a similar time frame as iNOS-YFP expression for both unactivated and IFNγ-activated samples (Figure 4B and C).

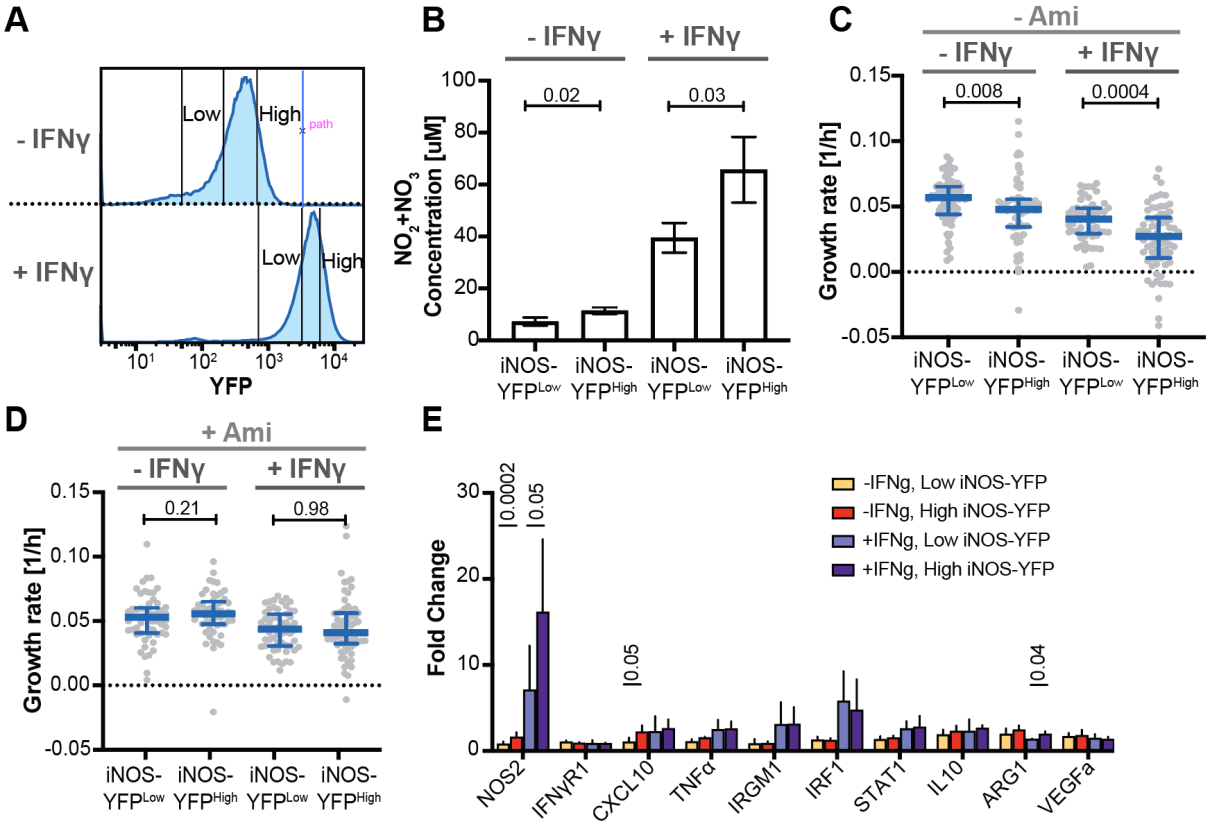


Figure 3: Single-cell variability of iNOS expression by macrophages contributes to heterogeneous growth rates of intracellular *M. tuberculosis*
Unactivated (−IFNγ) and activated (+IFNγ) RAW 264.7 macrophages that stably expressed YFP from the iNOS transcriptional promoter were flow-sorted into low- and high-fluorescence subpopulations prior to analysis. (A) Flow cytometry fluorescence profiles of iNOS-YFP-expressing macrophages. High-YFP and low-YFP gates are indicated. (B) The Griess assay was used to measure the cumulative concentration of reactive nitrogen species (NO_2 + NO_3) in the supernatants of macrophage subpopulations 24 h after sorting. P values were calculated using Student's t test. (C and D) Growth rates of intracellular *M. tuberculosis* in flow-sorted macrophage subpopulations were measured by time-lapse microscopy during 72 h. Macrophages were untreated (C) or treated with aminoguanidine to inhibit nitric oxide production (D). Each symbol represents a bacterial microcolony inside a single macrophage. Blue lines indicate median values and interquartile ranges. P values were calculated using a Mann-Whitney test. (E) Expression levels of selected genes involved in the IFNγ response or linked to macrophage polarization in flow-sorted macrophage subpopulations. Relative expression (fold change) was normalized to an unactivated and unsorted sample. NOS2, IFNγR1, CXCL10, IRGM1, IRF1, and STAT1 are involved in the IFNγ response and, along with tumor necrosis factor α (TNF-α), are markers for M1 polarization. VEGFa and ARG1 are markers for M2 polarization. P values were calculated using Student's t test (P values of >0.05 are not shown).

Finally, to assess the impact of infection on the fluctuation of iNOS-YFP expression at the single-cell level, we infected flow-sorted subpopulations of macrophages with tdTomato-expressing *M. tuberculosis* and tracked single infected cells over 72 h using time-lapse fluorescence microscopy. As observed in our population-based experiments, we found that iNOS-YFP expression levels fluctuated in single cells and macrophages sorted into iNOS-YFP^{low} and iNOS-YFP^{high}

populations converged over time and stabilized around the average level of gene expression found in the macrophage population prior to sorting (Figure 4D and E; Figure S6). These results confirmed that iNOS-YFP expression fluctuates independently of infection or IFNγ activation, although activation may influence the time scale of these fluctuations.

Despite the observed fluctuations in iNOS-YFP expression over time, we were able to measure significant differences in bacterial growth rates between iNOS-YFP^{high} and iNOS-YFP^{low} cells over the course of our experiments. These observations suggested that early exposure to different concentrations of intracellular RNS may be sufficient to drive differences in bacterial growth rates at early as well as late time points. We tested this hypothesis by comparing bacterial growth rates in unactivated iNOS-YFP^{high} and iNOS-YFP^{low} macrophages between 0 and 36 h and between 36 and 72 h. We found that intracellular bacterial growth rates were significantly different between iNOS-YFP^{high} and iNOS-YFP^{low} subpopulations during both the early and late time intervals (Figure S7), suggesting that differences in iNOS gene expression, even when limited to the early stages of infection, can have a long-lasting impact on bacterial growth rates.

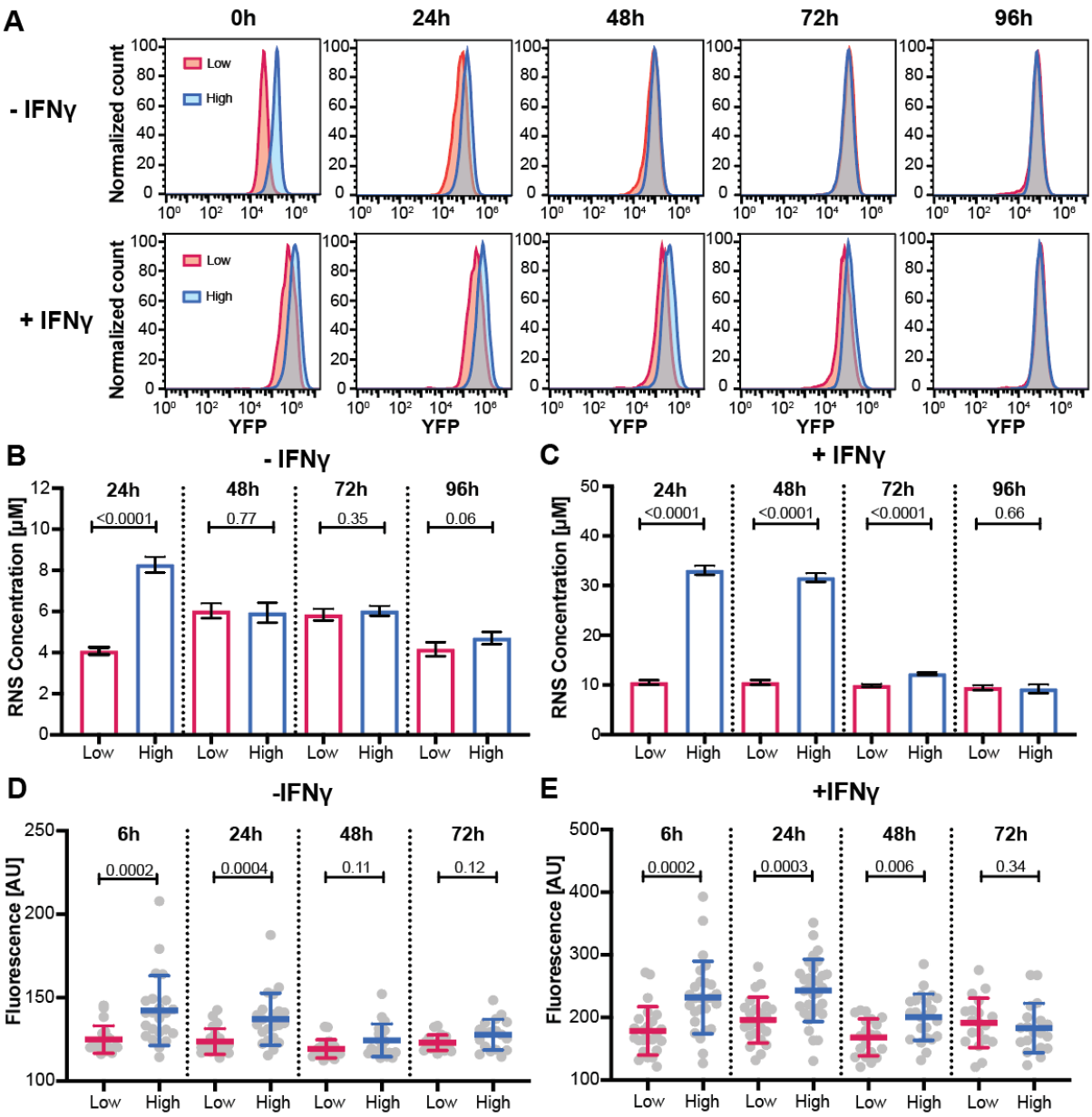


Figure 4: iNOS expression and RNS production fluctuate over time

Unactivated (–IFNγ) and activated (+IFNγ) RAW 264.7 macrophages that stably expressed YFP from the iNOS transcriptional promoter were flow-sorted into low- and high-fluorescence subpopulations prior to analysis. (A) Flow cytometry fluorescence profiles of iNOS-YFP-expressing macrophages 6, 24, 48, 72, and 96 h after sorting. (B and C) RNS concentrations in the supernatant of unactivated (B) and IFNγ-activated (C) iNOS-YFP-expressing macrophages 24, 48, 72, and 96 h after sorting. P values were calculated using Student's t test. (D and E) Single infected macrophages were identified and tracked using time-lapse microscopy. Shown are the fluorescence of *M. tuberculosis*-infected unactivated (D) and IFNγ-activated (E) iNOS-YFP-expressing macrophages 6, 24, 48, and 72 h after sorting as measured by microscopy. Each symbol represents a single infected macrophage. Blue lines indicate median values and interquartile ranges. P values were calculated using a Mann-Whitney test.

Discussion

During the course of an infection, *M. tuberculosis* encounters heterogeneous niches ranging from different intracellular compartments to different types of cells and lesions (Toniolo, Rutschmann et al., 2021). This interplay between bacteria and heterogeneous host environments likely plays a role in determining the outcome of infection, ranging from disease progression to sterilization.

Here, we focused on the initial phases of an *M. tuberculosis* infection, during which small numbers of bacteria interact with individual host cells, to determine whether preexisting phenotypic heterogeneity in macrophages could drive differential growth of intracellular *M. tuberculosis*, potentially leading to different infection outcomes. We observed that individual cells can express different levels of iNOS within a clonal population of macrophages; this heterogeneity was not linked to differences in macrophage polarization, nor to the expression of other IFNγ-related genes. Macrophages that expressed higher level of iNOS at the time of initial infection were more effective in controlling intracellular *M. tuberculosis* growth, suggesting that differences in expression levels of a single host antimicrobial gene could be sufficient to explain cell-to-cell variation in the control of intracellular bacteria. Our in vitro observations complemented a recent study showing that different populations of macrophages expressing different levels of iNOS coexist in the lungs of mice infected with *M. tuberculosis* and expression of iNOS correlates with expression of the bacterial stress marker *HspX* (Pisu et al., 2021). Although the role of iNOS in protection against tuberculosis in humans remains controversial, expression of iNOS has recently been observed in lung sections from tuberculosis patients and in explanted human alveolar macrophages infected with *M. tuberculosis* (Cho et al., 2020; Ufimtseva et al., 2021). Interestingly, human alveolar macrophages infected with *M. tuberculosis* show significant heterogeneity in the expression of several proinflammatory markers, including iNOS, which is correlated to their intracellular *M. tuberculosis* load (Ufimtseva et al., 2021). These studies suggest that heterogeneity in iNOS expression could have a role in disease progression *in vivo.*

Our observations that iNOS expression fluctuates in single macrophages and that flow-sorted iNOS-YFP^{high} and iNOS-YFP^{low} subpopulations converge toward an average level of iNOS expression over time **(Figure 4)** are consistent with previous evidence that gene expression may be essentially stochastic at the single-cell level. According to this framework, genes are transcribed in bursts of variable intensity that occur at random time intervals (Elowitz et al., 2002; Friedman et al., 2006; Mcadams & Arkin, 1997; Raj et al., 2006). Based on these observations, we hypothesize that in

individual macrophages iNOS expression might occur in bursts separated by silent intervals of variable duration. This model could explain our observation that flow-sorted subpopulations of iNOS-YFP^{high} and iNOS-YFPl^{ow} macrophages converged over time and eventually stabilized around the average level of iNOS expression found in the population prior to sorting. However, we cannot exclude that fluctuations in iNOS expression might reflect other mechanisms, such as cell cycle-dependent changes in gene expression or modifications in chromatin accessibility (Lannan et al., 2022).

Cell-to-cell variation in iNOS expression seems to account for most of the intermacrophage heterogeneity in *M. tuberculosis* growth rates observed in our experiments. However, we also found that two bacteria growing inside the same host cell may exhibit different growth rates. Intramacrophage differences in *M. tuberculosis* growth rates could reflect occupancy of more or less permissive intracellular compartments by individual bacteria within the same macrophage (Bakkum et al., 2020; Lienard et al., 2020; Miller et al., 2004; Rohde et al., 2007; Schnettger et al., 2017; Simeone et al., 2012; van der Wel et al., 2007; Welin et al., 2011). Alternatively, heterogeneous infection outcomes could also originate from the pathogen itself. Phenotypic heterogeneity in clonal bacterial populations is well documented and can be amplified by host stress, resulting in differences in bacterial fitness (Ackermann, 2015; Avraham et al., 2015; Bhaskar et al., 2014; Eldar & Elowitz, 2010; Manina et al., 2015). Cell-to-cell differences in the expression of bacterial virulence factors could also impact bacterial growth indirectly by driving different host-cell responses (Raffetseder et al., 2019). It is thus likely that heterogeneous single-cell growth of intracellular bacteria is due to the interplay of different host and bacterial factors.

Our finding that preexisting heterogeneity in host cells can have an impact on the growth of intracellular *M. tuberculosis* is particularly relevant for tuberculosis, because even a single bacterium infecting a single permissive host cell may be sufficient to initiate an infection (Riley et al., 1995; Saini et al., 2012). Macrophages have been reported to exhibit heterogeneity in the expression of many immune-related genes due to factors such as circadian rhythm, environmental variation, or age of the host, which could all impact how they respond to an initial infection (Bain & MacDonald, 2022; Keller et al., 2009; Lafuse et al., 2019; Melo et al., 2021). Our results suggest that differences in the expression of even a single antimicrobial gene, such as the gene for iNOS, could be sufficient to influence the outcome of infection. This conclusion could potentially be extended to any disease where the number of interacting host cells and bacteria is small at some stage of the infection (Moxon & Kussell, 2017). In such cases, chance encounters of the pathogen with host cells

expressing different levels of antibacterial defense mechanisms could determine whether infection is controlled or progresses to active disease.

Materials and Methods

Bone marrow-derived macrophages (BMDMs)

BMDMs were differentiated from frozen bone marrow stocks extracted from femurs of wild-type C57BL/6 mice or iNOS$^{-/-}$ mice (B6.129P2-Nos2tm1Lau/J mice from Jackson Laboratories, catalog number 002609). The bone marrow was cultured in petri dishes in BMDM differentiation medium (Dulbecco's modified Eagle's medium [DMEM] with 10% fetal bovine serum [FBS], 1% sodium pyruvate, 1% GlutaMax, and 20% L929 cell-conditioned medium [as a source of granulocyte-macrophage colony-stimulating factor]) for 7 days. The adherent cells were then gently lifted from the plate using a cell scraper and resuspended in BMDM culture medium (DMEM with 5% FBS, 1% sodium pyruvate, 1% GlutaMax, and 5% L929 cell-conditioned medium). The macrophages were then seeded in 35-mm ibidi µ-dishes or in 4-compartment ibidi µ-dishes and allowed to adhere for 4 h at 37°C, 5% CO_2 before use.

RAW 264.7 iNOS-YFP macrophage cell line

RAW 264.7 macrophages stably expressing YFP from a copy of the iNOS transcriptional promoter (Beaulieu et al., 2010) were cultured in DMEM with 10% FBS, 1% Glutamax, and 1% sodium pyruvate at 37°C, 5% CO_2. The macrophages were passaged every 3 days at a 1:4 ratio by gently lifting them off the culture flask with a cell scraper.

M. tuberculosis strains

GFP- and tdTomato-expressing *M. tuberculosis* Erdman strains were inoculated from frozen glycerol stocks in Middlebrook 7H9 (Difco) supplemented with 10% albumin-dextrose-saline (ADS), 0.5% glycerol, and 0.02% tyloxapol and cultured at 37°C with shaking.

Flow sorting of RAW 264.7 iNOS-YFP reporter macrophages

RAW 264.7 iNOS-YFP macrophages were detached from culture flasks with 10 mM EDTA. When required, macrophages were activated 24 h before detaching with 100 U/mL IFNγ. The cells were then collected by centrifugation, resuspended in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] with 1 mM EDTA), and sorted using a FACSaria Fusion system (the gating strategy is shown in Figure S3 in the supplemental material). Sorted cells were collected,

resuspended in DMEM with 10% FBS, 1% Glutamax, and 1% sodium pyruvate, seeded in μ-dishes, and allowed to adhere for at least 4 h before use.

Macrophage infections

For infection, 1 mL of *M. tuberculosis* culture at an optical density at 600 nm (OD_{600}) of 0.5 was pelleted and resuspended in 200 μL of macrophage medium. Bacteria were passed through a 5-μm filter to eliminate aggregates. The resulting single-cell suspension was used to infect BMDMs or RAW 264.7 iNOS-YFP macrophages at a multiplicity of infection (MOI) of 1:1. When two fluorescent strains of *M. tuberculosis* were used simultaneously, both were added at an MOI of 1:1. After 4 h of infection, macrophages were washed extensively with macrophage medium to remove extracellular bacteria. When required, 100 U/mL IFNγ was added to the macrophage medium 24 h before infection and maintained during the duration of the experiment. When required, 500 μM aminoguanidine was added to the culture medium at the time of infection and maintained thereafter.

Time-lapse microscopy of macrophages infected with *M. tuberculosis*

Infected BMDMs and RAW 264.7 iNOS-YFP macrophages were imaged with a DeltaVision microscope and a Nikon Ti2 microscope, respectively. A stage-top incubator (Okolab) was used to maintain the cells at 37°C in a humidified environment. Air mixed to 5% CO_2 was supplied using an Okolab gas mixer. Infected BMDMs were randomly selected and imaged for up to 168 h. Macrophage medium was refreshed every 3 days via custom tubing connected to the lid of the ibidi μ-dish. Infected BMDMs were imaged using a 60× oil-immersion objective at 2-h intervals; 3 × 1 μm z-stacks were acquired for each point. Bacteria were identified by fluorescence emission on the green (GFP) or red (tdTomato) channel using fluorescein isothiocyanate (excitation [Ex] 490/20, emission [Em] 525/36) and tetramethyl rhodamine isocyanate (Ex 555/25, Em 605/52) filters, respectively. Infected RAW 264.7 iNOS-YFP macrophages were imaged for 72 h using a 40× air objective at 1-h intervals; 3 × 1 μm or 5 × 1 μm z-stacks were acquired for each point. iNOS-YFP levels were quantified and tdTomato-expressing bacteria were imaged using GFP (Em 480/30, Ex 535/45) and mCherry (Em 560/40, Ex 635/60) dichroic filters, respectively. For both BMDMs and RAW 264.7 iNOS-YFP macrophages, at least 25 infected cells were imaged per condition.

Quantification of reactive nitrogen species (RNS) in cell culture medium

BMDMs and RAW 264.7 iNOS-YFP macrophages were seeded in triplicates in 96-well plates with 100 µL of their respective culture medium at a concentration of 10^6 cells/mL. When required, 100 U/mL IFNγ or 500 µM aminoguanidine was added to the culture medium. After 24 h of incubation at 37°C, 5% CO_2, 80 µL of culture supernatant was collected and centrifuged at 10,000 × g for 10 min. The concentration of RNS in the supernatant was measured using a nitrate/nitrite colorimetric assay kit (Abnova), as described by the manufacturer. Since RAW 264.7 macrophages divide approximately every 24 h, a different seeding strategy was used for time course experiments with these cells. Each population of flow-sorted RAW 264.7 iNOS-YFP macrophages was seeded in 4 wells of a 96-well plate at four different concentrations (10^6, 0.5×10^6, 0.25×10^6, or 0.125×10^6 cells/mL, all in 100 µL of medium) and measured at 24, 48, 72, and 96 h after sorting. This seeding strategy ensured that the samples used for different time points reached approximately the same number of cells. The plates were incubated at 37°C, 5% CO_2 between the different time points. When required, 100 U/mL of IFNγ was added to the medium of the cells directly after sorting.

Quantitative real-time PCR

Unactivated or preactivated (+100 U/mL IFNγ) RAW 264.7 iNOS-YFP macrophages were sorted as described above. Directly after sorting, the macrophages were collected by centrifugation and lysed, and RNA was extracted using a Qiagen RNeasy micro kit plus according to the manufacturer's instructions. DNase treatment was performed directly on the column during RNA extraction. The RNA was then reverse-transcribed with random hexamers using the SuperScript IV first-strand synthesis system (ThermoFisher). qRT-PCR mixtures were prepared using the SYBRGreen PCR master mix (Applied Biosystems) with 1 µM primers and 2 µL of cDNA. Reactions were run on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Amplicon specificity was confirmed by melting curve analysis. Primer sequences were obtained from Origene. Primers were synthesized by Microsynth, Switzerland (Table S1).

Flow cytometry time course

FACS-sorted unactivated or preactivated (+100 U/mL IFNγ) RAW 264.7 iNOS-YFP macrophage subpopulations were seeded following the same seeding strategy described for the time course experiments to quantify RNS. For each subpopulation,

5 wells of a 24-well plate were seeded with 350 µL of cells at 2×10^6, 10^6, 0.5×10^6, 0.25×10^6, or 0.125×10^6 cells/mL and analyzed by flow cytometry at 4, 24, 48, 72, and 96 h after sorting. The plates were incubated at 37°C, 5% CO_2 between the different time points. When required, 100 U/mL IFNγ was added to the medium of the cells directly after sorting. For analysis by flow cytometry, the cells were detached using trypsin, collected by centrifugation, resuspended in PBS plus 1 mM EDTA, and analyzed using a BD Accuri C6 flow cytometer.

Image analysis

Image analysis was performed using the FIJI version of the ImageJ software (Schindelin et al., 2012). All infected macrophages that survived for at least 24 h of imaging were analyzed. If a macrophage divided during the experiment, the daughter cell containing the bacteria was selected for continued analysis. If the bacterial microcolony was split between the two daughter cells, the analysis was stopped at this time point. All of the macrophages were imaged until the end of the experiment or until their death. The z-stacks acquired were projected into one image using a maximum intensity projection. A background subtraction was performed by subtracting from the fluorescence images a copy of the same images on which a Gaussian blur of 100-µm radius had been applied. Regions of interest corresponding to individual macrophages were manually drawn onto the phase images and transferred to the fluorescence images. A manual threshold was set on the fluorescence channel to segment the bacteria. The area above the threshold inside single macrophages was measured and used as a proxy for the number of intracellular bacteria for each time point. To quantify the growth rate of the intracellular bacteria, an exponential curve was fitted to the data. A similar method was used to measure the growth rate of bacteria identified as extracellular in BMDM infection experiments. Similarly, iNOS-YFP expression levels were quantified for each frame by transferring the manually drawn regions of interest corresponding to individual infected macrophages to the GFP fluorescence images. The average fluorescence intensity was measured for each individual macrophage and used as a proxy for iNOS-YFP expression levels. Cell death was identified using bright-field images. When macrophages die, they change shape (shrink), lose membrane integrity, and stop moving. Death events were identified by examining the subsequent time-lapse images for each cell (Figure S1). The time of death was manually annotated as the first time point at which death was observed. Out-of-focus images were manually excluded from analysis.

Supplementary Material

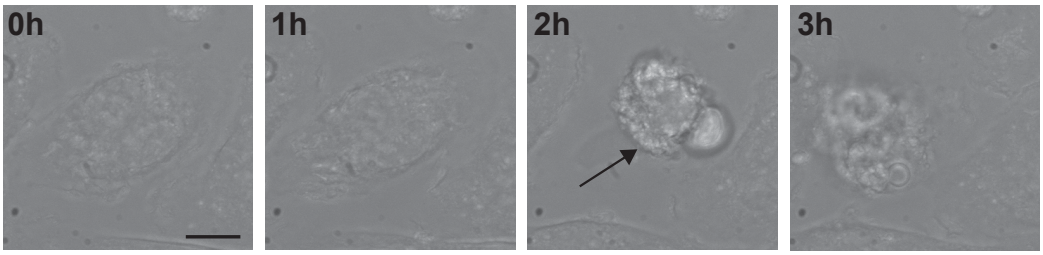


Figure S1: Macrophage time of death is identified on bright field images
Macrophages that die shrink, lyse and stop moving, and are easily identified on bright field images. Macrophage time of death is determined as the first frame on which a cell is identified as dead (black arrow). Scale bar = 10 μm.

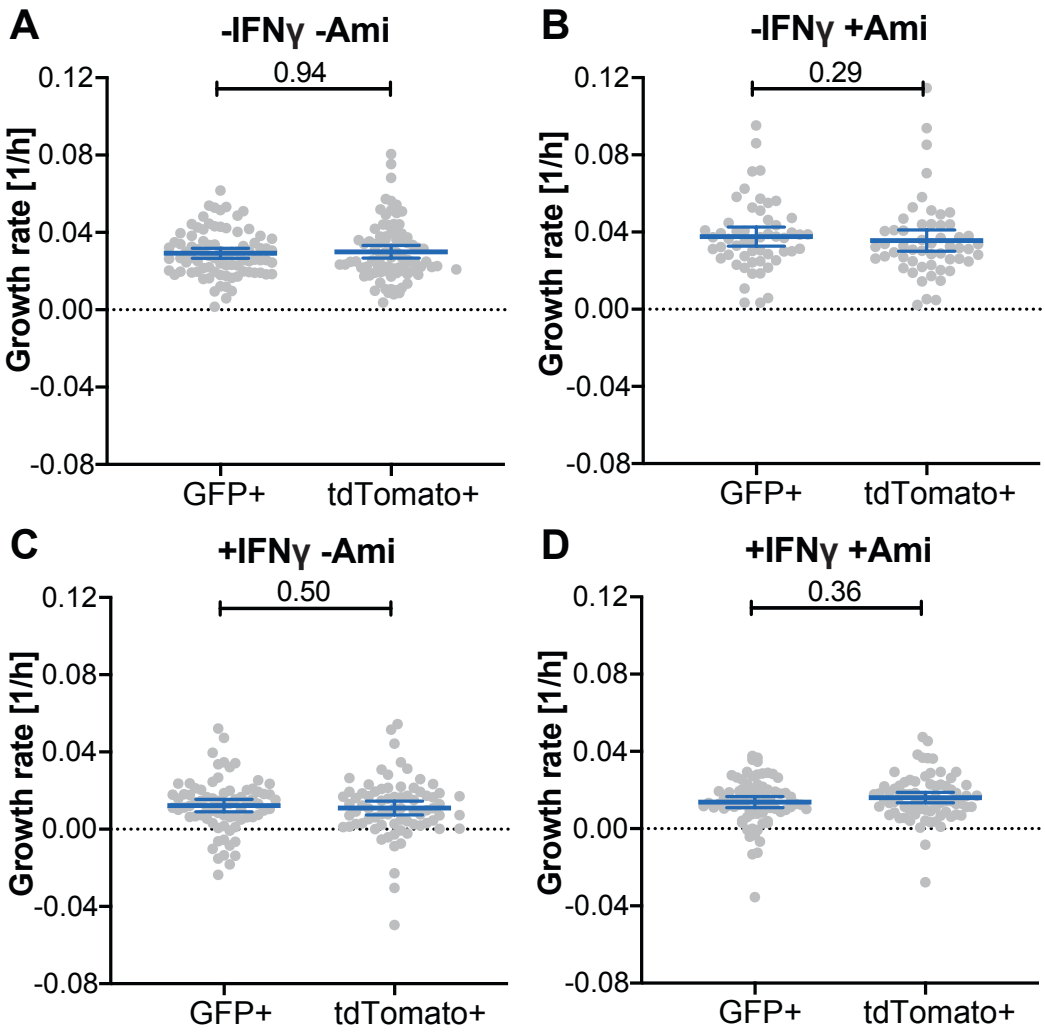


Figure S2: Strains of *M. tuberculosis* expressing GFP or tdTomato exhibit similar intracellular growth rates

Bone marrow-derived macrophages were simultaneously infected with *Mtb* strains expressing GFP or tdTomato and imaged by time-lapse microscopy at 1-hour or 2-hour intervals for 72 hours. **(A-D)** Intracellular growth rates of *Mtb* in unactivated macrophages (N=84 for red and for green) **(A)**, unactivated macrophages treated with aminoguanidine (N=54 for red and for green) **(B)**, IFNγ-activated macrophages (N=75 for red and for green) **(C)**, or IFNγ-activated macrophages treated with aminoguanidine (N=74 for red and for green) **(D)**. Each symbol represents a microcolony growing inside a single macrophage. Blue lines indicate median values and interquartile ranges. P values were calculated using a Mann-Whitney test.

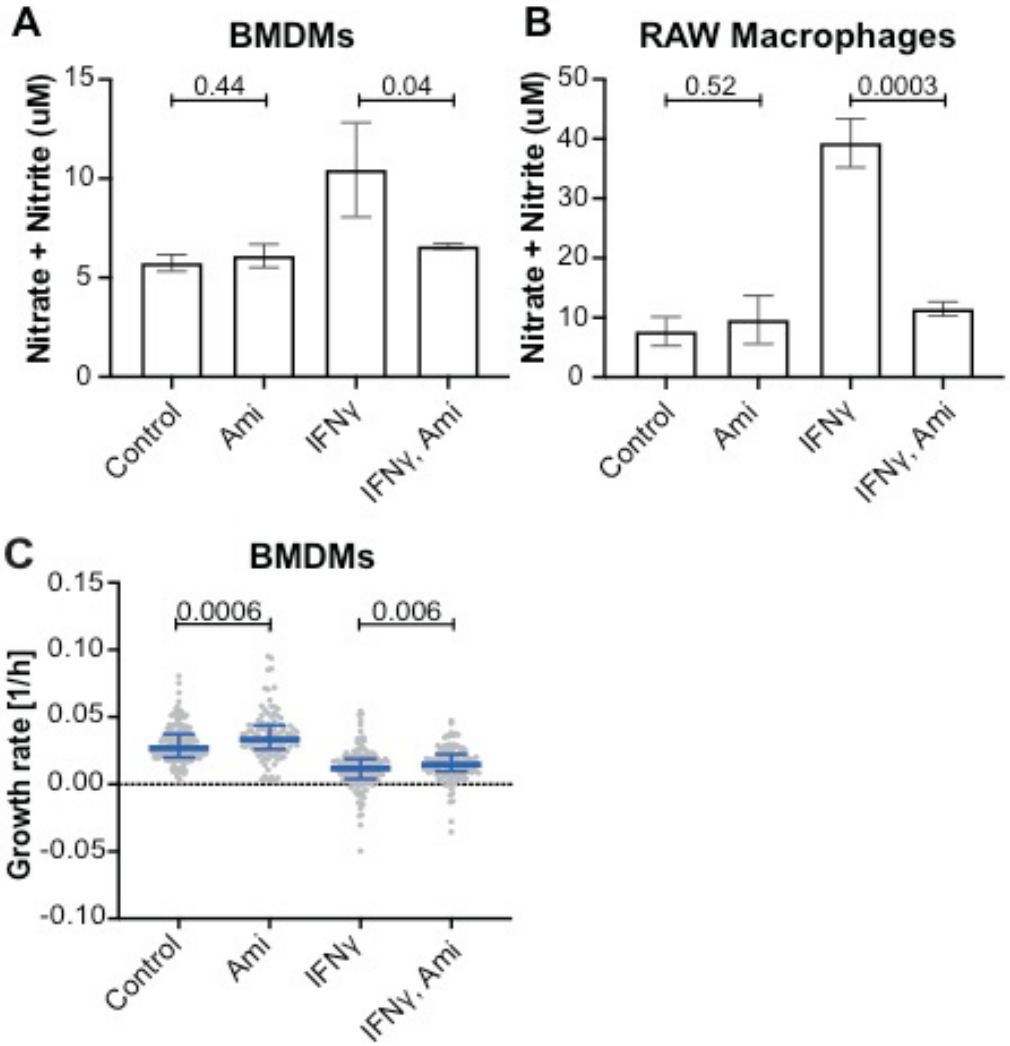


Figure S3: Aminoguanidine inhibits IFNγ-induced production of reactive nitrogen species (RNS) and macrophage control of intracellular bacteria

(A-B) Concentration of reactive nitrogen species (Nitrate + Nitrite) in the supernatant of bone marrow-derived macrophages (A) or RAW 264.7 macrophages (B), ± activation (IFNγ) and ± aminoguanidine (Ami) treatment to inhibit nitric oxide production. Error bars indicate standard deviations. P values were calculated using Student's t test. (C) Growth rates of intracellular *M. tuberculosis* in unactivated (-IFNγ) or pre-activated (+IFNγ) bone marrow derived macrophages (BMDMs), with or without aminoguanidine treatment (±Ami) to inhibit nitric oxide production. Each symbol represents a bacterial microcolony inside a single macrophage. Blue lines indicate median values and interquartile ranges. P values were calculated using a Mann-Whitney test.

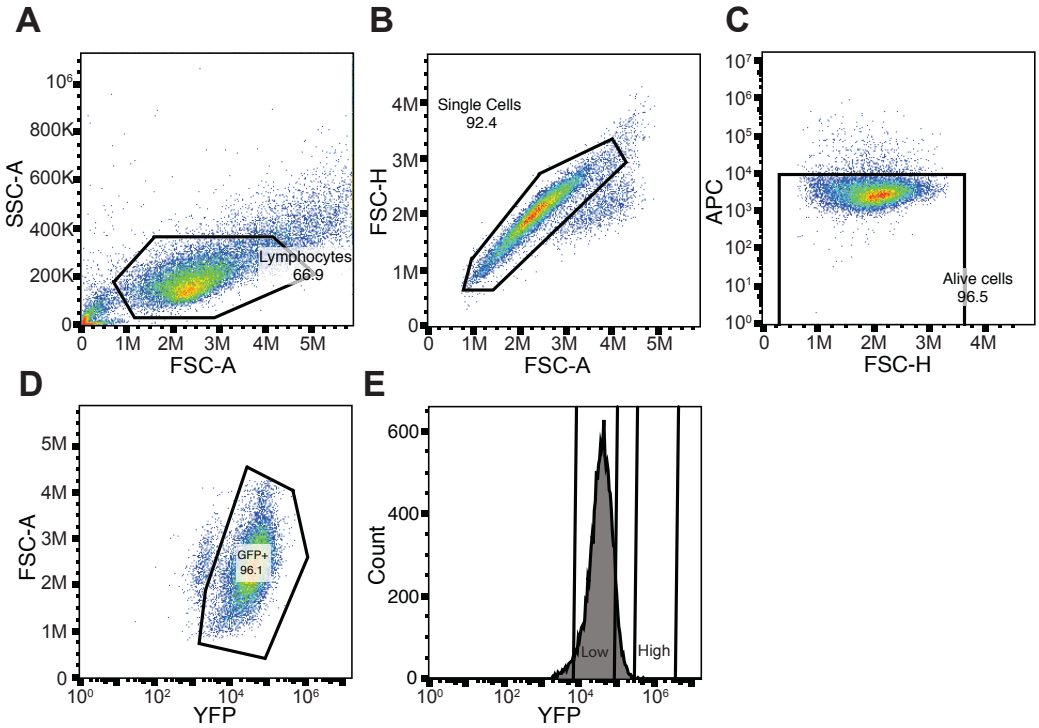


Figure S4: Gating strategies for fluorescence-activated cell sorting of iNOS-YFP reporter macrophages

(A,B) Individual RAW 264.7 macrophages were selected based on their forward scatter (FSC) and side scatter (SSC) (A) and by exclusion of doublets (B). (C,D) Live RAW 264.7 macrophages were selected as Sytox Red-negative (C) and YFP-positive (D) cells, which were subsequently used for flow sorting. (E) Gates were set to flow-sort iNOS-YFP^{low} and iNOS-YFP^{high} subpopulations comprising the 12% lowest and 12% highest cells in the distribution, respectively.

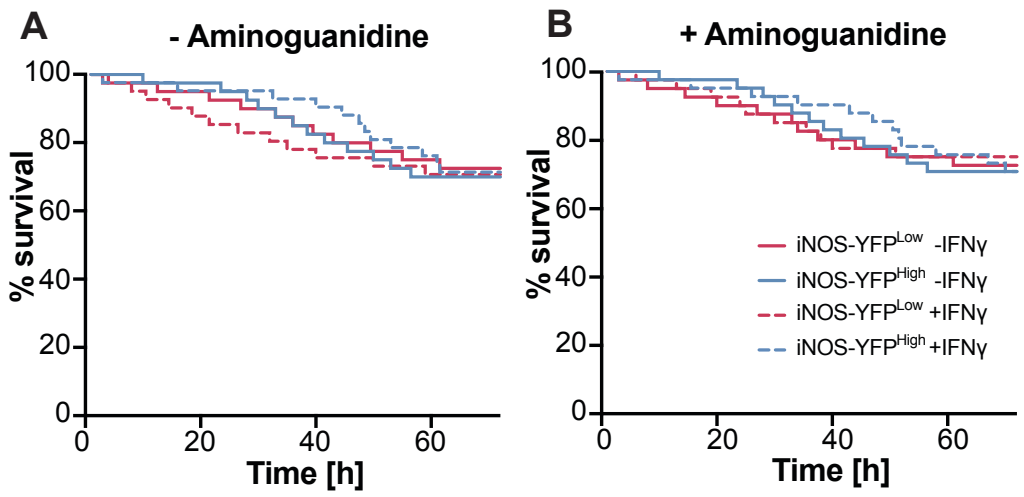


Figure S5: Survival of infected RAW 264.7 macrophages

Unactivated (-IFNγ) and activated (+IFNγ) RAW 264.7 macrophages that stably express YFP from the iNOS transcriptional promoter were flow-sorted into low- and high-fluorescence subpopulations and infected with *Mtb*. Single infected macrophages were identified and tracked using time-lapse microscopy, and their time of death was quantified. **(A-B)** Survival curves of infected macrophages untreated with Aminoguanidine **(A)** or treated with Aminoguanidine **(B).** In every condition, >70% of infected macrophages were alive at the end of the experiment (after 72 hours).

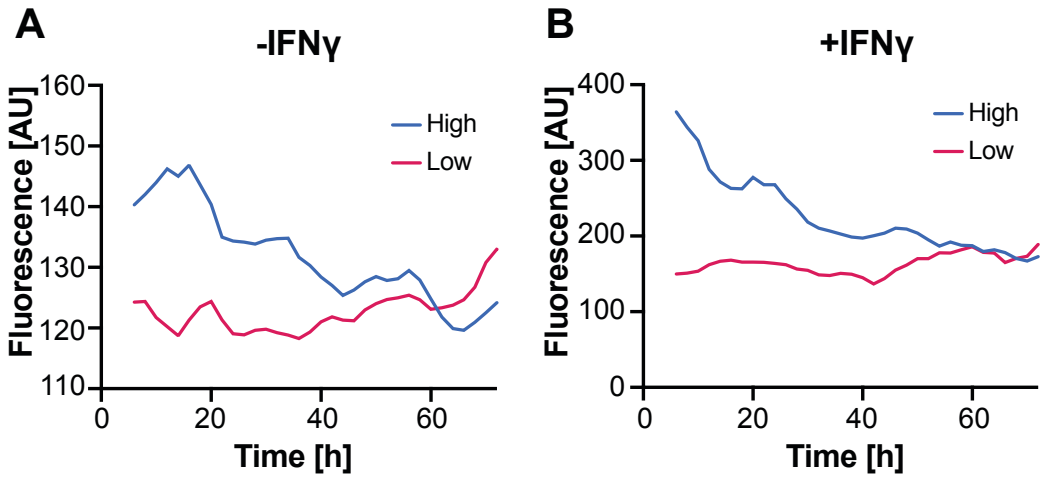


Figure S6: Single-cell fluorescence traces of iNOS-YFP reporter macrophages
Unactivated (-IFNγ) and activated (+IFNγ) RAW 264.7 macrophages that stably express YFP from the iNOS transcriptional promoter were flow-sorted into low- and high-fluorescence subpopulations and infected with *Mtb*. Single infected macrophages were identified and tracked using time-lapse microscopy. (A-B) Representative fluorescence profiles of single unactivated (A) or activated (B) macrophages originating from the iNOS-YFP^{High} or the iNOS-YFP^{Low} subpopulations.

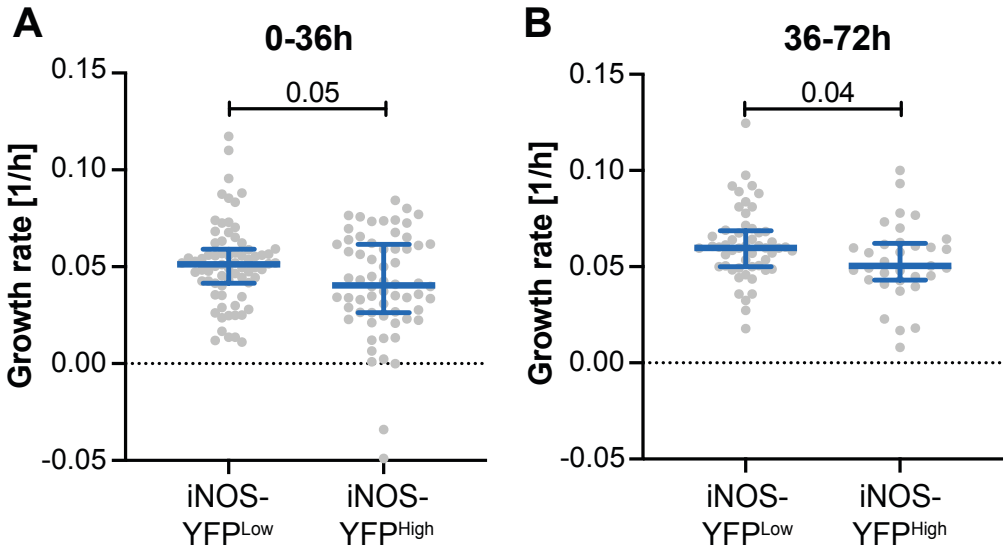


Figure S7: Growth rates of intracellular *M. tuberculosis* in iNOS-YFP^{low} and iNOS-YFP^{high} macrophages

Unactivated (-IFNγ) RAW 264.7 macrophages that stably express YFP from the iNOS transcriptional promoter were flow-sorted into low- and high-fluorescence subpopulations and infected with *Mtb*. The growth rate of intracellular Mtb was then measured by time-lapse microscopy. The bacterial growth rate was either quantified for the first 36 hours of the experiment (A) or from hours 36 to 72 (B). Blue lines indicate median values and interquartile ranges. P values were calculated using a Mann- Whitney test.

Table S1: Primers used for qRT-PCR characterization of iNOS-YFPlow and iNOS-YFP^{high} subpopulations of macrophages

NOS2
Forward Sequence GAGACAGGGAAGTCTGAAGCAC
Reverse Sequence CCAGCAGTAGTTGCTCCTCTTC

IRGM1
Forward Sequence CATTGCCTCTGAGCAGTTCAGC
Reverse Sequence CCTCTGATAGGACACTGGTGCT

IP10/CXCL10
Forward Sequence ATCATCCCTGCGAGCCTATCCT
Reverse Sequence GACCTTTTTTGGCTAAACGCTTTC

IFNGR1
Forward Sequence CTTGAACCCTGTCGTATGCTGG
Reverse Sequence TTGGTGCAGGAATCAGTCCAGG

IRF1
Forward Sequence TCCAAGTCCAGCCGAGACACTA
Reverse Sequence ACTGCTGTGGTCATCAGGTAGG

STAT1
Forward Sequence GCCTCTCATTGTCACCGAAGAAC
Reverse Sequence TGGCTGACGTTGGAGATCACCA

IL10
Forward Sequence CGGGAAGACAATAACTGCACCC
Reverse Sequence CGGTTAGCAGTATGTTGTCCAGC

VEGF
Forward Sequence CTGCTGTAACGATGAAGCCCTG
Reverse Sequence GCTGTAGGAAGCTCATCTCTCC

ARG1
Forward Sequence CATTGGCTTGCGAGACGTAGAC
Reverse Sequence GCTGAAGGTCTCTTCCATCACC

GAPDH
Forward Sequence CATCACTGCCACCCAGAAGACTG
Reverse Sequence ATGCCAGTGAGCTTCCCGTTCAG

Author Contributions

Ophélie Rutschmann: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Chiara Toniolo: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision.

John D. McKinney: Conceptualization, Writing – review & editing, Supervision, Funding Acquisition.

Acknowledgements

This work was supported by a grant to J.D.M. from the Swiss National Science Foundation (310030_207717). C.T. was supported by funding from the European Union's Horizon 2020 Research and Innovation program under Marie Skłodowska-Curie grant agreement 665667. This work was supported in part using the resources and services of the Flow Cytometry Core Facility at the School of Life Sciences of EPFL. We also thank Daniel Sage for help with image analysis and Carl Nathan for providing the RAW 264.7 iNOS-YFP reporter macrophages used in this study.

References

Ackermann M. 2015. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat Rev Microbiol* 13:497–508.

Avraham R, Haseley N, Brown D, Penaranda C, Jijon HB, Trombetta JJ, Satija R, Shalek AK, Xavier RJ, Regev A, Hung DT. 2015. Pathogen cell-to-cell variability drives heterogeneity in host immune responses. *Cell* 162:1309–1321.

Bain CC, MacDonald AS. 2022. The impact of the lung environment on macrophage development, activation and function: diversity in the face of adversity. *Mucosal Immunol* 15:223–234.

Bakkum T, Heemskerk MT, Bos E, Groenewold M, Oikonomeas-Koppasis N, Walburg KV, van Veen S, van der Lienden MJC, van Leeuwen T, Haks MC, Ottenhoff THM, Koster AJ, van Kasteren SI. 2020. Bioorthogonal correlative light-electron microscopy of Mycobacterium tuberculosis in macrophages reveals the effect of antituberculosis drugs on subcellular bacterial distribution. *ACS Cent Sci* 6:1997–2007.

Beaulieu AM, Rath P, Imhof M, Siddall ME, Roberts J, Schnappinger D, Nathan CF. 2010. Genome-wide screen for Mycobacterium tuberculosis genes that regulate host immunity. *PLoS One* 5:e15120.

Bhaskar A, Chawla M, Mehta M, Parikh P, Chandra P, Bhave D, Kumar D, Carroll KS, Singh A. 2014. Reengineering redox sensitive GFP to measure mycothiol redox potential of Mycobacterium tuberculosis during infection. *PLoS Pathog* 10:e1003902.

Bryson BD, Rosebrock TR, Tafesse FG, Itoh CY, Nibasumba A, Babunovic GH, Corleis B, Martin C, Keegan C, Andrade P, Realegeno S, Kwon D, Modlin RL, Fortune SM. 2019. Heterogeneous GM-CSF signaling in macrophages is associated with control of Mycobacterium tuberculosis. *Nat Commun* 10:e2329.

Cadena AM, Fortune SM, Flynn JL. 2017. Heterogeneity in tuberculosis. *Nat Rev Immunol* 17:691–702.

Chan J, Xing Y, Magliozzo RS, Bloom BR. 1992. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med* 175:1111–1122.

Cho HJ, Lim YJ, Kim J, Koh WJ, Song CH, Kang MW. 2020. Different macrophage polarization between drug-susceptible and multidrug-resistant pulmonary tuberculosis. *BMC Infect Dis* 20:81.

Cohen SB, Gern BH, Delahaye JL, Adams KN, Plumlee CR, Winkler JK, Sherman DR, Gerner MY, Urdahl KB. 2018. Alveolar macrophages provide an early Mycobacterium tuberculosis niche and initiate dissemination. *Cell Host Microbe* 24:439–446.e4.

Eldar A, Elowitz MB. 2010. Functional roles for noise in genetic circuits. *Nature* 467:167–173. Elowitz MB, Levine AJ, Siggia ED, Swain PS. 2002. Stochastic gene expression in a single cell. *Science* 297:1183–1186.

Friedman N, Cai L, Xie XS. 2006. Linking stochastic dynamics to population distribution: an analytical framework of gene expression. *Phys Rev Lett* 97:168302.

Gierahn TM, Wadsworth MH, Hughes TK, Bryson BD, Butler A, Satija R, Fortune S, Love JC, Shalek AK. 2017. Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. *Nat Methods* 14:395–398.

Houk VN, Baker JH, Sorensen K, Kent DC. 1968. The epidemiology of tuberculosis infection in a closed environment. *Arch Environ Health* 16:26–35.

Huang L, Nazarova EV, Tan S, Liu Y, Russell DG. 2018. Growth of Mycobacterium tuberculosis in vivo segregates with host macrophage metabolism and ontogeny. *J Exp Med* 215:1135–1152.

Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A, Maier B. 2009. A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci USA* 106:21407–21412.

Lafuse WP, Rajaram MVS, Wu Q, Moliva JI, Torrelles JB, Turner J, Schlesinger LS. 2019. Identification of an increased alveolar macrophage subpopulation in old mice that displays unique inflammatory characteristics and is permissive to Mycobacterium tuberculosis infection. *J Immunol* 203:2252–2264.

Lannan R, Maity A, Wollman R. 2022. Epigenetic fluctuations underlie gene expression timescales and variability. *Physiol Genomics* 54:220–229.

Lerner TR, Borel S, Greenwood DJ, Repnik U, Russell MR, Herbst S, Jones ML, Collinson LM, Griffiths G, Gutierrez MG. 2017. Mycobacterium tuberculosis replicates within necrotic human macrophages. *J Cell Biol* 216:583–594.

Lienard J, Nobs E, Lovins V, Movert E, Valfridsson C, Carlsson F. 2020. The Mycobacterium marinum ESX-1 system mediates phagosomal permeabilization and type I interferon production via separable mechanisms. *Proc Natl Acad Sci USA* 117:1160–1166.

Lin PL, Ford CB, Coleman MT, Myers AJ, Gawande R, Ioerger T, Sacchettini J, Fortune SM, Flynn JL. 2014. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nat Med* 20:75–79.

MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci USA* 94:5243–5248.

MacMicking JD, Taylor GA, McKinney JD. 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science* 302:654–659.

Mahamed D, Boulle M, Ganga Y, Mc Arthur C, Skroch S, Oom L, Catinas O, Pillay K, Naicker M, Rampersad S, Mathonsi C, Hunter J, Wong EB, Suleman M, Sreejit G, Pym AS, Lustig G, Sigal A. 2017. Intracellular growth of Mycobacterium tuberculosis after macrophage cell death leads to serial killing of host cells. *Elife* 6:e22028.

Manina G, Dhar N, McKinney JD. 2015. Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* 17:32–46.

Marakalala MJ, Raju RM, Sharma K, Zhang YJ, Eugenin EA, Prideaux B, Daudelin IB, Chen P-Y, Booty MG, Kim JH, Eum SY, Via LE, Behar SM, Barry CE, Mann M, Dartois V, Rubin EJ. 2016. Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nat Med* 22:531–538.

Martin CJ, Cadena AM, Leung VW, Lin PL, Maiello P, Hicks N, Chase MR, Flynn JL, Fortune SM. 2017. Digitally barcoding Mycobacterium tuberculosis reveals in vivo infection dynamics in the macaque model of tuberculosis. *mBio* 8:e00312-17.

McAdams HH, Arkin A. 1997. Stochastic mechanisms in gene expression. *Proc Natl Acad Sci USA* 94:814–819.

Melo EM, Oliveira VLS, Boff D, Galvão I. 2021. Pulmonary macrophages and their different roles in health and disease. *Int J Biochem Cell Biol* 141:106095.

Miller BH, Fratti RA, Poschet JF, Timmins GS, Master SS, Burgos M, Marletta MA, Deretic V. 2004. Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes during macrophage infection. *Infect Immun* 72:2872–2878.

Moxon R, Kussell E. 2017. The impact of bottlenecks on microbial survival, adaptation, and phenotypic switching in host-pathogen interactions. *Evolution* 71:2803–2816.
Pisu D, Huang L, Grenier JK, Russell DG. 2020. Dual RNA-Seq of Mtb-infected macrophages in vivo reveals ontologically distinct host-pathogen interactions. *Cell Rep* 30:335–350.e4.

Pisu D, Huang L, Narang V, Theriault M, Lê-Bury G, Lee B, Lakudzala AE, Mzinza DT, Mhango DV, Mitini-Nkhoma SC, Jambo KC, Singhal A, Mwandumba HC, Russell DG. 2021. Single cell

analysis of M. tuberculosis phenotype and macrophage lineages in the infected lung. *J Exp Med* 218:9.

Raffetseder J, Iakobachvili N, Loitto V, Peters PJ, Lerm M. 2019. Retention of EsxA in the capsule-like layer of Mycobacterium tuberculosis is associated with cytotoxicity and is counteracted by lung surfactant. *Infect Immun* 87:e00803-18.

Raj A, Peskin CS, Tranchina D, Vargas DY, Tyagi S. 2006. Stochastic mRNA synthesis in mammalian cells. *PLoS Biol* 4:e309.

Riley RL, Mills CC, Nyka W, Weinstock N, Storey PB, Sultan LU, Riley MC, Wells WF. 1995. Aerial dissemination of pulmonary tuberculosis. A two-year study of contagion in a tuberculosis ward. *Am J Epidemiol* 142:3–14.

Rohde K, Yates RM, Purdy GE, Russell DG. 2007. Mycobacterium tuberculosis and the environment within the phagosome. *Immunol Rev* 219:37–54.

Saini D, Hopkins GW, Seay SA, Chen CJ, Perley CC, Click EM, Frothingham R. 2012. Ultra-low dose of Mycobacterium tuberculosis aerosol creates partial infection in mice. *Tuberculosis (Edinb)* 92:160–165.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. FiJi. *Nat Methods* 9:676–682.

Schnettger L, Rodgers A, Repnik U, Lai RP, Pei G, Verdoes M, Wilkinson RJ, Young DB, Gutierrez MG. 2017. A Rab20-dependent membrane trafficking pathway controls M. tuberculosis replication by regulating phagosome spaciousness and integrity. *Cell Host Microbe* 21:619–628.e5.

Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, Brosch R, Enninga J. 2012. Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. *PLoS Pathog* 8:e1002507.

Simmons JD, Stein CM, Seshadri C, Campo M, Alter G, Fortune S, Schurr E, Wallis RS, Churchyard G, Mayanja-Kizza H, Boom WH, Hawn TR. 2018. Immunological mechanisms of human resistance to persistent Mycobacterium tuberculosis infection. *Nat Rev Immunol* 18:575–589.

Toniolo C, Rutschmann O, McKinney JD. 2021. Do chance encounters between heterogeneous cells shape the outcome of tuberculosis infections? *Curr Opin Microbiol* 59:72–78.

Ufimtseva EG, Eremeeva NI, Umpeleva TV, Vakhrusheva DV, Skornyakov SN. 2021. Mycobacterium tuberculosis load in host cells and the antibacterial activity of alveolar macrophages are linked and differentially regulated in various lung lesions of patients with pulmonary tuberculosis. *Int J Mol Sci* 22:3452.

van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, Brenner M, Peters PJ. 2007. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 129:1287–1298.

Weiss G, Schaible UE. 2015. Macrophage defense mechanisms against intracellular bacteria. *Immunol Rev* 264:182–203.

Welin A, Lerm M. 2012. Inside or outside the phagosome? The controversy of the intracellular localization of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 92:113–120.

Chapter 3. Activation of the non-canonical inflammasome by *Mycobacterium tuberculosis* promotes IL-1β secretion from infected macrophages

Ophélie Rutschmann, Chiara Toniolo & John D. McKinney

School of Life Sciences, EPFL, Lausanne, Switzerland

Manuscript in preparation

Abstract

The pro-inflammatory cytokine Interleukin-1 beta (IL-1β) plays a pivotal role in host defense against *Mycobacterium tuberculosis* by promoting inflammation and recruiting immune cells to the site of infection. Tight regulation of IL-1β secretion is critical to maintain a balance between immune defense and inflammation. Previous studies have demonstrated that IL-1β secretion can be mediated by the NLRP3 and AIM2 inflammasomes upon *M. tuberculosis* infection. Here we demonstrate that IL-1β can also be secreted upon activation of the non-canonical inflammasome in both murine and human macrophages infected with *M. tuberculosis*. Caspase-11, Caspase-4 and Caspase-5 undergo auto-proteolysis and activation in infected macrophages, resulting in IL-1β secretion. Secreted IL-1β can enhance the anti-mycobacterial properties of neighboring macrophages in a paracrine manner by promoting autophagy. Interestingly, Caspase-11 expression is induced in myeloid cells from the lungs of infected mice, suggesting a role for the control of *M. tuberculosis* infection in-vivo. Our findings thus identify a novel mechanism for IL-1β secretion in *M. tuberculosis*-infected cells and suggest a potential role for the non-canonical inflammasome in defense against mycobacterial infections.

Introduction

Mycobacterium tuberculosis is a highly effective pathogen that infects nearly one-third of the world's population. An *M. tuberculosis* infection begins when bacteria enter the lung alveoli, where they are phagocytosed by alveolar macrophages. This internalization event triggers protective immune mechanisms crucial for the control of the infection, among which the secretion of interleukin-1β (IL-1β). This pro-inflammatory cytokine plays a pivotal role in disease control by activating and recruiting immune cells to the site of infection (Silvério et al., 2021). Studies have demonstrated that mice lacking the ability to sense or secrete IL-1β exhibit heightened susceptibility to infection with *M. tuberculosis*, with increased bacterial burdens and enhanced mortality rates (Fremond, Togbe, et al., 2007; Juffermans et al., 2000; Saiga et al., 2012; Yamada et al., 2000).

As overproduction of IL-1β can induce lung damage, its secretion is tightly regulated and requires sensing of two different signals by the host cell (Rastogi & Briken, 2022; Silvério et al., 2021). The first signal can originate from extracellular bacterial components (such as LPS) or pro-inflammatory cytokines (such as TNFα or type 1 interferons). Upon sensing of this priming signal, the host cells upregulate the transcription of pro-IL-1β and other inflammasome-related genes. The processing of pro-IL-1β into its active form and its release from the cell requires the sensing of a second signal, which triggers the assembly of cytosolic molecular platforms known as inflammasomes (Martinon et al., 2002; Rastogi & Briken, 2022). In the case of an *M. tuberculosis* infection, two proteins are involved in initiating the assembly of the inflammasome: the cytosolic DNA sensor AIM2, and the pyrin-domain containing protein NLRP3. AIM2 detects intracellular *M. tuberculosis* DNA, while NLRP3 responds to other intracellular *M. tuberculosis* components or to infection-induced plasma membrane disruptions (Beckwith et al., 2020; Kurenuma et al., 2009; B. B. Mishra, Moura-alves, et al., 2010; Rastogi & Briken, 2022; Saiga et al., 2012; Wassermann et al., 2015; Yang et al., 2020). Upon sensing of these signals, AIM2 and NLRP3 associate with the protein ASC and the cysteine protease pro-caspase-1. The resulting inflammasome complex supports the cleavage and activation of caspase-1, which in turn cleaves pro-IL-1β into its active form, and GasderminD into its pore-forming N-terminus fragment. IL-1β is subsequently released from the cell through GasderminD pores in a process called pyroptosis, leading to lysis of the infected cell (Rastogi & Briken, 2022).

Alternatively, IL-1β secretion can be induced through the activation of a non-canonical inflammasome. This pathway, which involves Caspase-11 in mice and Caspase-4 and -5 in humans, is crucial for immune defense against gram-negative

bacteria. Upon infection, cytosolic LPS can bind directly to Caspase-11 and Caspase-4/-5, resulting in their oligomerization and auto-proteolysis. Activated Caspase-11/-4/-5 can subsequently cleave GasderminD into its N-terminus fragment, which triggers pyroptosis and facilitates IL-1β release (Kayagaki et al., 2015; B. L. Lee et al., 2018; J. Shi et al., 2014, 2015). The formation of GasderminD pores also promotes potassium efflux from the cell, which in turns leads to the activation of NLRP3 and the canonical inflammasome pathway (Rühl & Broz, 2015).

While the role of the non-canonical inflammasome in response to gram-negative bacterial infections is well-characterized, its involvement in the context of Tuberculosis is less clear. Preliminary evidence suggests that Caspase-11 is activated in cells infected with the non-pathogenic mycobacteria *M. smegmatis* expressing the tuberculosis protein PE_PGRS19, but to our knowledge, no experiments with pathogenic mycobacteria have been performed (Qian et al., 2022).

In this study, we investigated the mechanisms leading to non-canonical inflammasome activation and IL-1β secretion in human and murine macrophages infected with *M. tuberculosis*. We observe that Caspase-11 (in murine bone-marrow-derived macrophages) and Caspase-4/-5 (in human THP-1 macrophages) undergo processing and activation during *M. tuberculosis* infection, leading to IL-1β secretion and pyroptosis. We further show that IFNγ, a key cytokine implicated in host defense against *M. tuberculosis*, potentiates non-canonical inflammasome activation in both cell types. Interestingly, Caspase-11 and Caspase-4/-5 seem to act upstream of the canonical inflammasome pathway as their inhibition leads to a decrease in caspase-1 activity. Additionally, we reveal that IL-1β secreted by infected macrophages enhances neighboring macrophage's control over *M. tuberculosis* growth. Finally, we provide evidence that Caspase-11 is expressed in the lungs of infected mice, pointing towards a potential role for disease pathogenesis *in vivo*. To the best of our knowledge, our work represents the first evidence of non-canonical inflammasome activation in response to *M. tuberculosis* infection in both murine and human macrophages, shedding new light on the innate immune response against this pathogen.

Results

Caspase-11 is processed and proteolytically active in IFNγ-activated macrophages infected with *M. tuberculosis*.

The murine cysteine-aspartic protease 11 (Caspase-11) is expressed as an inactive precursor containing a caspase activation and recruitment domain (CARD) linked to a large (p20) and a small (p10) catalytically active subunit (Figure 1A). Caspase-11 oligomerizes and undergoes auto-proteolysis in response to intracellular LPS, which results in its activation and ultimately leads to IL-1β secretion and death of the cell by pyroptosis (Shi et al., 2014, 2015).

We assessed Caspase-11 processing 24 hours post *M. tuberculosis* infection by immunoblotting analysis of unactivated and IFNγ-activated bone marrow derived macrophages (BMDMs). Caspase-11 is cleaved in infected macrophages, as evidenced by the presence of a p32 subunit in the immunoblot (Figure 1B). Interestingly, this effect is strongly potentiated by IFNγ activation. To ascertain that Caspase-11 cleavage is associated with increased proteolytic activity, we treated the macrophages with a fluorescent substrate specific for Caspase-11 (Z-LEVD-AFC) and quantified fluorescence intensity 24 hours post-infection. *M. tuberculosis* infection leads to an increase in Caspase-11 activity, with this effect also potentiated by IFNγ activation (Figure 1C, supplementary Figure 1A).

IFNγ and *M. tuberculosis* synergistically upregulate Caspase-11 expression.

IFNγ can enhance non-canonical inflammasome activation by inducing the upregulation of several inflammasome-related genes, including Caspase-11 (Schauvliege et al., 2002). In line with this, we assessed the expression levels of inflammasome-related genes in both unactivated and IFNγ-activated BMDMs 6 hours post-infection. IFNγ induces the expression of Caspase-11, Aim2, IL-18 and Caspase-1 in both uninfected and infected macrophages (Figure 1D, Supplementary Figure 2A). Remarkably, the combination of *M. tuberculosis* infection and IFNγ-activation has a synergistic effect on Caspase-11 expression, and this effect seems to be mediated by the sensing of *M. tuberculosis* lipids (Figure 1D, Supplementary Figure 2B). Taken together, our results suggest that Caspase-11 is processed and activated in macrophages infected with *M. tuberculosis*, and that IFNγ can act as a priming signal by inducing the upregulation of Caspase-11.

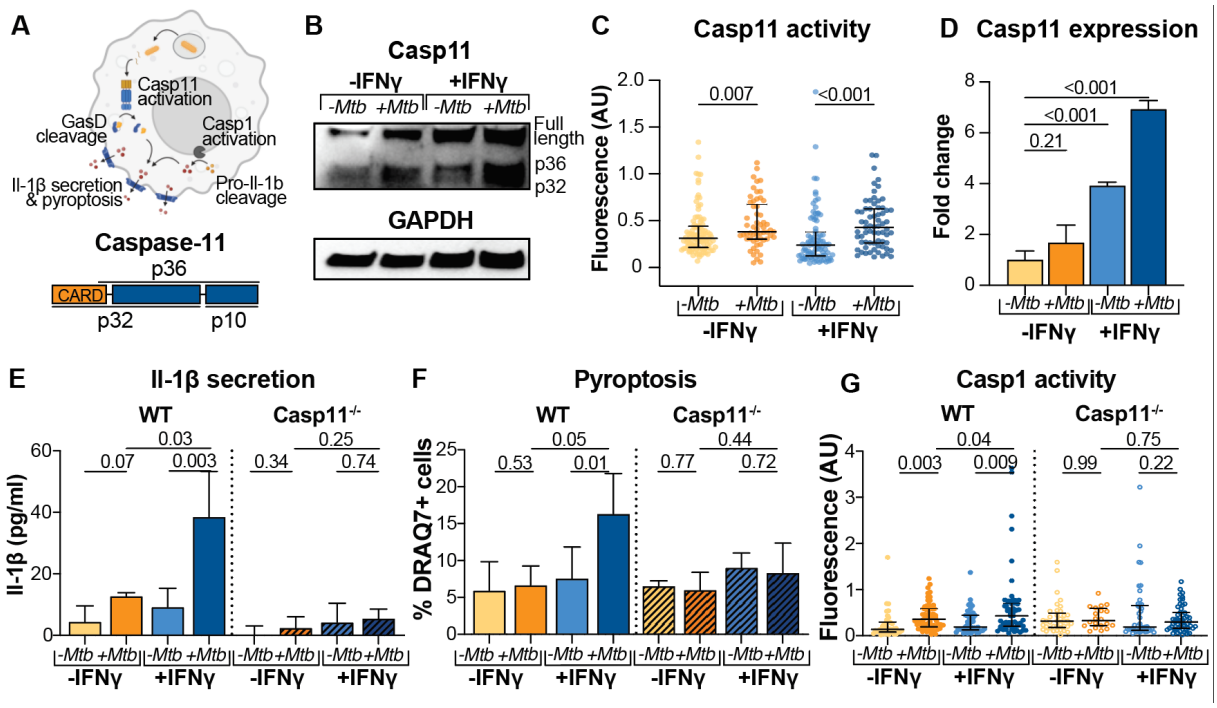


Figure 1: Caspase-11 mediates IL-1β secretion and pyroptosis in IFNγ-activated macrophages infected with *M. tuberculosis*

(A) Caspase-11 is a murine protease composed of a caspase activation and recruitment domain (CARD, orange) and two catalytic subunits (blue). Upon sensing LPS, Caspase-11 oligomerizes and undergoes auto-proteolysis, which leads to its activation, subsequent secretion of IL-1β, and death of the cell by pyroptosis.

(B-D) Unactivated (-IFNγ) and activated (+IFNγ) WT BMDMs were infected with *M. tuberculosis*. (B) Caspase-11 processing was assessed 24 hours post-infection by immunoblotting analysis of pooled cell lysates and supernatants. (C) Caspase-11 activity was quantified 24 hours post-infection using the fluorescent substrate for Caspase-11 Z-LEVD-AFC and fluorescence microscopy. Each dot represents a single macrophage. (D) Caspase-11 expression levels were measured 6 hours post-infection by qPCR.

(E-G) Unactivated (-IFNγ) and activated (+IFNγ) WT and Caspase-11$^{-/-}$ BMDMs were infected with *M. tuberculosis.* (E) The concentration of IL-1β in the supernatant 24 hours post-infection was quantified using an ELISA. (F) Macrophages were incubated with DRAQ7 and the apoptosis inhibitor Z-DEVD-FMK and imaged for 36 hours using time-lapse microscopy. The percentage of DRAQ7 positive was quantified and used as an indicator of pyroptosis. (G) Caspase-1 activity was measured 24 hours post-infection using the fluorescent substrate for Caspase-1 Z-YVAD-AFC and fluorescence microscopy. Each dot represents a single macrophage. P-values were calculated using a Student t-test (D-F) or a Mann-Whitney test (C&G).

Caspase-11 mediates IL-1β secretion in macrophages infected with *M. tuberculosis.*

We next investigated the role of Caspase-11 in mediating IL-1β secretion, pyroptosis and canonical inflammasome activation in macrophages infected with *M. tuberculosis*. We observe that in WT macrophages, the concentration of secreted IL-1β correlates with Caspase-11 activity, with IFNγ-activated macrophages secreting more IL-1β than unactivated macrophages upon infection with *M. tuberculosis* (Figure

1E). In Caspase-11$^{-/-}$ BMDMs however, IL-1β secretion upon infection with *M. tuberculosis* is abolished (Figure 1E).

In macrophages infected with gram-negative bacteria, Caspase-11 activation triggers death of the cell by pyroptosis. To investigate this in *M. tuberculosis*-infected macrophages, we incubated the cells with DRAQ7, a marker for cell death, and imaged them for 36 hours. To exclude the possibility of cell death by apoptosis, we added the apoptosis inhibitor Z-DEVD-FMK to the medium throughout the experiment, as previously described (Beckwith et al., 2020). *M. tuberculosis* infection results in an increase in the total number of DRAQ7-positive cells, but only when the macrophages are activated with IFNγ (Figure 1F). Caspase-11 contributes to this process, as infected Caspase-11$^{-/-}$ macrophages do not undergo more pyroptosis than uninfected cells (Figure 1F).

Finally, we investigated whether Caspase-11 acts upstream of the canonical inflammasome, as previously suggested, by measuring Caspase-1 activity in infected cells using the fluorescent substrate FAM-YVAD-FMK. In WT BMDMs, *M. tuberculosis* infection results in increased Caspase-1 activity in both unactivated and IFNγ-activated macrophages (Figure 1G). However, in Caspase-11$^{-/-}$ BMDMs, no increase in Caspase-1 activity is observed upon infection, suggesting that Caspase-11 acts upstream of Caspase-1 in *M. tuberculosis*-infected macrophages (Figure 1G).

IL-1β secretion by infected macrophages increases growth control of *M. tuberculosis* by bystander macrophages.

IL-1β is a pro-inflammatory cytokine that activates macrophages and enhances their anti-mycobacterial properties (Pilli, Arko-mensah, et al., 2012) (Supplementary Figure 3A-B). We hypothesized that IL-1β secreted by macrophages infected with *M. tuberculosis* can act in a paracrine manner to increase the anti-mycobacterial properties of neighboring macrophages. To investigate this, we used a transwell system to separate infected macrophages (on top of the transwell) from their uninfected neighbors (in the well below) (Figure 2A).

We first assessed whether there is a difference in the control of *M. tuberculosis* growth between bystander BMDMs that have sensed IL-1β secreted by infected macrophages and naïve BMDMs that have not been exposed to this signal. We either infected the "signaler" macrophages on top of the transwell with a fluorescent strain of *M. tuberculosis* or left them uninfected, and subsequently infected the "receiver" macrophages underneath 24 hours later. We then used fluorescence time-lapse microscopy to determine the growth rate of single intracellular bacterial microcolonies

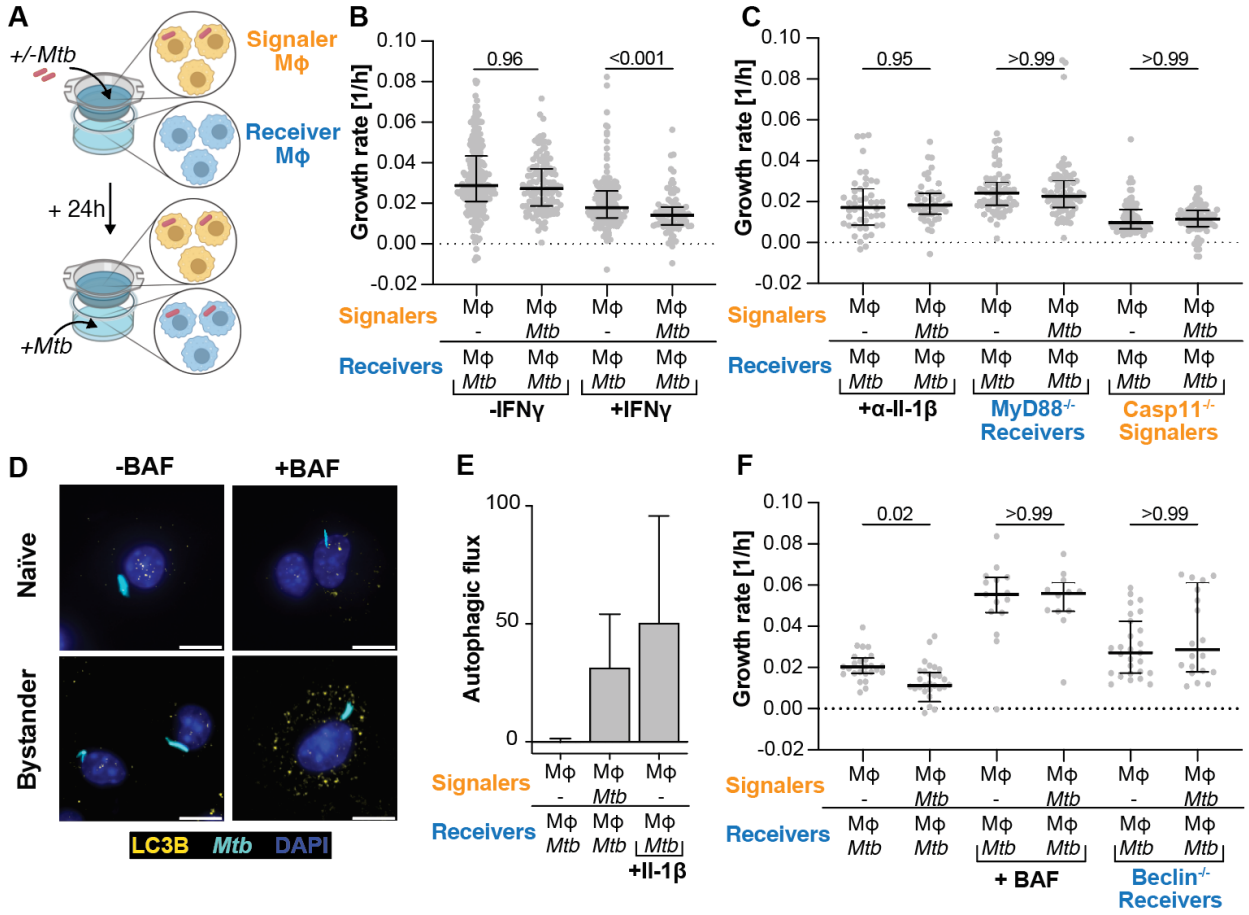


Figure 2: Caspase-11-dependent Il-1β secretion leads to increased control of *M. tuberculosis growth* by bystander macrophages

(A) A transwell system is used to separate "signaler" macrophages (top, yellow) from "receiver" macrophages (bottom, blue). Signaler macrophages are either infected with M. tuberculosis or left uninfected and incubated with uninfected receiver macrophages for 24 hours. Subsequently, receiver macrophages are infected with *M. tuberculosis* and the growth rate of intracellular bacterial microcolonies is quantified using time lapse microscopy. In each experiment, naïve receiver macrophages (under uninfected signaler macrophages) are compared to bystander receiver macrophages (under infected signaler macrophages). When required, IFNγ is added at the beginning of the experiment. Each dot represents the growth rate of a M. tuberculosis microcolony growing in a single macrophage.

(B) The growth rate of *M. tuberculosis* microcolonies within unactivated and IFNγ-activated receiver BMDMs was quantified using time-lapse microscopy. (C) The growth rate of *M. tuberculosis* microcolonies inside IFNγ-activated WT receiver BMDMs treated with neutralizing antibodies against Il-1β, IFNγ-activated MyD88$^{-/-}$ receiver BMDMs, and IFNγ-activated WT BMDMs bystanders of Caspase-11$^{-/-}$ signaler BMDMs was quantified using time-lapse microscopy.

(D-E) Autophagic flux was assessed by LC3b-II immunostaining in naïve receiver macrophages (under uninfected signaler macrophages), bystander receiver macrophages (under infected signaler macrophages) and macrophages treated with 200 ng/ml IL-1β. Autophagic flux was defined as the difference in the mean number of LC3b-II punctae between Bafilomycin-treated macrophages and untreated macrophages. (F) The growth rate of *M. tuberculosis* microcolonies inside IFNγ-activated WT receiver BMDMs, IFNγ-activated WT receiver BMDMs treated with Bafilomycin, and Beclin$^{-/-}$ receiver macrophages was quantified using time-lapse microscopy. P-values were calculated using a Student t-test (E) or a Mann-Whitney test (B-C; F).

within the receiver macrophages (Figure 2A). Interestingly, bystander BMDMs control *M. tuberculosis* growth better than naïve BMDMs, but only in the presence of IFNγ (Figure 2B).

To validate the role of IL-1β in enhancing bacterial growth control by bystander macrophages, we repeated the experiments adding neutralizing antibodies against IL-1β in the culture medium. Under these conditions, bystander BMDMs no longer exhibit improved control over *M. tuberculosis* growth compared to naïve macrophages (Figure 2C, Supplementary Figure 3C). Furthermore, bystander MyD88$^{-/-}$ BMDMs, which cannot respond to IL-1β, also fail to exhibit enhanced *M. tuberculosis* growth control. Similarly, WT macrophages bystander of Caspase-11$^{-/-}$ signaler BMDMs, which do not secrete IL-1β, also do not exhibit enhanced *M. tuberculosis* growth control (Figure 2C). Taken together, our results suggest that Caspase-11-dependent IL-1β secretion by infected macrophages can enhance the anti-mycobacterial properties of bystander macrophages.

Enhanced growth control of *M. tuberculosis* by bystander macrophages is mediated by increased autophagy.

IL-1β has been shown to induce autophagy in macrophages, a mechanism crucial for host defense against *M. tuberculosis* (Pilli, Arko-mensah, et al., 2012). We hypothesized that bystander macrophages can control *M. tuberculosis* growth better than naïve macrophages thanks to increased autophagy. To test this hypothesis, we quantified autophagic flux in bystander and naïve macrophages infected with *M. tuberculosis* by immunostaining. Interestingly, infected bystander macrophages display an increase in autophagic flux in comparison to naïve macrophages (Figure 2D-E). Additionally, inhibiting autophagy using Bafilomycin or using autophagy-deficient Beclin$^{-/-}$ receiver macrophages abolishes the differences in *M. tuberculosis* growth control between bystander and naïve macrophages. This suggests that bystander macrophages control *M. tuberculosis* growth better than naïve macrophages thanks to an increase in their autophagic capacity (Figure 2F, Supplementary Figure 4A).

Caspase-11 gene expression is upregulated upon infection with *M. tuberculosis in-vivo.*

To assess the relevance of our findings in-vivo, we analyzed a publicly available single-cell RNA sequencing dataset published by Pisu et al. (2021). In their study, Pisu and colleagues infected C57BL/6 mice for 21 days with *M. tuberculosis* and collected infected and uninfected (bystander) CD45+ cells from the lungs for

sequencing. As a control, CD45+ cells from the lungs of uninfected mice were also analyzed. The authors processed and analyzed the dataset and clustered the cells into different subpopulations (reproduced in Figure 3A).

We first determined the percentage of CD45+ cells expressing Caspase-11 in the lungs of uninfected and infected mice. Interestingly, we observed a baseline presence of Caspase-11+ cells in the lungs of uninfected mice, with this proportion significantly increasing upon infection with *M. tuberculosis* (4.3% in uninfected mice compared to 19.6% in infected mice, Figure 3B). Caspase-11 expression is not restricted to CD45+ cells actively harvesting bacteria but is also evident in uninfected bystander cells (20.7% of infected and 16.6% of bystander CD45+ cells express Caspase-11, Figure 3B). In uninfected mice, the primary population of cells expressing Caspase-11 consists of alveolar macrophages, while in infected mice

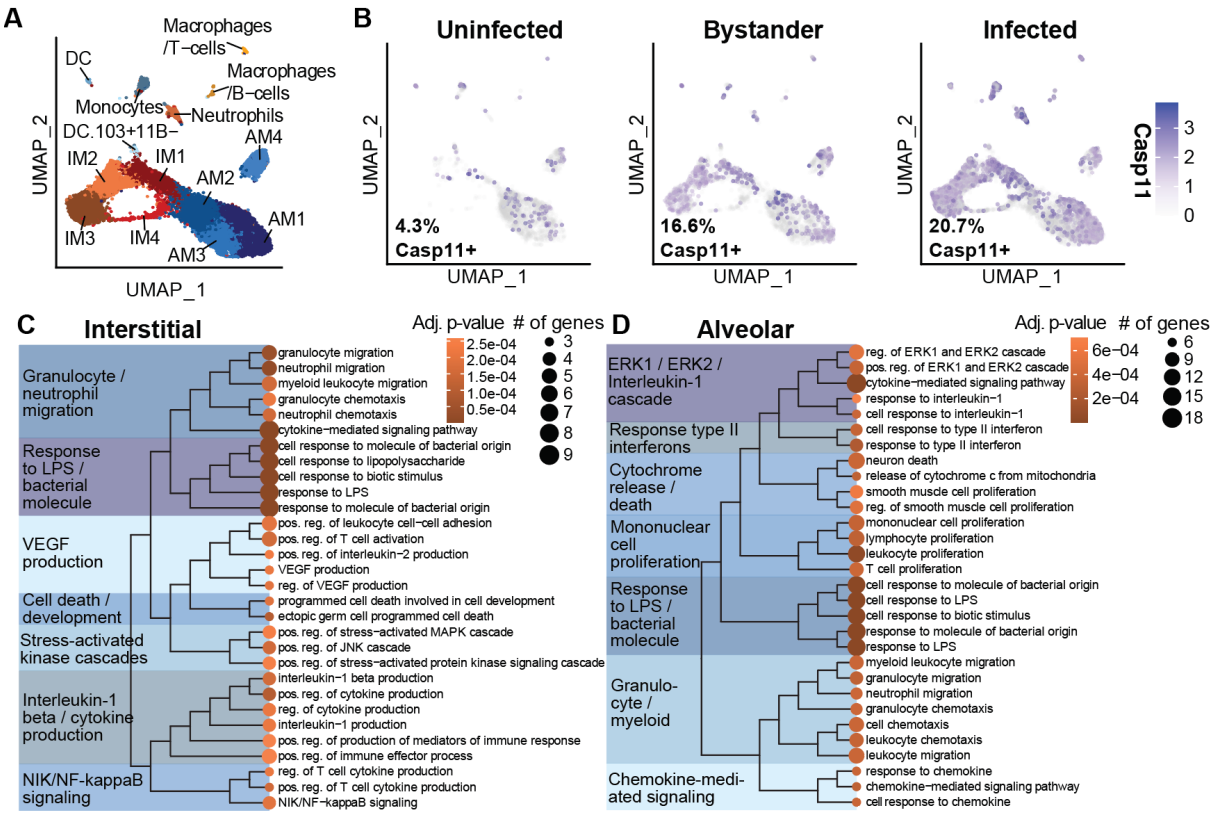


Figure 3: Caspase-11 gene expression is upregulated upon infection with *M. tuberculosis* in-vivo.
(A) Reproduction of a Umap plot showing unbiased clustering of CD45+ cells in the dataset, as analyzed by Pisu et al., (2021). (B) Umap plots showing Caspase-11 expression levels (in log-normalized counts) in CD45+ cells extracted from the lungs of uninfected mice (Uninfected) or from mice infected with M. tuberculosis (Bystander & Infected). (C-D) Gene set enrichment analysis comparing Caspase-11+ and Caspase-11- interstitial (C) and alveolar (D) macrophages infected with M. tuberculosis. The top 30 significantly over-represented Gene Ontology (GO, BP) terms were aggregated using semantic similarity for easier visualization.

there are more Caspase-11+ interstitial macrophages (Supplementary Figure 5A-B). Interestingly, within infected mice, the subpopulation of alveolar macrophages exhibiting the strongest Caspase-11 expression is the AM_1 subpopulation, which has been characterized as pro-inflammatory by Pisu and colleagues.

We then performed a pathway enrichment analysis to gain insight on the differences between Caspase-11+ and Caspase-11- macrophages infected with *M. tuberculosis*. In both interstitial and alveolar macrophage populations, Caspase-11+ and Caspase-11- macrophages differ in their pro-inflammatory signature (Figure 3C-D). Specifically, Caspase-11+ macrophages show upregulation of genes involved in the MAPK/ERK and NF-kB signaling pathway, as well as genes involved in the secretion of pro-inflammatory cytokines such as IL-1β and VEGF (Figure 3C-D).

Caspase-4 and Caspase-5 are processed and proteolytically active in human macrophages infected with *M. tuberculosis.*

Caspase-11 has two orthologs in humans, Caspase-4 and Caspase-5. Given the slight variations in the mechanisms of non-canonical inflammasome activation between human and murine cells, we explored whether Caspase-4 and Caspase-5 processing and activity could also be observed in human THP-1 macrophages. By immunoblotting analysis of unactivated and IFNγ-activated THP-1 macrophages infected with *M. tuberculosis*, we confirmed that Caspase-4 and Caspase-5 are processed in infected THP-1 macrophages, with this effect being potentiated by IFNγ-activation (Figure 4A). Using the fluorescent substrate for Caspase-4/-5 Z-LEVD-AFC, we further confirmed that the increased processing of Caspase-4/-5 correlates with increased proteolytic activity in infected macrophages (Figure 4B).

We then investigated whether IFNγ could also act as a priming signal for non-canonical inflammasome activation in human macrophages by quantifying the expression of inflammasome-related genes in unactivated and IFNγ-activated macrophages 6 hours post infection. Caspase-4 expression is not upregulated by IFNγ-activation or *M. tuberculosis* infection, while Caspase-5 is slightly upregulated upon IFNγ-activation (Figure 4C-D, Supplementary Figure 6A). This suggests that the mechanism leading to IFNγ-potentiation of Caspase-4/-5 activity in human macrophages does not seem to be directly linked to the upregulation of Caspase-4 or Caspase-5 expression, in contrast to what was observed in murine macrophages.

Caspase-4/-5 mediated IL-1β secretion in human macrophages infected with *M. tuberculosis.*

We investigated the role of Caspase-4/-5 in mediating IL-1β secretion, pyroptosis and canonical inflammasome activation in human macrophages infected with *M. tuberculosis*. We observe that in THP-1 macrophages, the concentration of secreted IL-1β correlates with Caspase-4/-5 activity, with IFNγ-activated macrophages secreting more IL-1β than unactivated macrophages upon infection with *M. tuberculosis* (Figure 4E). In THP-1 macrophages treated with the Caspase-4/-5 inhibitor ICH-2 however, IL-1β secretion upon infection with *M. tuberculosis* is greatly diminished (Figure 4E, Supplementary Figure 7A).

Caspase-4/-5 activation has been reported to trigger death of the cell by pyroptosis upon infection with gram-negative bacteria. To investigate this in *M. tuberculosis*-infected macrophages, we incubated the cells with DRAQ7, a marker for cell death, and imaged them for 36 hours, as described above. *M. tuberculosis* infection results in a slight increase in the total number of DRAQ7-positive cells, but only when the macrophages are activated with IFNγ (Figure 4F). Caspase-4/-5 contribute to this process, as infected THP-1 macrophages treated with a Caspase-4/-5 inhibitor do not die more of pyroptosis than uninfected cells (Figure 4F).

Finally, we investigated whether Caspase-4/-5 act upstream of the canonical inflammasome by measuring Caspase-1 activity in infected cells using the fluorescent substrate FAM-YVAD-FMK. In untreated THP-1 macrophages, *M. tuberculosis* infection results in increased Caspase-1 activity in both unactivated and IFNγ-activated macrophages (Figure 4G). However, in macrophages treated with the Caspase-4/-5 inhibitor, no increase in Caspase-1 activity is observed upon infection, suggesting that Caspase-4/-5 acts upstream of Caspase-1 in *M. tuberculosis*-infected macrophages, similarly to what was observed in murine macrophages (Figure 4G).

IL-1β secretion by infected human macrophages increases growth control of *M. tuberculosis* by bystander macrophages.

We next investigated the role of secreted IL-1β in enhancing *M. tuberculosis* growth control by human bystander macrophages. As described with murine macrophages, we used a transwell system to investigate whether there is a difference in the control of *M. tuberculosis* growth between bystander THP-1 macrophages that have sensed IL-1β secreted by infected macrophages and naïve THP-1 macrophages that have not been exposed to this signal. We observe that bystander

THP-1 macrophages control *M. tuberculosis* growth better than naïve THP-1 macrophages, both in the presence and absence of IFNγ (Figure 4H).

To validate the role of IL-1β we repeated the experiment adding neutralizing antibodies against IL-1β to the culture medium. Under these conditions, bystander THP-1 macrophages no longer exhibit improved control over *M. tuberculosis* growth compared to naïve macrophages, both in the presence and the absence of IFNγ (Figure 4I). Finally, to confirm the role of Caspase-4/-5, we repeated the experiment adding a Caspase-4/-5 inhibitor to the culture medium. Under these conditions, bystander THP-1 macrophages also no longer exhibit improved control over *M. tuberculosis* growth compared to naïve macrophages (Figure 4J). Taken together, these results suggest that Caspase-4/-5-dependent IL-1β secretion by infected human macrophages acts in a paracrine manner to enhance the anti-mycobacterial properties of bystander macrophages, as was observed in murine macrophages.

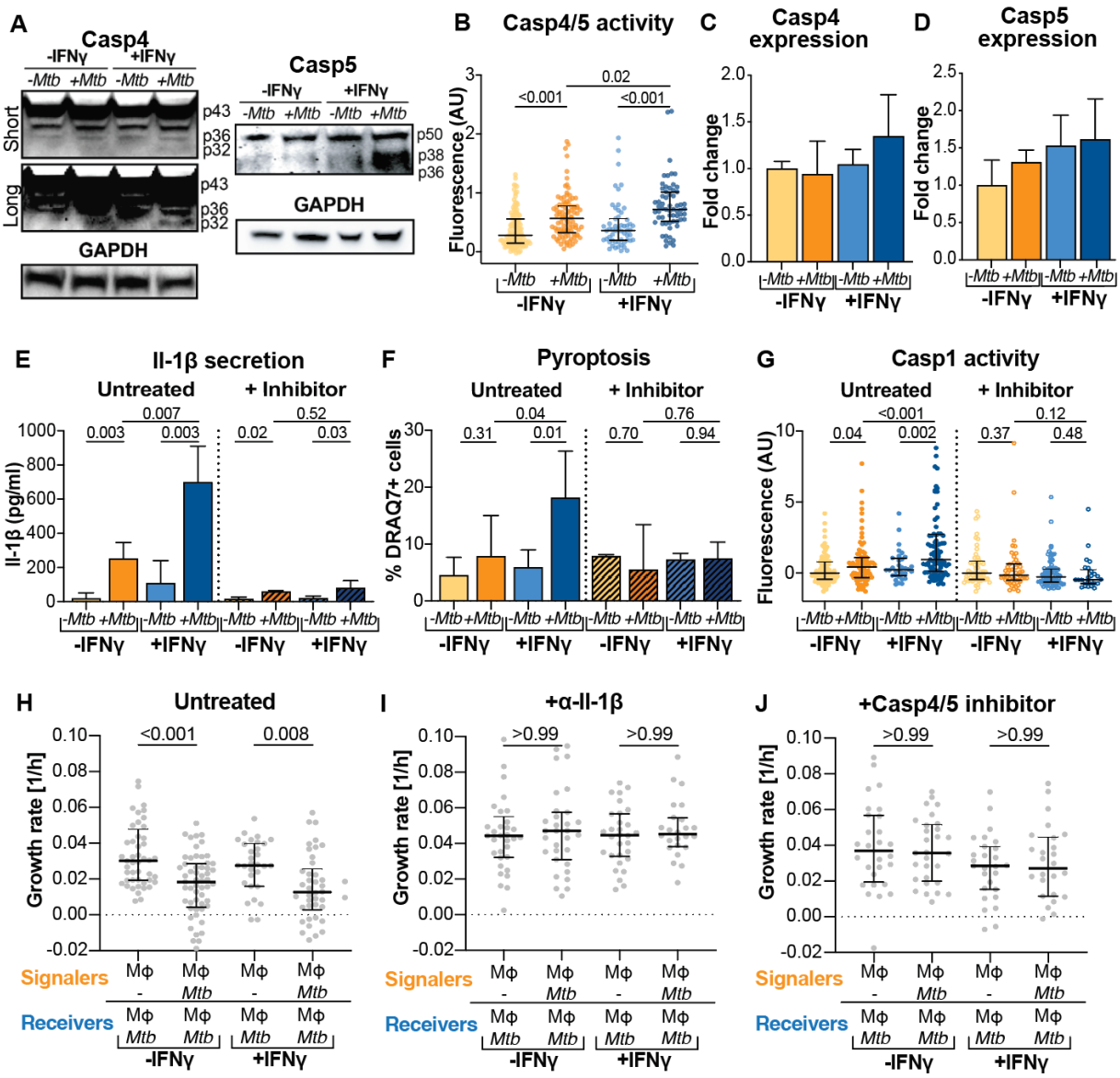


Figure 4: Caspase-4 and Caspase-5 mediate IL-1β secretion and pyroptosis in THP-1 macrophages infected with *M. tuberculosis*

(A-D) Unactivated (-IFNγ) and activated (+IFNγ) THP-1 macrophages were infected with *M. tuberculosis.* (A) Caspase-4 and Caspase-5 processing were assessed 24 hours post-infection by immunoblotting analysis of pooled cell lysates and supernatants. (B) Caspase-4/-5 activity was quantified 24 hours post-infection using the fluorescent substrate for Caspase-4/-5 Z-LEVD-AFC and fluorescence microscopy. Each dot represents a single macrophage. Caspase-4 (C) and Caspase-5 (D) expression levels were measured 6 hours post-infection by qPCR.

(E-G) Unactivated (-IFNγ) and activated (+IFNγ) THP-1 macrophages either untreated or treated with the Caspase-4/-5 inhibitor ICH-2 were infected with *M. tuberculosis*. (E) The concentration of IL-1β in the supernatant 24 hours post-infection was quantified using ELISA. (F) Macrophages were incubated with DRAQ7 and the apoptosis inhibitor Z-DEVD-FMK and imaged for 36 hours using time-lapse microscopy. The percentage of DRAQ7 positive was quantified and used as an indicator of pyroptosis. (G) Caspase-1 activity was measured 24 hours post-infection using the fluorescent substrate for Caspase-1 Z-YVAD-AFC and fluorescence microscopy. Each dot represents a single macrophage.

(H-J) A transwell system is used to separate "signaler" macrophages (top, yellow) from "receiver" macrophages (bottom, blue). Signaler macrophages are either infected with *M.*

tuberculosis or left uninfected and incubated with uninfected receiver macrophages for 24 hours. Subsequently, receiver macrophages are infected with *M. tuberculosis* and the growth rate of intracellular bacterial microcolonies is quantified using time lapse microscopy. In each experiment, naïve receiver macrophages (under **uninfected** signaler macrophages) are compared to bystander receiver macrophages (under **infected** signaler macrophages). When required, IFNγ is added at the beginning of the experiment. Each dot represents the growth rate of a *M. tuberculosis* microcolony growing in a single macrophage. **(H)** The growth rate of *M. tuberculosis* microcolonies within unactivated and IFNγ-activated receiver macrophages was quantified using time-lapse microscopy. **(I)** The growth rate of *M. tuberculosis* microcolonies within unactivated and IFNγ-activated receiver macrophages treated with neutralizing antibodies against Il-1β was quantified using time-lapse microscopy. **(J)** The growth rate of *M. tuberculosis* microcolonies within unactivated and IFNγ-activated receiver macrophages treated with the Caspase-4/-5 inhibitor ICH-2 was quantified using time-lapse microscopy. P-values were calculated using a Student t-test **(C-F)** or a Mann-Whitney test **(C&G-J).**

Discussion

The non-canonical inflammasome, characterized by the activation of Caspase-11 in mice and Caspases-4/-5 in humans, has emerged as a critical component of the host immune response to gram-negative bacterial infections (Thurston et al., 2016; W. Wang et al., 2017). Caspase-11 and Caspase-4/-5 can directly detect the presence of intracellular LPS and trigger a cascade of events leading to pyroptosis and the release of the pro-inflammatory cytokine IL-1β (Broz et al., 2012; J. Shi et al., 2014). Involvement of the non-canonical inflammasome for defense against mycobacteria, which lack LPS, has recently been suggested, but only experiments with the non-pathogenic mycobacteria *M. smegmatis* have been performed up to now (Qian et al., 2022).

In our study, we demonstrated the involvement of Caspase-11 (in murine macrophages) and Caspase-4/-5 (in human macrophages) in the defense against *M. tuberculosis*. We observe that these caspases are processed and activated upon *M. tuberculosis* infection, leading to IL-1β secretion. Furthermore, our results suggest that Caspase-11 and Caspase-4/-5 act upstream of the canonical inflammasome, as inhibiting them leads to a decrease in Caspase-1 activity. One possible explanation for this observation is that Caspase-11 and Caspase-4/-5 could induce NLRP3 inflammasome assembly and subsequent activation of Caspase-1 through the formation of GasderminD pores and potassium efflux from the cell (Rivers-Auty & Brough, 2015; Rühl & Broz, 2015; Schmid-Burgk et al., 2015).

In contrast to what has been reported for gram-negative bacteria, infection with *M. tuberculosis* only results in a modest increase in pyroptosis in IFNγ-activated macrophages, and no increase in unactivated cells. This phenotype is particularly pronounced in human macrophages, with unactivated cells showing a robust Caspase-4/-5-dependent IL-1β secretion upon infection without displaying enhanced pyroptosis. While pyroptosis and cytokine secretion are usually coupled, recent studies have shown that the scale can tip in both directions under specific conditions (reviewed in Li & Jiang, 2023). In comparison to Gram-negative bacteria, *M. tuberculosis* thus seems to elicit a more pronounced “hyperactive” cell state characterized by IL-1β secretion with little cell death.

Interestingly, our results show that Caspase-11 and Caspases-4/-5 activation is potentiated by IFNγ, a key cytokine involved in host defense against intracellular pathogens (Weiss & Schaible, 2015). In murine macrophages, this potentiation could partly be attributed to IFNγ-induced upregulation of Caspase-11 expression. In human macrophages however, the mechanism seems to be distinct, as IFNγ induces

only a minor upregulation of Caspase-5, and no upregulation of Caspase-4. Recent studies have highlighted the importance of Guanylate-binding proteins (GBPs) in enhancing non-canonical inflammasome activation, particularly in the context of IFNγ signaling (Dilucca et al., 2021; Meunier & Broz, 2015; Santos et al., 2020). GBPs play a critical role in disrupting pathogen-containing vacuoles, potentially facilitating the exposure of bacterial ligands to cytosolic sensors (Meunier et al., 2014). Furthermore, several GBPs have been shown to participate in the immune response against mycobacteria (Kim et al., 2011; Olive et al., 2023). Thus, in the context of *M. tuberculosis* infection, IFNγ might also enhances non-canonical inflammasome activation through the induction of GBPs.

One intriguing aspect of our findings is that *M. tuberculosis* infection can activate Caspase-11 and Caspases-4/-5 in the absence of LPS. We were however unable to elucidate whether this results from direct binding of an *M. tuberculosis* component to these inflammatory caspases, or if this is due to an indirect mechanism. Further research should focus on investigating if Caspase-11 and Caspase-4/-5 can bind components of the mycobacterial cell wall. Notably, it would be interesting to investigate the role of mycobacterial lipids, as several are established virulence factors that can be recognized by a wide range of host cell receptors.

As Caspase-11 and Caspase-4/-5 are key players in the secretion of IL-1β, a potent pro-inflammatory cytokine important for the control of *M. tuberculosis* infection *in vivo*, their activation during infection may have significant implications for tuberculosis disease pathogenesis (Fremond, Doz, et al., 2007; Jayaraman et al., 2013; Pilli, Arko-mensah, et al., 2012). Our results indicate that secreted IL-1β has paracrine effects on neighboring macrophages, enhancing their control over *M. tuberculosis* growth. Furthermore, our analysis revealed that Caspase-11 gene expression is induced in the lungs of mice infected with *M. tuberculosis*. However, it is important to note that gene expression does not necessarily indicate protein activation. Future investigations should focus on assessing Caspase-11 protein cleavage and activity in cells extracted from the lungs of infected mice to confirm its functional relevance *in vivo*. Furthermore, studies with knock-out animals should also be performed to understand the role of the non-canonical inflammasome *in vivo*. Indeed, mice lacking both Caspase-1 and Caspase-11 are slightly more susceptible to infection with *M. tuberculosis*, and this effect is strongly amplified in mice also lacking the phagocyte oxidase (Mayer-Barber et al., 2010; Thomas & Olive, 2023). To our knowledge however, no research has been done studying the susceptibility of single Caspase-11 mutant mice to *M. tuberculosis*, which would be necessary to fully understand the role of the non-canonical inflammasome *in vivo*.

In conclusion, our study provides insights into the activation and function of Caspase-11 (in murine macrophages) and Caspases-4/-5 (in human macrophages) during *M. tuberculosis* infection. We highlighted the critical role of these caspases in IL-1β secretion and the subsequent paracrine enhancement of growth control in neighboring macrophages. Our findings open avenues for further investigation into the role of non-canonical inflammasome signaling during *M. tuberculosis* infection and provide valuable insights into host defense mechanisms against this pathogen.

Materials and Methods

Bone marrow derived macrophage (BMDM) differentiation and culture

The bone marrow was harvested from the femurs of wildtype and mutant C57BL/6 mice (See Table 1 for list of mutant mice used). All mice were female and between 6 and 12 weeks old at the time of sacrifice, except for the Beclin$^{f/fl}$ LysM-Cre^{+} mice which were male. The harvested bone marrow was aliquoted and cryopreserved. To differentiate BMDMs, an aliquot of the cryopreserved bone marrow was thawed and cultured for 7 days in BMDM differentiation medium (Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% GlutaMax, and 20% L929 cell-conditioned medium as a source of granulocyte-macrophage colony-stimulating factor). After 7 days, the adherent cells were gently detached from the plate using a cell scraper, resuspended in BMDM culture medium (DMEM with 5% FBS, 1% sodium-pyruvate, 1% GlutaMax and 5% L929-cell-conditioned medium), and seeded for further experiments.

THP-1 macrophages culture and differentiation

THP-1 macrophages were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% fetal bovine serum (FBS), 1% Glutamax and 1% Sodium Pyruvate. THP-1 cells were differentiated into macrophages by incubation with 100 nM Phorbol 12-myristate 13-acetate (PMA, Stemcell technologies) for 24 hours followed by 24 hours of incubation in RPMI culture medium without PMA.

Mycobacterium tuberculosis cultures

tdTomato-expressing WT *M. tuberculosis* Erdman was inoculated from frozen glycerol stocks in Middlebrook 7H9 (Difco) supplemented with 10% ADC (Difco), 0.5% glycerol, and 0.02% tyloxapol and cultured at 37°C with shaking.

M. tuberculosis lipid extraction

20 ml of tdTomato-expressing *M. tuberculosis* Erdman were grown to OD600 0.5 and spun down for 10 minutes at 5000 g. Supernatant was removed and the bacterial pellet was resuspended in 5 ml of 10:1 methanol:0.3% NaCl. 5 ml of petroleum ether was added to the sample and the tube was vortexed vigorously for 3 minutes. The sample was spun at 2000 g for 10 minutes at 4°C, and the top layer was collected. The procedure was repeated once more by adding 5 ml petroleum ether to the remaining bottom layer and spinning again. The top layer was also collected, and 10

ml of chloroform was added to the extract. The sample was then air-dried at room temperature overnight, and the lipid were resuspended in BMDM culture medium.

Macrophage infections

BMDMs were seeded at a density of 100'000 cells/ml in 12 or 96 well plates and let to rest for 2 hours. THP-1 monocytes were seeded at a density of 100'000 cells/ml in 12 or 96 well plates and differentiated as described above. For infections, 1ml of *M. tuberculosis* culture at an OD600 of 0.5-0.8 was pelleted and resuspended in 200µL of macrophage culture medium. The bacteria were passed through a 5 µm filter to eliminate aggregates and the resulting single-cell suspension was used to infect the macrophages at a multiplicity of infection (MOI) of 1:1. After 5 hours of incubation (for the BMDMs) or after 12 hours of incubation (for the THP-1 macrophages), the infected cells were washed with macrophage culture medium to remove any extracellular bacteria. When required, 100 U/ml murine IFNγ (Peprotech), 100 U/ml human IFNγ (Peprotech), 0.02-2 ng/ml murine IL-1β (Peprotech), 0.2-2 ng/ml human IL-1β (Peprotech), a 1:1000 dilution of DRAQ7 (BioLegend), 2 µl/ml of the caspase-3 inhibitor Z-DEVD-FMK (Sigma Aldrich), 10 uM Nigericin (InvivoGen), 10 uM ICH-2 (Sigma Aldrich), 10 µg/ml of neutralizing antibodies against murine IL-1β (Bxcell, clone B122), or 10 µg/ml of neutralizing antibodies against human IL-1β (InvivoGen, clone 4H5) were added to the cells at the time of infection and kept in the medium during the course of the experiment.

M. tuberculosis growth rate quantification

Infected macrophages were imaged with a Nikon Ti2 microscope. A stage-top incubator (Okolab) was used to maintain the cells at 37°C in a humidified environment. Air mixed to 5% CO_2 was supplied using an Okolab gas mixer. Infected macrophages were randomly selected and imaged using a 40X air objective at 1-hour intervals for a duration of 72 hours. 5 to 7 x 0.75 µm z-stacks were acquired for each point. The bacteria were identified through fluorescence emission in the red (tdTomato-expressing *M. tuberculosis*) channels using an mCherry (Em 560/40, Ex 635/60) dichroic filter. For each experiment, at least 25 infected cells were imaged per condition.

Image analysis was performed using the FIJI version of the ImageJ software (Schindelin et al., 2012) as described in (Rutschmann et al. 2022). All infected macrophages that stayed alive for at least 24 hours of imaging were analyzed. In the rare case of macrophage division during the experiment, the daughter cell containing the bacteria was selected for further analysis. If the bacterial microcolony was split between the two daughter cells, the analysis was stopped at this time-point. All of the

macrophages were imaged until the end of the experiment or until their death. The z-stacks acquired were projected into one image using a maximum intensity projection. A background subtraction was performed by subtracting from the fluorescence images a copy of the same images on which a Gaussian blur of 20 µm radius had been applied. Regions of interest corresponding to individual macrophages were manually drawn onto the phase images and transferred to the fluorescence images. A manual threshold was set on the fluorescent channel to segment the bacteria. The area above the threshold inside single macrophages was measured and used as a proxy for the number of intracellular bacteria for each time point. To quantify the growth rate of the intracellular bacteria, an exponential curve was fitted to the data. A similar method was used to measure the growth rate of bacteria identified as extracellular in BMDM infection experiments. Similarly, iNOS-YFP expression levels were quantified for each frame by transferring the manually drawn regions of interest corresponding to individual infected macrophages to the GFP fluorescence images. The average fluorescence intensity was measured for each individual macrophage and used as a proxy for iNOS-YFP expression levels. For all the analysis, visibly out-of-focus images were manually excluded.

Transwell experiments

Macrophages were seeded at a density of 100'000 cells /ml in glass-bottom 24- or 96-well plates and in the corresponding 0.4 mm pore-size polycarbonate transwell inserts (Corning Inc.). For infection, 1 ml of *M. tuberculosis* culture at an optical density at 600 nm (OD600) of 0.5 was pelleted and resuspended in 200 µl of macrophage culture medium. Bacteria were passed through a 5-µm filter to eliminate aggregates and the resulting single-cell suspension was used to infect the macrophages on top of the transwell at a multiplicity of infection (MOI) of 1:1. After 5 hours, the infected macrophages were washed with macrophage culture medium to remove extracellular bacteria and the transwells were moved on top of their corresponding wells. If necessary, both the cells in the transwell and in the well underneath were activated with 100 U/ml of IFNg. Neutralizing antibodies against IL-1β were also added at this point at a concentration of 2 ng/ml when required.
After 24 hours, the transwells were removed from their wells and the macrophages underneath were infected with *M. tuberculosis* at an MOI of 1:1 as described above. After 5 hours, the infected macrophages were washed extensively and the transwells placed back on top of their respective wells. 1/3 of the medium was refreshed and the infected macrophages were imaged for 72 hours. These experiments were also performed with uninfected transwells as a control. Quantification of the growth rate of

M. tuberculosis microcolonies growing inside the macrophages in the bottom wells (the receiver macrophages) was performed as described above.

Pyroptosis analysis

Macrophages were incubated with a 1:1000 dilution of DRAQ7 (BioLegend) and 2 µl/ml of the caspase-3 inhibitor Z-DEVD-FMK (Sigma Aldrich) and imaged with a Nikon Ti2 microscope. A stage-top incubator (Okolab) was used to maintain the cells at 37°C in a humidified environment. Air mixed to 5% CO_2 was supplied using an Okolab gas mixer. Macrophages were randomly selected and imaged using a 40X air objective at 1-hour intervals for a duration of 72 hours. 5 to 7 x 0.75 µm z-stacks were acquired for each point. The bacteria were identified through fluorescence emission in the red (tdTomato-expressing *M. tuberculosis*) channels using an mCherry (Em 560/40, Ex 635/60) dichroic filter. DRAQ7 positive cells were identified through fluorescence emission in the Cy5 channel (Em 692/40, Ex 624/40).
Image analysis was performed using the FIJI version of the ImageJ software (Schindelin et al., 2012). For the conditions with uninfected macrophages, all macrophages in the field of view were analyzed. In the case of infection, only infected macrophages were analyzed. Individual macrophages were followed and the frame during which a macrophage turned DRAQ7-positive was noted as the time of death. For each experiment, at least 50 cells were analyzed per condition.

Caspase-1 and Caspase-11/-4/-5 activity staining

Infected and uninfected macrophages were washed twice with PBS and incubated for 1 hour at 37°C with Z-LEVD-AFC (Caspase-11/-4/-5 activity staining, Enzo) or FAM-YVAD-FMK (Abcam) in reaction buffer (Caspase-1 assay kit, ab219935, Abcam). The cells were then washed twice in PBS and Caspase activity was measured by emission in the GFP channel (Em 520/36, Ex 472/30). Bacteria were identified through fluorescence emission in the red (tdTomato-expressing *M. tuberculosis*) channels using an mCherry (Em 560/40, Ex 635/60) dichroic filter. Macrophages were randomly selected and imaged using a 40X air objective. 15 x 0.25 µm z-stacks were acquired for each point.
Image analysis was performed using the FIJI version of the ImageJ software (Schindelin et al., 2012). For the conditions with uninfected macrophages, all macrophages in the field of view were analyzed. In the case of infection, only infected macrophages were analyzed. For each point, the z-stacks acquired were projected into one image (Sum Slices projection). A background subtraction was performed by subtracting from the fluorescence images a copy of the same images on which a Gaussian blur of 30 µm radius had been applied. A circle of 30 µm radius was

manually drawn in the GFP channel centered on each cell, and the Raw Integral Density was measured and used for quantification of caspase activity. All obtained values were divided by 10^6 for graphical representation.

Quantification of autophagic flux

Uninfected and infected macrophages were fixed for 2 h with 4% paraformaldehyde 24 h post-infection. Both untreated macrophages and macrophages treated with 100 nM Bafilomycin A1 (Invivogen) for 2 hours were processed. Fixed samples were then permeabilized for 15 min with 2% BSA, 2% saponine, 0.1% Triton x-100 in PBS at RT. The samples were then blocked for 1 h with 2% BSA in PBS at RT. Anti-LC3b rabbit primary antibodies (Abcam) were diluted 1:200 in 2% BSA in PBS and used for primary staining of the cells overnight at 4°C. The samples were then washed and incubated with a secondary goat anti-rabbit antibody conjugated to Alexa Fluor 647 (ThermoFisher Scientific) diluted 1:200 in 2% BSA in PBS for 1h at RT. The samples were then imaged using fluorescence microscopy. Bacteria were identified through fluorescence emission in the red (tdTomato-expressing *M. tuberculosis*) channel. LC3b staining was visualized using the Cy5 channel. Macrophages were randomly selected and imaged using a 60X oil objective. 12 x 0.3 µm z-stacks were acquired for each point. The average number of autophagosomes in each sample was then quantified using the FIJI version of the ImageJ software (Schindelin et al., 2012). For the conditions with uninfected macrophages, all macrophages in the field of view were analyzed. In the case of infection, only infected macrophages were analyzed. A background subtraction was first performed on each z-stack by subtracting from the fluorescence image a copy of the same image on which a Gaussian blur of 5 µm radius had been applied. The processed z-stacks were then projected into one image (Maximum intensity projection). A threshold was applied on the resulting image, and the number of particles above the threshold was counted for each cell and used to determine the number of autophagosome per cell. The difference in the mean number of autophagosome per cell between the Bafilomycin-treated condition and the untreated condition was then calculated and used as a measure for autophagic flux. Experiments were performed in triplicates.

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

RNA extraction from macrophages was performed using a combination of Trizol/Chloroform extraction and the Qiagen RNeasy micro kit plus. Briefly, macrophages were lysed by adding 800 µl of Trizol to the cells. 200 µl of Chloroform was added to the lysate and the resulting mixture was incubated for 5 min at room

temperature followed by centrifugation at 13'000 x g at 4°C. The aqueous phase containing the RNA was collected, mixed with 350 µl of 70% Ethanol and transferred to a quiagen RNeasy MinElute Spin Column. The subsequent steps for RNA extraction were performed according to the manufacturer's instructions. During the extraction process, a DNAse treatment was performed directly on the column to remove contaminating DNA. Following RNA extraction, reverse transcription was performed using the SuperScript IV first-strand synthesis system with random hexamers as primers (ThermoFisher). The cDNA obtained was used for quantitative real-time PCR (qRT-PCR). The qRT-PCR reactions were prepared using the SYBRGreen PCR Master Mix (Applied Biosystems) with 1 µM primers and 2 µl of cDNA. The reactions were run on an QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Amplicon specificity was confirmed by melting-curve analysis. The sequence for all primers were obtained from Origene and the primers were synthesized by Microsynth, Switzerland (Table 2).

Protein extraction and immunoblotting

Proteins from cell lysates (prepared using RIPA buffer) and from supernatants were filtered through a 0.2 µm pore-size filter. Protein quantification was performed using a Qbit assay. The samples were then mixed with NuPAGE™ LDS Sample Buffer and 0.1% SDS and heated up at 70°C for 10 minutes before being loaded onto a NuPAGE 4-12% bis-tris gel (Invitrogen). Electrophoresis was performed according to the manufacturer's instructions. The separated proteins were then transferred to a nitrocellulose membrane using the iBlot dry blotting system (Invitrogen). The membrane was blocked with Tris-buffered saline (TBS) containing 2% bovine serum albumin (BSA) for 1 hour at room temperature and incubated overnight at 4°C with the primary antibody diluted in TBS-Tween (TBS + 0.1% Tween-20) with 2% BSA. All primary antibodies were used at a dilution of 1:1000 (See Table 3 for list of antibodies used). Following primary antibody incubation, the membrane was washed 3x with TBS and incubated with an HRP-conjugated secondary antibody diluted 1:1000 in TBS-Tween with 2% BSA for 1 hour at room temperature. The membrane was then washed twice with TBS-tween and 3x with TBS, incubated with the chemiluminescent peroxidase substrate-1 (Sigma-Aldrich) and imaged using an appropriate detection system.

ELISA

Cell culture supernatants were collected from macrophages 24 hours after infection. IL-1β concentration was measured using a mouse IL-1β SimpleStep ELISA kit

(Abcam) or a human IL-1β SimpleStep ELISA kit (Abcam) according to the manufacturer's instructions.

sc-RNAseq data analysis

The Seurat object from (Pisu et al., 2021) was downloaded and analyzed using Seurat (v. 3.1.4). Pathway enrichment analysis was performed using clusterProfiler. The analysis was performed separately for interstitial macrophages and alveolar macrophages. Briefly, infected cells were sorted into cells expressing Caspase-11 and cells not expressing Caspase-11. A list was created containing all genes that were differentially expressed between the two populations, selecting only genes with an adjusted P value <0.05 (non-parametric Wilcoxon rank-sum test, p-value adjustment performed using a Bonferroni correction, as implemented in Seurat (Stuart et al., 2019). A Gene Ontology (GO) enrichment analysis was then performed using the clusterProfiler package (choosing the Biological Process category). Closely related GO terms were aggregated using semantic similarity for easier visualization. Only the top 30 GO terms were chosen for analysis. For the violin plots and feature plots, data visualization was performed using the log-normalized counts.

Table 1: List of mice used in this study

Strain	Description	Source	Citation
WT	C57BL/6 background, wild type.	Bred in-house	-
Caspase-11$^{-/-}$	Casp11 exon 5 in ES cells was flanked by loxP sites and deleted with Cre recombinase. These mice are in a C57BL/6 background.	Mice were a gift from prof. Broz (UNIL, Switzerland)	Kayagaki et al. 2011
MyD88$^{-/-}$	The Myd88-deficient allele encodes a deletion of exon 3 of the MyD88 gene, which is predicted to generate a null allele (C57BL/6 background).	Jackson Laboratories B6.129P2(SJL)-Myd88tm1.1Defr/J, strain #009088	Hou, Reizis, and DeFranco 2008
Beclin$^{f/fl}$ LysM-Cre^{+} mice	These mice lack Beclin1 expression in myeloid cells. (C57BL/6 background). Beclin1 is a central component of the phosphatidylinositol-3-kinase (PI3K) complex and is implicated in autophagosome formation.	Bone marrow extracted from these mice was a gift from prof. Stallings (Washington University School of Medicine)	Sanjuan et al. 2007

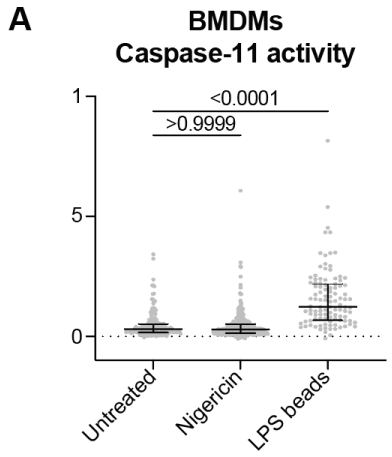
Table 2: List of qPCR primers

Mouse primers:	Primer sequence
GAPDH_fw_mouse	CATCACTGCCACCCAGAAGACTG
GAPDH_rv_mouse	ATGCCAGTGAGCTTCCCGTTCAG
Caspase-11_fw_mouse	GTGGTGAAAGAGGAGCTTACAGC
Caspase-11_rv_mouse	GCACCAGGAATGTGCTGTCTGA
Caspase-1_fw_mouse	GGCACATTTCCAGGACTGACTG
Caspase-1_rv_mouse	GCAAGACGTGTACGAGTGGTTG
NLRP3_fw_mouse	TCACAACTCGCCCAAGGAGGAA
NLRP3_rv_mouse	AAGAGACCACGGCAGAAGCTAG
IL-1β_fw_mouse	TGGACCTTCCAGGATGAGGACA
IL-1β_rv_mouse	GTTCATCTCGGAGCCTGTAGTG
AIM2_fw_mouse	AGGCTGCTACAGAAGTCTGTCC
AIM2_rv_mouse	TCAGCACCGTGACAACAAGTGG
IL-18_fw_mouse	GACAGCCTGTGTTCGAGGATATG
IL-18_rv_mouse	TGTTCTTACAGGAGAGGGTAGAC
Human primers:	
GAPDH_fw_human	GTCTCCTCTGACTTCAACAGCG
GAPDH_rv_human	ACCACCCTGTTGCTGTAGCCAA
Caspase-1_fw_human	GCTGAGGTTGACATCACAGGCA
Caspase-1_rv_human	TGCTGTCAGAGGTCTTGTGCTC
NLRP3_fw_human	GGACTGAAGCACCTGTTGTGCA
NLRP3_rv_human	TCCTGAGTCTCCCAAGGCATTC
IL-1β_fw_human	CCACAGACCTTCCAGGAGAATG
IL-1β_rv_human	GTGCAGTTCAGTGATCGTACAGG
AIM2_fw_human	GCTGCACCAAAAGTCTCTCCTC
AIM2_rv_human	CTGCTTGCCTTCTTGGGTCTCA
IL-18_fw_human	GATAGCCAGCCTAGAGGTATGG
IL-18_rv_human	CCTTGATGTTATCAGGAGGATTCA
Caspase-4_fw_human	GGGATGAAGGAGCTACTTGAGG
Caspase-4_rv_human	CCAAGAATGTGCTGTCAGAGGAC
Caspase-5_fw_human	ACAACCGCAACTGCCTCAGTCT
Caspase-5_rv_human	GAATCTGCCTCCAGGTTCTCAG

Table 3: List of important reagents

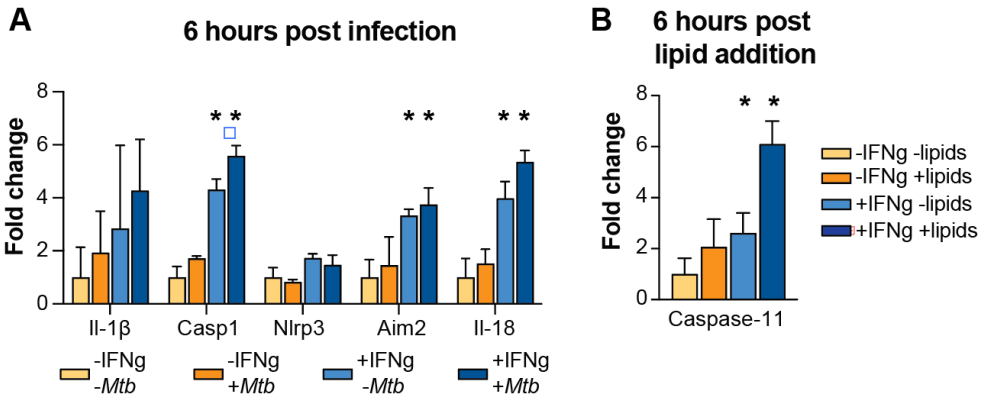
Reagent type	Description	Source
Recombinant protein	Murine recombinant IFNγ	
Recombinant protein	Human recombinant IFNγ	Peprotech
Recombinant protein	Murine recombinant IL-1β	Peprotech
Recombinant protein	Human recombinant IL-1β	Peprotech
Dye	DRAQ7	Biolegends
Dye	Ac-LEVD-AFC	Enzo
Assay kit	Caspase-1 assay kit	Abcam (ab219935)
Inhibitor	Z-DEVD-FMK	Sigma Aldrich
Inhibitor	ICH-2	Sigma Aldrich
Antibody	Neutralizing antibodies against murine IL-1β	BxCell (B122)
Antibody	Neutralizing antibodies against human IL-1β	Invivogen (4H5)
Antibody	Recombinant Anti-Caspase-11 antibody	Abcam (EPR18628)
Antibody	Recombinant Anti-Caspase-4 antibody	Abcam (EPR20921-83)
Antibody	Recombinant Anti-Caspase-5 antibody	Cell Signaling Technology (D3G4W)
Antibody	Recombinant Anti-GAPDH antibody	Abcam (EPR16891)
Antibody	Recombinant Anti-LC3b antibody	Abcam (EPR18709)
Other	Nigericin	Invivogen
Other	Bafilomycin A1	Invivogen
Chemical	Phorbol 12-myristate 13-acetate (PMA)	Stemcell technologies
ELISA kit	Human IL-1 beta ELISA Kit	Abcam (ab214025)
ELISA kit	Mouse IL-1 beta ELISA Kit	Abcam (ab197742)

Supplementary information



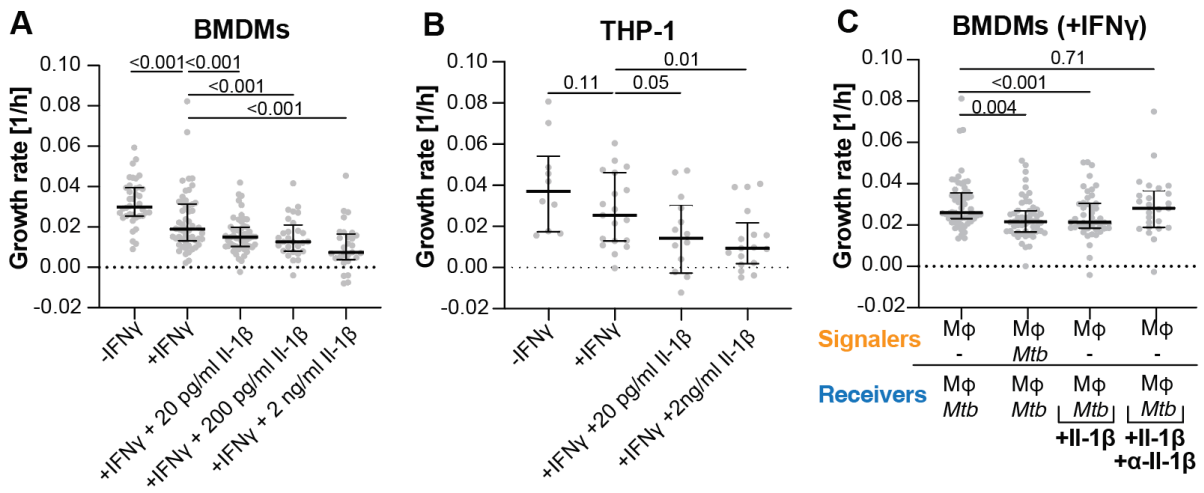
Supplementary Figure 1: The substrate Z-LEVD-AFC becomes fluorescent upon cleavage by Caspase-11, but not Caspase-1

(A) BMDMs were incubated for 5 hours with 10 uM Nigericin (a Caspase-1 activator) or with LPS-coated beads (a Caspase-11 activator). Caspase-11 activity was measured using the fluorescent substrate for Caspase-11 Z-LEVD-AFC and fluorescence microscopy. Each dot represents a single macrophage. P-values were calculated using a Mann-Whitney test. Bars represent the median and interquartile range.



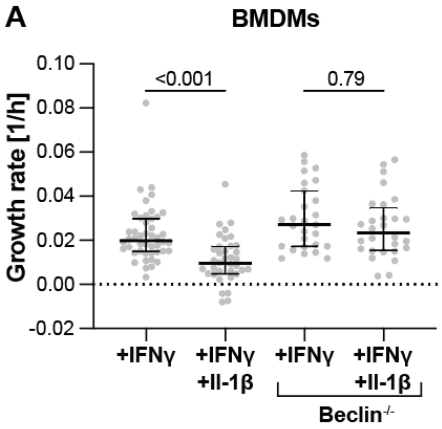
Supplementary Figure 2: Effect of IFNγ and *M. tuberculosis* infection on the expression of inflammasome-related genes in bone marrow-derived macrophages.

(A) Expression levels of inflammasome-related genes for unactivated (-IFNγ) and activated (+IFNγ) BMDMs uninfected or infected with M. tuberculosis. **(B)** Caspase-11 expression levels for unactivated (-IFNγ) and activated (+IFNγ) BMDMs untreated or treated with *M. tuberculosis* lipids. Relative gene expression is depicted as normalized to the unactivated, uninfected sample. Stars indicate p-values smaller than 0.05, as determined by a Student t-test.



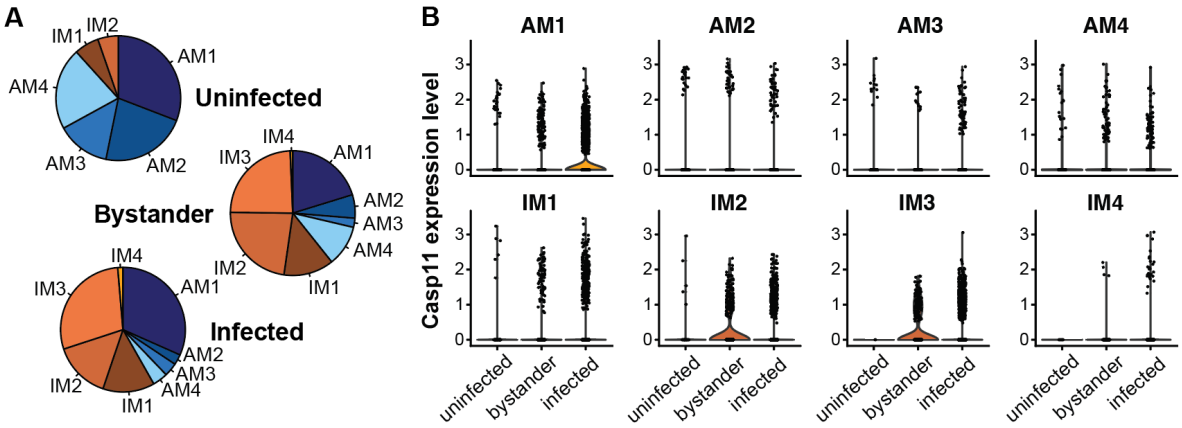
Supplementary Figure 3: Il-1β enhances control of *M. tuberculosis* growth by murine and human macrophages activated with IFNγ.

(A) Bone marrow-derived macrophages (BMDMs) were infected with *M. tuberculosis* and treated with IFN-γ and 20 pg/ml, 200 pg/ml, or 2 ng/ml Il-1β. (B) THP-1 macrophages were infected with *M. tuberculosis* and treated with IFN-γ 20 pg/ml or 2 ng/ml Il-1β. The growth rate of intracellular *M. tuberculosis* microcolonies was assessed using fluorescence time-lapse microscopy. (C) IFN-γ-activated "signaler" and "receiver" BMDMs were seeded in a transwell system with signalers on the top and receivers on the bottom. Signaler macrophages were either infected with *M. tuberculosis* or left uninfected. After 24 hours, receiver macrophages were infected with *M. tuberculosis*, and the growth rate of intracellular bacterial microcolonies was assessed using fluorescence time-lapse microscopy. When required, macrophages were treated with 200 pg/ml Il-1β and neutralizing antibodies against Il-1β. P-values were calculated using a Mann-Whitney test. Bars represent the median and interquartile range.



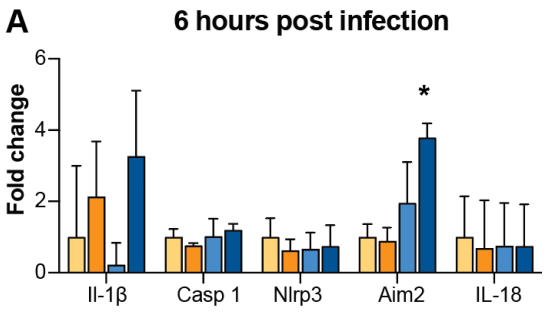
Supplementary Figure 4: Beclin$^{-/-}$ BMDMs do not respond to IL-1β.

(A) WT and Beclin BMDMs were infected with *M. tuberculosis* and treated with IFN-γ and 2 ng/ml IL-1β. 1β. The growth rate of intracellular *M. tuberculosis* microcolonies was assessed using fluorescence time-lapse microscopy. Each dot represents the growth rate of a *M. tuberculosis* microcolony growing in a single macrophage. P-values were calculated using a Mann-Whitney test. Bars represent the median and interquartile range.



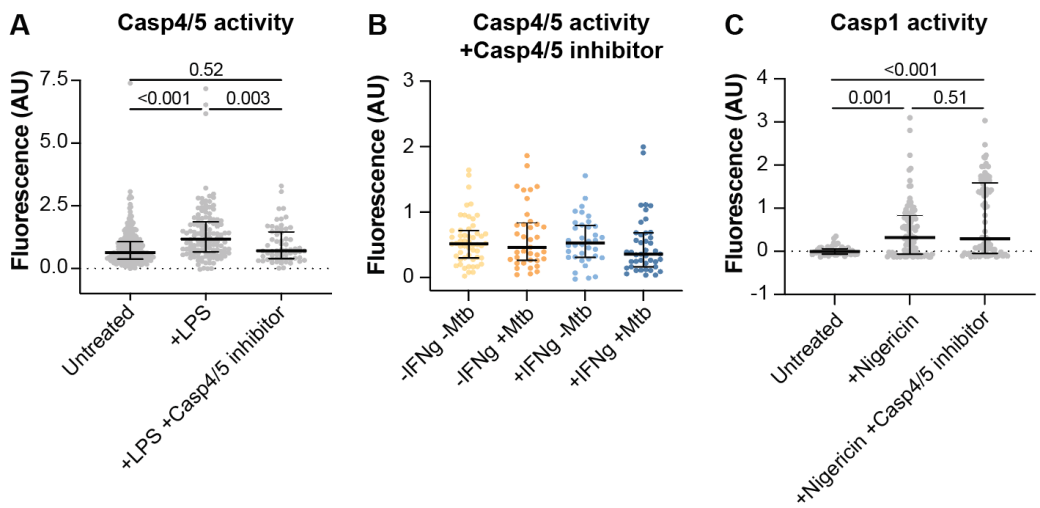
Supplementary Figure 5: Analysis of a publicly available sc-RNAseq dataset (Pisu et al., 2021).

(A) Distribution of Caspase-11+ macrophages in the lungs of uninfected mice and mice infected with *M. tuberculosis*. In uninfected mice, most Caspase-11+ macrophages are alveolar macrophages, while in infected mice, there is a stronger proportion of interstitial macrophages. (B) Violin plots showing Caspase-11 expression levels (in log-normalized counts) in the different alveolar and interstitial macrophage subpopulations (as identified by Pisu et al., 2021).



Supplementary Figure 6: Effect of IFNγ and *M. tuberculosis* infection on the expression of inflammasome-related genes in THP-1 macrophages

(A) Expression levels of inflammasome-related genes for unactivated (-IFNγ) and activated (+IFNγ) THP-1 macrophages uninfected or infected with *M. tuberculosis.* Relative gene expression is depicted as normalized to the unactivated, uninfected sample. Stars indicate p-values smaller than 0.05, as determined by a Student t-test.



Supplementary Figure 7: The Caspase-4/-5 inhibitor ICH-2 selectively inhibits Caspase-4/-5, but not Caspase-1

(A) THP-1 macrophages were incubated with LPS beads for 4 hours. Caspase-4/-5 activity was measured using the fluorescent substrate for Caspase-4/-5 Z-LEVD-AFC and fluorescence microscopy. Each dot represents a single macrophage. (B) Unactivated (-IFNγ) and activated (+IFNγ) THP-1 macrophages were infected with *M. tuberculosis* in presence of the Caspase-4/-5 inhibitor ICH-2. Caspase-4/-5 activity was measured 24 hours post activation using the fluorescent substrate for Caspase-4/-5 Z-LEVD-AFC and fluorescence microscopy. Each dot represents a single macrophage. (B) THP-1 macrophages were left untreated, treated with Nigericin (a Caspase-1 activator) or treated with a combination of Nigericin and the Caspase-4/-5 inhibitor ICH-2. Caspase-1 activity was measured using the fluorescent substrate specific for Caspase-1 Z-YVAD-AFC, and fluorescence microscopy. Each dot represents a single macrophage. P-values were calculated using a Mann-Whitney test. Bars represent the median and interquartile range.

Author Contributions

Ophélie Rutschmann: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Chiara Toniolo: Conceptualization, Investigation.

John D. McKinney: Conceptualization, Supervision, Funding Acquisition.

References

Beckwith, K. S., Beckwith, M. S., Ullmann, S., Sætra, R. S., Kim, H., Marstad, A., Åsberg, S. E., Strand, T. A., Haug, M., Niederweis, M., Stenmark, H. A., & Flo, T. H. (2020). Plasma membrane damage causes NLRP3 activation and pyroptosis during Mycobacterium tuberculosis infection. *Nature Communications*, 11(1).

Broz, P., Ruby, T., Belhocine, K., Bouley, D. M., Kayagaki, N., Dixit, V. M., & Monack, D. M. (2012). Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. *Nature*, 490(7419), 288–291.

Chen, J. M., Zhang, M., Rybniker, J., Boy-Röttger, S., Dhar, N., Pojer, F., & Cole, S. T. (2013). Mycobacterium tuberculosis EspB binds phospholipids and mediates EsxA-independent virulence. *Molecular Microbiology*, 89(6), 1154–1166.

Dilucca, M., Ramos, S., Shkarina, K., Santos, J. C., & Broz, P. (2021). Guanylate-Binding Protein-Dependent Noncanonical Induced Multinucleated Giant Cell Formation. MBio, 12(4).
Fremond, C. M., Doz, E., Rose, S., Vasseur, V., Maillet, I., Jacobs, M., Ryffel, B., & Quesniaux, V. F. J. (2007). IL-1 Receptor-Mediated Signal Is an Essential Component tuberculosis Infection. *The Journal of Immunology*, 18(31), 1178.

Fremond, C. M., Togbe, D., Doz, E., Rose, S., Vasseur, V., Maillet, I., Jacobs, M., Ryffel, B., Valerie, F. J., Fremond, C. M., Doz, E., Rose, S., Vasseur, V., Maillet, I., Jacobs, M., Ryffel, B., & Quesniaux, V. F. J. (2007). IL-1 receptor mediated signal is an essential component of MyD88-Dependent Innate response to Mycobacterium tuberculosis infection. *The Journal of Immunology*, 179, 1178–1189.

Hou, B., Reizis, B., & DeFranco, A. L. (2008). Toll-like Receptors Activate Innate and Adaptive Immunity by using Dendritic Cell-Intrinsic and -Extrinsic Mechanisms. *Immunity*, 29(2), 272–282.

Jayaraman, P., Sada-Ovalle, I., Nishimura, T., Anderson, A. C., Kuchroo, V. K., Remold, H. G., & Behar, S. M. (2013). IL-1 beta promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *The Journal of Immunology*, 190(8), 4196–4204.

Juffermans, N. P., Florquin, S., Camoglio, L., Verbon, A., Kolk, A. H., Speelman, P., Deventer, S. J. H. Van, & van der Poll, T. (2000). Interleukin-1 Signaling Is Essential for Host Defense during Murine Pulmonary Tuberculosis. *The Journal of Infectious Diseases*, 182, 902–908.

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., Cuellar, T., Haley, B., Roose-Girma, M., Phung, Q. T., Liu, P. S., Lill, J. R., Li, H., Wu, J., Kummerfeld, S., Zhang, J., Lee, W. P., Snipas, S. J., Salvesen, G. S., … Dixit, V. M. (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*, 526(7575), 666–671.

Kayagaki, N., Warming, S., Lamkanfi, M., Walle, L. Vande, Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., Zhang, J., Lee, W. P., Roose-Girma, M., & Dixit, V. M. (2011). Non-canonical inflammasome activation targets caspase-11. *Nature*, 479(7371), 117–121.

Kim, B.-H., Shenoy, A. R., Kumar, P., Das, R., Tiwari, S., & MacMicking, J. D. (2011). A Family of IFN-g–Inducible 65-kD GTPases Protects Against Bacterial Infection. *Science*, 332.

Kurenuma, T., Kawamura, I., Hara, H., Uchiyama, R., Daim, S., Dewamitta, S. R., Sakai, S., Tsuchiya, K., Nomura, T., & Mitsuyama, M. (2009). The RD1 Locus in the Mycobacterium tuberculosis Genome Contributes to Activation of Caspase-1 via Induction of Potassium Ion Efflux in Infected Macrophages. *Infection and Immunity*, 77(9), 3992–4001.

Lee, B. L., Stowe, I. B., Gupta, A., Kornfeld, O. S., Roose-Girma, M., Anderson, K., Warming, S., Zhang, J., Lee, W. P., & Kayagaki, N. (2018). Caspase-11 auto-proteolysis is crucial for noncanonical inflammasome activation. *Journal of Experimental Medicine*, 215(9), 2279–2288.

Li, Y., & Jiang, Q. (2023). Uncoupled pyroptosis and IL-1β secretion downstream of inflammasome signaling. *Frontiers in Immunology*, 14(April).

MacMicking, J. D., Taylor, G. A., & McKinney, J. D. (2003). Immune Control of Tuberculosis by IFN-γ-inducible LRG-47. *Science*, 302(5645), 654–659.

Martinon, F., Burns, K., Boveresses, C., & Epalinges, C.-. (2002). The Inflammasome : A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-beta. *Molecular Cell*, 10, 417–426.

Mayer-Barber, K. D., Barber, D. L., Shenderov, K., White, S. D., Wilson, M. S., Cheever, A., Kugler, D., Hieny, S., Caspar, P., Wilson, S., Cheever, A., Kugler, D., Hieny, S., Caspar, P., Nunez, G., Schlueter, D., Flavell, R. A., Sutterwala, F. S., & Sher, A. (2010). Cutting Edge: Caspase-1 Independent IL-1 β Production Is Critical for Host Resistance to Mycobacterium tuberculosis and Does Not Require TLR Signaling In Vivo. *The Journal of Immunology*, 184, 3326–3330.

Meunier, E., & Broz, P. (2015). Interferon-induced guanylate-binding proteins promote cytosolic lipopolysaccharide detection by caspase-11. *DNA and Cell Biology,* 34(1), 1–5.

Meunier, E., Dick, M. S., Dreier, R. F., Schürmann, N., Broz, D. K., Warming, S., Roose-Girma, M., Bumann, D., Kayagaki, N., Takeda, K., Yamamoto, M., & Broz, P. (2014). Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature*, 509(7500), 366–370.

Mishra, B. B., Moura-alves, P., Sonawane, A., Hacohen, N., Griffiths, G., Moita, L. F., & Anes, E. (2010). Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3 / ASC inflammasome. *Cellular Microbiology*, 12(8), 1046–1063.

Olive, A. J., Smith, C. M., Baer, C. E., Coers, J., & Sassetti, C. M. (2023). Mycobacterium tuberculosis Evasion of Guanylate Binding Protein-Mediated Host Defense in Mice Requires the ESX1 Secretion System. *International Journal of Molecular Sciences*, 24(3), 1–13.

Pilli, M., Arko-mensah, J., Ponpuak, M., Roberts, E., Master, S., Mandell, M., Dupont, N., Ornatowski, W., Jiang, S., Bruun, J., Hansen, T. E., Johansen, T., & Deretic, V. (2012). TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. *Immunity*, 37(2), 223–234.

Pisu, D., Huang, L., Narang, V., Theriault, M., Lê-Bury, G., Lee, B., Lakudzala, A. E., Mzinza, D. T., Mhango, D. V., Mitini-Nkhoma, S. C., Jambo, K. C., Singhal, A., Mwandumba, H. C., & Russell, D. G. (2021). Single cell analysis of M. tuberculosis phenotype and macrophage lineages in the infected lung. *Journal of Experimental Medicine*, 218(9).

Qian, J., Hu, Y., Zhang, X., Chi, M., Xu, S., Wang, H., & Zhang, X. (2022). Mycobacterium tuberculosis PE_PGRS19 Induces Pyroptosis through a Non-Classical Caspase-11/GSDMD Pathway in Macrophages. *Microorganisms*, 10(12).

Rastogi, S., & Briken, V. (2022). Interaction of Mycobacteria With Host Cell Inflammasomes. *Frontiers in Immunology*, 13, 791136.

Rivers-Auty, J., & Brough, D. (2015). Potassium efflux fires the canon: Potassium efflux as a common trigger for canonical and noncanonical NLRP3 pathways. *European Journal of Immunology*, 45(10), 2758–2761.

Rühl, S., & Broz, P. (2015). Caspase-11 activates a canonical NLRP3 inflammasome by promoting K+ efflux. *European Journal of Immunology*, 45(10), 2927–2936.

Rutschmann, O., Toniolo, C., & McKinney, J. D. (2022). Preexisting Heterogeneity of Inducible Nitric Oxide Synthase Expression Drives Differential Growth of Mycobacterium tuberculosis in Macrophages. *MBio*, 13(5).

Saiga, H., Kitada, S., Shimada, Y., Kamiyama, N., Okuyama, M., Makino, M., Yamamoto, M., & Takeda, K. (2012). Critical role of AIM2 in Mycobacterium tuberculosis infection. *International Immunology*, 24(10), 637–644.

Sanjuan, M. A., Dillon, C. P., Tait, S. W. G., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J. L., Withoff, S., & Green, D. R. (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature*, 450(7173), 1253–1257.

Santos, J. C., Boucher, D., Schneider, L. K., Demarco, B., Dilucca, M., Shkarina, K., Heilig, R., Chen, K. W., Lim, R. Y. H., & Broz, P. (2020). Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. *Nature Communications*, 11(1).

Schauvliege, R., Vanrobaeys, J., Schotte, P., & Beyaert, R. (2002). Caspase-11 gene expression in response to lipopolysaccharide and interferon-γ requires nuclear factor-κB and signal transducer and activator of transcription (STAT) 1. *Journal of Biological Chemistry*, 277(44), 41624–41630.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682.

Schmid-Burgk, J. L., Gaidt, M. M., Schmidt, T., Ebert, T. S., Bartok, E., & Hornung, V. (2015). Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *European Journal of Immunology*, 45(10), 2911–2917.

Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., & Shao, F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature*, 526(7575), 660–665.

Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., Hu, L., & Shao, F. (2014). Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature*, 514(7521), 187–192.

Silvério, D., Gonçalves, R., Appelberg, R., & Saraiva, M. (2021). Advances on the Role and Applications of Interleukin-1 in Tuberculosis. MBio, 12(6), 1–13.

Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., Hao, Y., Stoeckius, M., Smibert, P., & Satija, R. (2019). Comprehensive Integration of Single-Cell Data. *Cell*, 177(7), 1888-1902.e21.

Thomas, S. M., & Olive, A. J. (2023). High Lethality of Mycobacterium tuberculosis Infection in Mice Lacking the Phagocyte Oxidase and Caspase1/11. *Infection and Immunity*.

Thurston, T. L. M., Matthews, S. A., Jennings, E., Alix, E., Shao, F., Shenoy, A. R., Birrell, M. A., & Holden, D. W. (2016). Growth inhibition of cytosolic Salmonella by caspase-1 and caspase-11 precedes host cell death. *Nature Communications*, 7, 1–15.

Wang, W., Shao, Y., Li, S., Xin, N., Ma, T., Zhao, C., & Song, M. (2017). Caspase-11 plays a protective role in pulmonary Acinetobacter baumannii infection. *Infection and Immunity,* 85(10).

Wassermann, R., Gulen, M. F., Sala, C., Hornung, V., Cole, S. T., Wassermann, R., Gulen, M. F., Sala, C., Perin, S. G., Lou, Y., & Rybniker, J. (2015). Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1. *Cell Host and Microbe*, 17(6), 799–810.

Weiss, G., & Schaible, U. E. (2015). Macrophage defense mechanisms against intracellular bacteria. *Immunological Reviews*, 264(1), 182–203.

Yamada, H., Mizumo, S., Horai, R., Iwakura, Y., & Sugawara, I. (2000). Protective role of interleukin-1 in mycobacterial infection in IL-1 α/β double-knockout mice. *Laboratory Investigation,* 80(5), 759–767.

Yang, Y., Xu, P., He, P., Shi, F., Tang, Y., Guan, C., Zen, H., Zhou, Y., Song, Q., Zhou, B., Jiang, S., Shao, C., Sun, J., Yang, Y., Wang, X., & Song, H. (2020). Mycobacterial PPE13 activates inflammasome by interacting with the NATCH and LRR domains of NLRP3. *The FASEB Journal,* 34(9).

Chapter 4. Conclusion & Future Perspectives

In my thesis, I focused on two projects studying the interactions between macrophages and *M. tuberculosis*. **In a first project**, we used a combination of time-lapse microscopy and fluorescent reporters to study heterogeneity in the interactions between single macrophages and *M. tuberculosis*. Our results showed that heterogeneity in the expression of a single gene, the one coding for iNOS, is sufficient to explain why some macrophages control *M. tuberculosis* growth better than others. These results emphasize the importance of considering cellular individuality when studying host-pathogen interactions, especially for diseases such as tuberculosis where the minimum infective dose can be extremely low. In these cases, due to the low number of bacteria implicated, random encounters between "strong" or "weak" bacteria and "strong" or "weak" macrophages could determine the outcome of an initial infection and whether an individual is able to clear the disease or not (as reviewed in Toniolo et al., 2021). Phenotypic heterogeneity has been little studied so far in the field of infection biology in general and in the field of tuberculosis pathogenesis in particular, partially due to the technical difficulties involved with working with a BSL3-level microorganism. With this project, we were able to bring new data to the field that highlight the necessity of considering heterogeneity in the host and pathogen when studying the pathophysiology of an infectious disease.

One limitation of our study is that it was performed exclusively *in vitro*. However, thanks to recent advances in single-cell techniques such as sc-RNAseq or proteomics of single cells, our results can be linked to studies performed *in vivo*. Indeed, our observations are in line with a recent study that shows that sub-populations of macrophages expressing different levels of iNOS exist in the lungs of *M. tuberculosis*-infected mice (Pisu et al., 2021). Interestingly, the authors also observed that expression of iNOS in infected macrophages correlates with bacterial stress. Nonetheless, it is important to note that this study only provides a static snapshot of host cell and bacterial heterogeneity at a given point in disease progression, whereas many of these phenotypes are highly dynamic. Technical improvements in *in vivo* imaging techniques and/or the combination of time-lapse microscopy with endpoint 'omic' techniques would be necessary to achieve the temporal resolution required to fully characterize the evolution of cell-to-cell heterogeneity over time.

In a second project, we characterized non-canonical inflammasome activation in macrophages infected with *M. tuberculosis*. The non-canonical inflammasome is a crucial component of the host's defense against gram-negative bacteria, yet its implication in defense against pathogenic mycobacteria had not been previously

established. Our study demonstrates the involvement of Caspase-11 (in murine macrophages) and Caspase-4/-5 (in human macrophages) in the immune response to *M. tuberculosis*. We showed that these inflammatory caspases are activated upon infection with *M. tuberculosis*, leading to IL-1β secretion. Furthermore, we established that IL-1β secreted from infected cells can potentiate the anti-mycobacterial properties of neighboring cells. Our study thus identifies a novel mechanism for IL-1β secretion in macrophages infected with *M. tuberculosis* and suggests a role for the non-canonical inflammasome in defense against pathogenic mycobacteria.

Nevertheless, our study leaves several significant questions unanswered. Firstly, the mechanisms leading to Caspase-11/-4/-5 activation in response to *M. tuberculosis* infection require further investigation. In our study, we did not ascertain whether Caspase-11/-4/-5 are activated by direct sensing of a *M. tuberculosis* component, as is the case with LPS, or if an indirect mechanism leads to their activation. Studying the role of *M. tuberculosis* lipids in activating the non-canonical inflammasome would be of particular interest. Indeed, mycobacterial lipids are known virulence factors and can be recognized by a variety of cell host receptors. Furthermore, Caspase-11 has been shown to be able to bind to host-derived oxidized phospholipids (Zanoni et al., 2016). Interestingly, Caspase-11 activation upon phospholipid recognition leads to a robust secretion of IL-1β without pyroptosis, which is similar to what we observe in our study. One way to address this question would be to coat beads with individual mycobacterial lipids and quantify if uptake of these beads leads to IL-1β secretion and Caspase-11/-4/-5 activation in macrophages. If we do find that a mycobacterial lipid leads to non-canonical inflammasome activation, we can proceed to investigate if it can directly bind to purified Caspase-11/-4/-5.

Furthermore, the precise mechanism through which IFNγ potentiates non-canonical inflammasome activation remains elusive. In murine macrophages, this could be partly attributed to upregulation of Caspase-11 gene expression. In human macrophages however, the mechanism appears to be distinct as IFNγ does not induce a strong upregulation of neither Caspase-4 nor Caspase-5. To investigate this further, it would be interesting to look at the role of Guanylate-binding proteins (GBPs). Indeed, these proteins have been shown to enhance non-canonical inflammasome activation in the context of gram-negative bacterial infections by disrupting pathogen-containing vacuoles (Meunier et al., 2014; Santos et al., 2020; Wand et al., 2020). Several GBPs have also been shown to contribute to host defense against mycobacteria (Kim et al., 2011). Interestingly, *M. tuberculosis* seems to be able to evade GBP-mediated immunity thanks to its ESX-1 secretion system (Olive et al., 2023). It would thus be interesting to explore the role of GBPs in mediating non-canonical inflammasome activation in response to infection with *M. tuberculosis.* One

way to investigate this would be by performing immunostainings to see if we can observe colocalization of GBPs with intracellular *M. tuberculosis*. Additionally, we could use RNA interference to selectively silence the expression of different GBPs and observe if we still can measure Caspase-11/-4/-5 activation in the infected macrophages.

Another limit of our experimental setup is that we cannot differentiate between the roles of Caspase-4 and Caspase-5 due to the non-specific nature of the inhibitor used in our experiments. To address this problem, we could repeat the experiments using Caspase-4 or Caspase-5 KO THP-1 macrophages, which would allow us to differentiate between the individual contributions of these enzymes. Alternatively, we could use RNA interference to selectively inhibit either Caspase-4 or Caspase-5, thus enabling a more precise understanding of the respective function of each of these proteins.

Lastly, the translation of our findings to an *in vivo* context remains to be determined. Future investigations should focus on assessing if Caspase-11 is cleaved and active in cells extracted from the lungs of infected mice. Furthermore, studies with knock-out animals could also be performed to understand the role of the non-canonical inflammasome *in vivo*. Indeed, mice lacking both Caspase-1 and Caspase-11 exhibit a slight increase in susceptibility to *M. tuberculosis* infection, and this effect is strongly exacerbated in mice that also lack the CYBB subunit of the phagocyte oxidase (Mayer-Barber et al., 2010; Thomas & Olive, 2023). However, to date, no research has been done to study the susceptibility of single Caspase-11 mutant mice to *M. tuberculosis*, which would be necessary to fully understand the role of the non-canonical inflammasome *in vivo*.

Bibilography

Abdallah, A. M., Bestebroer, J., Savage, N. D. L., de Punder, K., van Zon, M., Wilson, L., Korbee, C. J., van der Sar, A. M., Ottenhoff, T. H. M., van der Wel, N. N., Bitter, W., & Peters, P. J. (2011). Mycobacterial Secretion Systems ESX-1 and ESX-5 Play Distinct Roles in Host Cell Death and Inflammasome Activation. *The Journal of Immunology*, *187*(9), 4744–4753.

Abengozar-Muela, M., Esparza, M. V., Garcia-Ros, D., Vásquez, C. E., Echeveste, J. I., Idoate, M. A., Lozano, M. D., Melero, I., & de Andrea, C. E. (2020). Diverse immune environments in human lung tuberculosis granulomas assessed by quantitative multiplexed immunofluorescence. *Modern Pathology*, *33*(12), 2507–2519.

Abrahams, K. A., & Besra, G. S. (2021). Synthesis and recycling of the mycobacterial cell envelope. *Current Opinion in Microbiology*, *60*(i), 58–65.

Ackermann, M. (2015). A functional perspective on phenotypic heterogeneity in microorganisms. *Nature Reviews Microbiology*, *13*(8), 497–508.

Adams, L. B., Dinauer, M. C., Morgenstern, D. E., & Krahenbuhl, J. L. (1997). Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. *Tubercle and Lung Disease*, *78*(5–6), 237–246.

Aegerter, H., Lambrecht, B. N., & Jakubzick, C. V. (2022). Biology of lung macrophages in health and disease. *Immunity*, *55*(9), 1564–1580.

Agarwal, P., Gordon, S., & Martinez, F. O. (2021). Foam Cell Macrophages in Tuberculosis. *Frontiers in Immunology*, *12*(December), 1–8.

Aguilo, J. I., Alonso, H., Uranga, S., Marinova, D., Arbués, A., de Martino, A., Anel, A., Monzon, M., Badiola, J., Pardo, J., Brosch, R., & Martin, C. (2013). ESX-1-induced apoptosis is involved in cell-to-cell spread of Mycobacterium tuberculosis. *Cellular Microbiology*, *15*(12), 1994–2005.

Aguilo, N., Uranga, S., Mata, E., Tarancon, R., Gómez, A. B., Marinova, D., Otal, I., Monzón, M., Badiola, J., Montenegro, D., Puentes, E., Rodríguez, E., Vervenne, R. A. W., Sombroek, C. C., Verreck, F. A. W., & Martín, C. (2020). Respiratory Immunization With a Whole Cell Inactivated Vaccine Induces Functional Mucosal Immunoglobulins Against Tuberculosis in Mice and Non-human Primates. *Frontiers in Microbiology*, *11*(June), 1–15.

Ahmad, F., Rani, A., Alam, A., Zarin, S., Pandey, S., Singh, H., Hasnain, S. E., & Ehtesham, N. Z. (2022). Macrophage: A Cell With Many Faces and Functions in Tuberculosis. *Frontiers in Immunology*, *13*(May), 1–18.

Allen, S. S., Cassone, L., Lasco, T. M., & McMurray, D. N. (2004). Effect of Neutralizing Transforming Growth Factor β1 on the Immune Response against Mycobacterium tuberculosis in Guinea Pigs. *Infection and Immunity*, *72*(3), 1358–1363.

Altare, F., Durandy, A., Lammas, D., Emile, J. F., Lamhamedi, S., Le Deist, F., Drysdale, P., Jouanguy, E., Döffinger, R., Bernaudin, F., Jeppsson, O., Gollob, J. A., Meinl, E., Segal, A. W., Fischer, A., Kumararatne, D., & Casanova, J. L. (1998). Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science*, *280*(5368), 1432–1435.

Alves da Silva, D. A., Da Silva, M. V., Oliveira Barros, C. C., Dias Alexandre, P. B., Timóteo, R. P., Catarino, J. S., Sales-Campos, H., Machado, J. R., Rodrigues, D. B. R., Oliveira, C. J., & Rodrigues, V. (2018). TNF-α blockade impairs in vitro tuberculous granuloma formation and down modulate Th1, Th17 and Treg cytokines. *PLoS ONE*, *13*(3), 1–13.

Antonelli, L. R. V., Rothfuchs, A. G., Gonçalves, R., Roffê, E., Cheever, A. W., Bafica, A., Salazar, A. M., Feng, C. G., & Sher, A. (2010). Intranasal poly-IC treatment exacerbates tuberculosis in mice through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. *Journal of Clinical Investigation*, *120*(5), 1674–1682.

Armstrong, J. A., & D'Arcy Hart, P. (1971). Response of cultured macrophages to mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *The Journal of Experimental Medicine*, *134*(3), 713–740.

Augenstreich, J., Arbues, A., Simeone, R., Haanappel, E., Wegener, A., Sayes, F., Le Chevalier, F., Chalut, C., Malaga, W., Guilhot, C., Brosch, R., & Astarie-Dequeker, C. (2017). ESX-1 and phthiocerol dimycocerosates of Mycobacterium tuberculosis act in concert to cause phagosomal rupture and host cell apoptosis. *Cellular Microbiology*, *19*(7), 1–19.

Augenstreich, J., Haanappel, E., Sayes, F., Simeone, R., Guillet, V., Mazeres, S., Chalut, C., Mourey, L., Brosch, R., Guilhot, C., & Astarie-Dequeker, C. (2020). Phthiocerol Dimycocerosates From Mycobacterium tuberculosis Increase the Membrane Activity of Bacterial Effectors and Host Receptors. *Frontiers in Cellular and Infection Microbiology*, *10*(August), 1–10.

Austin, C. M., Ma, X., & Graviss, E. A. (2008). Common nonsynonymous polymorphisms in the NOD2 gene are associated with resistance or susceptibility to tuberculosis disease in African Americans. *Journal of Infectious Diseases*, *197*(12), 1713–1716.

Avraham, R., Haseley, N., Brown, D., Penaranda, C., Jijon, H. B., Trombetta, J. J., Satija, R., Shalek, A. K., Xavier, R. J., Regev, A., & Hung, D. T. (2015). Pathogen cell-to-cell variability drives heterogeneity in host immune responses. *Cell*, *162*, 1309–1321.

Awuh, J. A., & Flo, T. H. (2017). Molecular basis of mycobacterial survival in macrophages. *Cellular and Molecular Life Sciences*, *74*(9), 1625–1648.

Aylan, B., Bernard, E. M., Pellegrino, E., Botella, L., Fearns, A., Athanasiadi, N., Bussi, C., Santucci, P., & Gutierrez, M. G. (2023). ATG7 and ATG14 restrict cytosolic and phagosomal Mycobacterium tuberculosis replication in human macrophages. *Nature Microbiology*, *8*(5), 803–818.

Bafica, A., Scanga, C. A., Feng, C. G., Leifer, C., Cheever, A., & Sher, A. (2005). TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *Journal of Experimental Medicine*, *202*(12), 1715–1724.

Bain, C. C., & MacDonald, A. S. (2022). The impact of the lung environment on macrophage development, activation and function: diversity in the face of adversity. *Mucosal Immunology*, *15*(2), 223–234.

Bakkum, T., Heemskerk, M. T., Bos, E., Groenewold, M., Oikonomeas-koppasis, N., Walburg, K. V, Veen, S. Van, Lienden, M. J. C. Van Der, Leeuwen, T. Van, Haks, M. C., Ottenho, T. H. M., Koster, A. J., & Kasteren, S. I. Van. (2020). Bioorthogonal Correlative Light-Electron Microscopy of Mycobacterium tuberculosis in Macrophages Reveals the Effect of Antituberculosis Drugs on Subcellular Bacterial Distribution. *ACS Central Science*, *6*, 1997–2007.

Barnes, P. F., Leedom, J. M., Chan, L. S., Wong, S. F., Shah, J., Vachon, L. A., Overturf, G. D., & Modlin, R. L. (1988). Predictors of short-term prognosis in patients with pulmonary tuberculosis. *Journal of Infectious Diseases*, *158*(2), 366–371.

Barrios-Payán, J., Saqui-Salces, M., Jeyanathan, M., Alcántara-Vazquez, A., Castañon-Arreola, M., Rook, G., & Hernandez-Pando, R. (2012). Extrapulmonary locations of mycobacterium tuberculosis DNA during latent infection. *Journal of Infectious Diseases*, *206*(8), 1194–1205.

Basu, J., Shin, D. M., & Jo, E. K. (2012). Mycobacterial signaling through toll-like receptors. *Frontiers in Cellular and Infection Microbiology*, *2*(November), 145.

Basu, S., Fowler, B. J., Kerur, N., Arnvig, K. B., & Rao, N. A. (2018). NLRP3 inflammasome activation by mycobacterial ESAT-6 and dsRNA in intraocular tuberculosis. *Microbial Pathogenesis*, *114*(November 2017), 219–224.

Beaulieu, A. M., Rath, P., Imhof, M., Siddall, M. E., Roberts, J., Schnappinger, D., & Nathan, C. F. (2010). Genome-Wide Screen for Mycobacterium tuberculosis Genes That Regulate Host Immunity. *Plos One*, *5*(12), 1–14.

Beckwith, K. S., Beckwith, M. S., Ullmann, S., Sætra, R. S., Kim, H., Marstad, A., Åsberg, S. E., Strand, T. A., Haug, M., Niederweis, M., Stenmark, H. A., & Flo, T. H. (2020). Plasma membrane damage causes NLRP3 activation and pyroptosis during Mycobacterium tuberculosis infection. *Nature Communications*, *11*(1).

Behler, F., Steinwede, K., Balboa, L., Ueberberg, B., Maus, R., Kirchhof, G., Yamasaki, S., Welte, T., & Maus, U. A. (2012). Role of Mincle in Alveolar Macrophage-Dependent Innate Immunity against Mycobacterial Infections in Mice. *The Journal of Immunology*, *189*(6), 3121–3129.

Behr, M. A., Edelstein, P. H., & Ramakrishnan, L. (2018). Revisiting the timetable of tuberculosis. *British Medical Journal*, *2738*, 1–10.

Behr, M. A., Edelstein, P. H., & Ramakrishnan, L. (2019). Is Mycobacterium tuberculosis infection life long ? *British Medical Journal*, *5770*, 1–7.

Bekker, L. G., Maartens, G., Steyn, L., & Kaplan, G. (1998). Selective increase in plasma tumor necrosis factor-α and concomitant clinical deterioration after initiating therapy in patients with

severe tuberculosis. *Journal of Infectious Diseases*, *178*(2), 580–584.

Berry, M. P. R., Graham, C. M., McNab, F. W., Xu, Z., Bloch, S. A. A., Oni, T., Wilkinson, K. A., Banchereau, R., Skinner, J., Wilkinson, R. J., Quinn, C., Blankenship, D., Dhawan, R., Cush, J. J., Mejias, A., Ramilo, O., Kon, O. M., Pascual, V., Banchereau, J., … O'Garra, A. (2010). An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature*, *466*(7309), 973–977.

Bhaskar, A., Chawla, M., Mehta, M., Parikh, P., Chandra, P., Bhave, D., Kumar, D., Carroll, K. S., & Singh, A. (2014). Reengineering Redox Sensitive GFP to Measure Mycothiol Redox Potential of Mycobacterium tuberculosis during Infection. *PLoS Pathogens*, *10*(1).

Bhat, K. H., Srivastava, S., Kotturu, S. K., Ghosh, S., & Mukhopadhyay, S. (2017). The PPE2 protein of Mycobacterium tuberculosis translocates to host nucleus and inhibits nitric oxide production. *Scientific Reports*, *7*(August 2015), 1–13.

Blair, H. A., & Scott, L. J. (2015). Delamanid: a review of its use in patients with multidrug-resistant tuberculosis. *Drugs*, *75*(1), 91–100.

Blanc, L., Daudelin, I. B., Podell, B. K., Chen, P. Y., Zimmerman, M., Martinot, A. J., Savic, R. M., Prideaux, B., & Dartois, V. (2018). High-resolution mapping of fluoroquinolones in TB rabbit lesions reveals specific distribution in immune cell types. *ELife*, *7*, 1–21.

Boogaard, J. Van Den, Kibiki, G. S., Kisanga, E. R., Boeree, M. J., & Aarnoutse, R. E. (2009). New drugs against tuberculosis : problems , progress , and evaluation of agents in clinical development. *Antimicobial Agents and Chemotherapy*, *53*(3), 849–862.

Bourigault, M. L., Segueni, N., Rose, S., Court, N., Vacher, R., Vasseur, V., Erard, F., Bert, M. Le, Garcia, I., Iwakura, Y., Jacobs, M., Ryffel, B., & Quesniaux, V. F. J. (2013). Relative contribution of il-1α, il-1β and tnf to the host response to mycobacterium tuberculosis and attenuated m. Bovis bcg. *Immunity, Inflammation and Disease*, *1*(1), 47–62.

Bowdish, D. M. E., Sakamoto, K., Kim, M. J., Kroos, M., Mukhopadhyay, S., Leifer, C. A., Tryggvason, K., Gordon, S., & Russell, D. G. (2009). MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis. *PLoS Pathogens*, *5*(6).

Bozzano, F., Costa, P., Passalacqua, G., Dodi, F., Ravera, S., Pagano, G., Canonica, G. W., Moretta, L., & De Maria, A. (2009). Functionally relevant decreases in activatory receptor expression on NK cells are associated with pulmonary tuberculosis in vivo and persist after successful treatment. *International Immunology*, *21*(7), 779–791.

Braverman, J., & Stanley, S. A. (2017). Nitric Oxide modulates macrophage responses to Mycobacterium tuberculosis infection through activation of HIF-1 α and repression of NF- κB. *The Journal of Immunology*, *17*, 1–10.

Broz, P., Ruby, T., Belhocine, K., Bouley, D. M., Kayagaki, N., Dixit, V. M., & Monack, D. M. (2012). Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. *Nature*, *490*(7419), 288–291.

Bryson, B. D., Rosebrock, T. R., Tafesse, F. G., Itoh, C. Y., Nibasumba, A., Babunovic, G. H., Corleis, B., Martin, C., Keegan, C., Andrade, P., Realegeno, S., Kwon, D., Modlin, R. L., & Fortune, S. M. (2019). Heterogeneous GM-CSF signaling in macrophages is associated with control of Mycobacterium tuberculosis. *Nature Communications*, *10*(1).

Buchmeier, N., & Fahey, R. C. (2006). The mshA gene encoding the glycosyltransferase of mycothiol biosynthesis is essential in Mycobacterium tuberculosis Erdman. *FEMS Microbiology Letters*, *264*(1), 74–79.

Bulut, Y., Michelsen, K. S., Hayrapetian, L., Naiki, Y., Spallek, R., Singh, M., & Arditi, M. (2005). Mycobacterium tuberculosis heat shock proteins use diverse toll-like receptor pathways to activate pro-inflammatory signals. *Journal of Biological Chemistry*, *280*(22), 20961–20967.

Bussi, C., & Gutierrez, M. G. (2019). Mycobacterium tuberculosis infection of host cells in space and time. *FEMS Microbiology Review*, *43*, 341–361.

Cadena, A. M., Fortune, S. M., & Flynn, J. L. (2017). Heterogeneity in tuberculosis. *Nature Reviews Immunology*, *17*(11), 691–702.

Camacho, L. R., Ensergueix, D., Perez, E., Gicquel, B., & Guilhot, C. (1999). Identification of a virulence gene cluster of Mycobacterium tuberculosis by signature-tagged transposon

mutagenesis. *Molecular Microbiology*, *34*(2), 257–267.

Cambier, C. J., Banik, S. M., Buonomo, J. A., & Bertozzi, C. R. (2020). Spreading of a mycobacterial cell surface lipid into host epithelial membranes promotes infectivity. *ELife*, *9*, 1–68.

Cambier, C. J., Falkow, S., & Ramakrishnan, L. (2014). Host evasion and exploitation schemes of Mycobacterium tuberculosis. *Cell*, *159*(7), 1497–1509.

Cambier, C. J., Takaki, K. K., Larson, R. P., Hernandez, R. E., Tobin, D. M., Urdahl, K. B., Cosma, C. L., & Ramakrishnan, L. (2014). Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature*, *505*(7482), 218–222.

Cardoso, N. C., Oosthuizen, C. B., Peton, N., & Singh, V. (2021). Drug Repurposing for Tuberculosis. In S. K. Saxena (Ed.), *Drug Repurposing*. IntechOpen.

Carlsson, F., Kim, J., Dumitru, C., Barck, K. H., Carano, R. A. D., Sun, M., Diehl, L., & Brown, E. J. (2010). Host-detrimental role of Esx-1-mediated inflammasome activation in Mycobacterial infection. *PLoS Pathogens*, *6*(5), 1–12.

Carmona, J., Cruz, A., Moreira-Teixeira, L., Sousa, C., Sousa, J., Osorio, N. S., Saraiva, A. L., Svenson, S., Kallenius, G., Pedrosa, J., Rodrigues, F., Castro, A. G., & Saraiva, M. (2013). Mycobacterium tuberculosis Strains Are Differentially Recognized by TLRs with an Impact on the Immune Response. *PLoS ONE*, *8*(6), 1–10.

Carow, B., Hauling, T., Qian, X., Kramnik, I., Nilsson, M., & Rottenberg, M. E. (2019). Spatial and temporal localization of immune transcripts defines hallmarks and diversity in the tuberculosis granuloma. *Nature Communications*, *10*(1), 1–15.

Castillo, E. F., Dekonenko, A., Arko-Mensah, J., Mandell, M. A., Dupont, N., Jiang, S., Delgado-Vargas, M., Timmins, G. S., Bhattacharya, D., Yang, H., Hutt, J., Lyons, C. R., Dobos, K. M., & Deretic, V. (2012). Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proceedings of the National Academy of Sciences*, *109*, 3168–3176.

Chai, Q., Wang, L., Liu, C. H., & Ge, B. (2020). New insights into the evasion of host innate immunity by Mycobacterium tuberculosis. *Cellular and Molecular Immunology*, *17*(9), 901–913.

Chakarov, S., Lim, H. Y., Tan, L., Lim, S. Y., See, P., Lum, J., Zhang, X., Foo, S., Nakamizo, S., Duan, K., Kong, W. T., Gentek, R., Balachander, A., Carbajo, D., Bleriot, C., Malleret, B., Kit, J., Tam, C., Baig, S., … Ginhoux, F. (2019). Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches. *Science*, *363*, 1–12.

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

Chan, J., Tanaka, K., Carroll, D., Flynn, J., & Bloom, B. R. (1995). Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. *Infection and Immunity*, *63*(2), 736–740.

Chen, J. M., Boy-Röttger, S., Dhar, N., Sweeney, N., Buxton, R. S., Pojer, F., Rosenkrands, I., & Cole, S. T. (2012). EspD is critical for the virulence-mediating ESX-1 secretion system in Mycobacterium tuberculosis. *Journal of Bacteriology*, *194*(4), 884–893.

Chen, J. M., Zhang, M., Rybniker, J., Boy-Röttger, S., Dhar, N., Pojer, F., & Cole, S. T. (2013). Mycobacterium tuberculosis EspB binds phospholipids and mediates EsxA-independent virulence. *Molecular Microbiology*, *89*(6), 1154–1166.

Chen, Z., Wang, W., Liang, J., Wang, J., Feng, S., & Zhang, G. (2015). Association between toll-like receptors 9 (TLR9) gene polymorphism and risk of pulmonary tuberculosis: Meta-analysis. *BMC Pulmonary Medicine*, *15*(1), 1–10.

Cheng, S., Sun, C., Lao, W., & Kang, H. (2019). Association of VAMP8 rs1010 Polymorphism with Host Susceptibility to Pulmonary Tuberculosis in a Chinese Han Population. *Genetic Testing and Molecular Biomarkers*, *23*(5), 299–303.

Cho, H. J., Lim, Y. J., Kim, J., Koh, W. J., Song, C. H., & Kang, M. W. (2020). Different macrophage polarization between drug-susceptible and multidrug-resistant pulmonary tuberculosis. *BMC Infectious Diseases*, *20*(1), 1–10.

Cho, T., Khatchadourian, C., Nguyen, H., Dara, Y., Jung, S., & Venketaraman, V. (2021). A review of the BCG vaccine and other approaches toward tuberculosis eradication. *Human Vaccines and Immunotherapeutics*, *17*(8), 2454–2470.

Choi, H. S., Rai, P. R., Chu, H. W., Cool, C., & Chan, E. D. (2002). Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, *166*(2), 178–186.

Chowdhury, R. R., Vallania, F., Yang, Q., Lopez, C. J., Darboe, F., Penn-nicholson, A., Rozot, V., Nemes, E., Malherbe, S. T., Ronacher, K., Walzl, G., Hanekom, W., Davis, M. M., Winter, J., Chen, X., & Scriba, T. J. (2018). A multi-cohort study of the immune factors associated with M. tuberculosis infection outcomes. *Nature*, *560*(7720), 644–648.

Cinelli, M. A., Do, H. T., Miley, G. P., & Silverman, R. B. (2020). Inducible nitric oxide synthase: Regulation, structure, and inhibition. *Medicinal Research Reviews*, *40*(1), 158–189.

Clark, S., Lanni, F., Marinova, D., Rayner, E., Martin, C., & Williams, A. (2017). Revaccination of Guinea pigs with the live attenuated Mycobacterium tuberculosis vaccine MTBVAC improves BCG's protection against tuberculosis. *Journal of Infectious Diseases*, *216*(5), 525–533.

Cohen, S. B., Gern, B. H., Delahaye, J. L., Adams, K. N., Plumlee, C. R., Winkler, J. K., Sherman, D. R., Gerner, M. Y., & Urdahl, K. B. (2018). Alveolar Macrophages Provide an Early Mycobacterium tuberculosis Niche and Initiate Dissemination. *Cell Host and Microbe*, *24*(3), 439-446.e4.

Collins, A. C., Cai, H., Li, T., Franco, L. H., Li, X. D., Nair, V. R., Scharn, C. R., Stamm, C. E., Levine, B., Chen, Z. J., & Shiloh, M. U. (2015). Cyclic GMP-AMP Synthase Is an Innate Immune DNA Sensor for Mycobacterium tuberculosis. *Cell Host and Microbe*, *17*(6), 820–828.

Conradie, F., Diacon, A. H., Ngubane, N., Howell, P., Everitt, D., Crook, A. M., Mendel, C. M., Egizi, E., Moreira, J., Timm, J., McHugh, T. D., Wills, G. H., Bateson, A., Hunt, R., Van Niekerk, C., Li, M., Olugbosi, M., & Spigelman, M. (2020). Treatment of Highly Drug-Resistant Pulmonary Tuberculosis. *New England Journal of Medicine*, *382*(10), 893–902.

Converse, P. J., Dannenberg, A. M., Estep, J. E., Sugisaki, K., Abe, Y., Schofield, B. H., & Pitt, M. L. M. (1996). Cavitary tuberculosis produced in rabbits by aerosolized virulent tubercle bacilli. *Infection and Immunity*, *64*(11), 4776–4787.

Cooper, A. M., Magram, J., Ferrante, J., & Orme, I. M. (1997). Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *Journal of Experimental Medicine*, *186*(1), 39–45.

Cooper, A. M., Segal, B. H., Frank, A. A., Holland, S. M., & Orme, I. M. (2000). Transient loss of resistance to pulmonary tuberculosis in p47(phox-/-) mice. *Infection and Immunity*, *68*(3), 1231–1234.

Cooper, A. M., Solache, A., & Khader, S. A. (2007). Interleukin-12 and tuberculosis: an old story revisited. *Current Opinion in Immunology*, *19*(4), 441–447.

Corleis, B., & Dorhoi, A. (2020). Early dynamics of innate immunity during pulmonary tuberculosis. *Immunology Letters*, *221*(November 2019), 56–60.

Corleis, B., Korbel, D., Wilson, R., Bylund, J., Chee, R., & Schaible, U. E. (2012). Escape of Mycobacterium tuberculosis from oxidative killing by neutrophils. *Cellular Microbiology*, *14*(7), 1109–1121.

Correia-Neves, M., Sundling, C., Cooper, A., & Källenius, G. (2019). Lipoarabinomannan in Active and Passive Protection Against Tuberculosis. *Frontiers in Immunology*, *10*(September).

Court, N., Vasseur, V., Vacher, R., Frémond, C., Shebzukhov, Y., Yeremeev, V. V., Maillet, I., Nedospasov, S. A., Gordon, S., Fallon, P. G., Suzuki, H., Ryffel, B., & Quesniaux, V. F. J. (2010). Partial Redundancy of the Pattern Recognition Receptors, Scavenger Receptors, and C-Type Lectins for the Long-Term Control of Mycobacterium tuberculosis Infection . *The Journal of Immunology*, *184*(12), 7057–7070.

Cox, J. S., Chen, B., Mcneil, M., & Jr, W. R. J. (1999). Complex lipid determines tissue-specific replication of Mycobacterium tuberculosis in mice. *Nature*, *402*, 79–83.

Cronan, M. R., Beerman, R. W., Rosenberg, A. F., Saelens, J. W., Johnson, M. G., Oehlers, S. H., Sisk, D. M., Jurcic Smith, K. L., Medvitz, N. A., Miller, S. E., Trinh, L. A., Fraser, S. E., Madden, J. F., Turner, J., Stout, J. E., Lee, S., & Tobin, D. M. (2016). Macrophage Epithelial Reprogramming Underlies Mycobacterial Granuloma Formation and Promotes Infection. *Immunity*, *45*(4), 861–876.

Dai, G., & McMurray, D. N. (1999). Effects of modulating TGF-β1 on immune responses to

mycobacterial infection in guinea pigs. *Tubercle and Lung Disease*, *79*(4), 207–214.

Darrah, P. A., Zeppa, J. J., Maiello, P., Hackney, J. A., Wadsworth, M. H., Hughes, T. K., Pokkali, S., Swanson, P. A., Grant, N. L., Rodgers, M. A., Kamath, M., Causgrove, C. M., Laddy, D. J., Bonavia, A., Casimiro, D., Lin, P. L., Klein, E., White, A. G., Scanga, C. A., … Seder, R. A. (2020). Prevention of tuberculosis in macaques after intravenous BCG immunization. *Nature*, *577*(7788), 95–102.

Dartois, V. A., & Rubin, E. J. (2022). Anti-tuberculosis treatment strategies and drug development: challenges and priorities. *Nature Reviews Microbiology*, *20*(11), 685–701.

Darwin, K. H., Ehrt, S., Gutierrez-Ramo, J.-C., Weich, N., & Nathan, C. F. (2003). The proteasome of Mycobacterium tuberculosis is required for resistance to nitric oxide. *Science*, *302*, 1963–1967.

Day, N. J., Santucci, P., & Gutierrez, M. G. (2023). Host cell environments and antibiotic efficacy in tuberculosis. *Trends in Microbiology*, *xx*(xx), 1–10.

Dean, G. S., Rhodes, S. G., Coad, M., Whelan, A. O., Cockle, P. J., Clifford, D. J., Hewinson, R. G., & Vordermeier, H. M. (2005). Minimum infective dose of Mycobacterium bovis in cattle. *Infection and Immunity*, *73*(10), 6467–6471.

Decout, A., Silva-Gomes, S., Drocourt, D., Blattes, E., Rivière, M., Prandi, J., Larrouy-Maumus, G., Caminade, A. M., Hamasur, B., Källenius, G., Kaur, D., Dobos, K. M., Lucas, M., Sutcliffe, I. C., Besra, G. S., Appelmelk, B. J., Gilleron, M., Jackson, M., Vercellone, A., … Nigou, J. (2018). Deciphering the molecular basis of mycobacteria and lipoglycan recognition by the C-type lectin Dectin-2. *Scientific Reports*, *8*(1), 1–11.

Delogu, G., Sali, M., & Fadda, G. (2013). The biology of Mycobacterium tuberculosis infection. *Mediterr J Hematol Infect Dis*, *5*, 1–8.

Deretic, V., & Wang, F. (2023). Autophagy is part of the answer to tuberculosis. *Nature Microbiology*, *8*(5), 762–763.

Derrick, S. C., Kolibab, K., Yang, A., & Morris, S. L. (2014). Intranasal administration of Mycobacterium bovis BCG induces superior protection against aerosol infection with Mycobacterium tuberculosis in mice. *Clinical and Vaccine Immunology*, *21*(10), 1443–1451.

Dhiman, R., Indramohan, M., Barnes, P. F., Nayak, R. C., Paidipally, P., Rao, L. V. M., & Vankayalapati, R. (2009). IL-22 Produced by Human NK Cells Inhibits Growth of Mycobacterium tuberculosis by Enhancing Phagolysosomal Fusion . *The Journal of Immunology*, *183*(10), 6639–6645.

Dilucca, M., Ramos, S., Shkarina, K., Santos, J. C., & Broz, P. (2021). Guanylate-Binding Protein-Dependent Noncanonical Induced Multinucleated Giant Cell Formation. *MBio*, *12*(4).

Divangahi, M., Mostowy, S., Coulombe, F., Kozak, R., Guillot, L., Veyrier, F., Kobayashi, K. S., Flavell, R. A., Gros, P., & Behr, M. A. (2008). NOD2-Deficient Mice Have Impaired Resistance to Mycobacterium tuberculosis Infection through Defective Innate and Adaptive Immunity . *The Journal of Immunology*, *181*(10), 7157–7165.

Domaszewska, T., Scheuermann, L., Hahnke, K., Mollenkopf, H., Dorhoi, A., Kaufmann, S. H. E., & Weiner, J. (2017). Concordant and discordant gene expression patterns in mouse strains identify best-fit animal model for human tuberculosis. *Scientific Reports*, *7*(1), 1–13.

Donald, P. R., Diacon, A. H., Lange, C., Demers, A. M., Von Groote-Biddlingmeier, F., & Nardell, E. (2018). Droplets, dust and Guinea pigs: An historical review of tuberculosis transmission research, 1878-1940. *International Journal of Tuberculosis and Lung Disease*, *22*(9), 972–982.

Drage, M. G., Pecora, N. D., Hise, A. G., Febbraio, M., Silverstein, R. L., Golenbock, D. T., Boom, W. H., & Harding, C. V. (2009). TLR2 and its co-receptors determine responses of macrophages and dendritic cells to lipoproteins of Mycobacterium tuberculosis. *Cellular Immunology*, *258*(1), 29–37.

Drage, M. G., Tsai, H. C., Pecora, N. D., Cheng, T. Y., Arida, A. R., Shukla, S., Rojas, R. E., Seshadri, C., Moody, D. B., Boom, W. H., Sacchettini, J. C., & Harding, C. V. (2010). Mycobacterium tuberculosis lipoprotein LprG (Rv1411c) binds triacylated glycolipid agonists of Toll-like receptor 2. *Nature Structural and Molecular Biology*, *17*(9), 1088–1095.

Drennan, M. B., Nicolle, D., Quesniaux, V. J. F., Jacobs, M., Allie, N., Mpagi, J., Frémond, C., Wagner, H., Kirschning, C., & Ryffel, B. (2004). Toll-Like Receptor 2-Deficient Mice Succumb to Mycobacterium tuberculosis Infection. *American Journal of Pathology*, *164*(1), 49–57.

Dussurget, O., Stewart, G., Neyrolles, O., Pescher, P., Young, D., & Marchal, G. (2001). Role of Mycobacterium tuberculosis copper-zinc superoxide dismutase. *Infection and Immunity*, *69*(1), 529–533.

Ehrt, S., & Schnappinger, D. (2009). Mycobacterial survival strategies in the phagosome: Defence against host stresses. *Cellular Microbiology*, *11*(8), 1170–1178.

Ehrt, S., Schnappinger, D., Bekiranov, S., Drenkow, J., Shi, S., Gingeras, T. R., Gaasterland, T., Schoolnik, G., & Nathan, C. (2001). Reprogramming of the macrophage transcriptome in response to interferon gamma and Mycobacterium tuberculosis : signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *Journal of Experimental Medicine*, *194*, 1123–1139.

Eldar, A., & Elowitz, M. B. (2010). Functional roles for noise in genetic circuits. *Nature*, *467*(7312), 167–173.

Elowitz, M. B., Levine, A. J., Siggia, E. D., & Swain, P. S. (2002). Stochastic gene expression in a single cell. *Science*, *297*, 1183–1187.

Etna, M. P., Giacomini, E., Severa, M., & Coccia, E. M. (2014). Pro-and anti-inflammatory cytokines in tuberculosis: A two-edged sword in TB pathogenesis. *Seminars in Immunology*, *26*(6), 543–551.

Fatima, S., Kumari, A., Das, G., & Dwivedi, V. P. (2020). Tuberculosis vaccine: A journey from BCG to present. *Life Sciences*, *252*(March).

Fernandes-Alnemri, T., Yu, J. W., Datta, P., Wu, J., & Alnemri, E. S. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*, *458*(7237), 509–513.

Ferwerda, G., Girardin, S. E., Kullberg, B. J., Le Bourhis, L., De Jong, D. J., Langenberg, D. M. L., Van Crevel, R., Adema, G. J., Ottenhoff, T. H. M., Van Der Meer, J. W. M., & Netea, M. G. (2005). NOD2 and toll-like receptors are nonredundant recognition systems of Mycobacterium tuberculosis. *PLoS Pathogens*, *1*(3), 0279–0285.

Firmani, M. A., & Riley, L. W. (2002). Reactive nitrogen intermediates have a bacteriostatic effect on Mycobacterium tuberculosis in vitro. *Journal of Clinical Microbiology*, *40*(9), 3162–3166.

Flynn, B. J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A., & Bloom, B. R. (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *Journal of Experimental Medicine*, *178*, 2249–2254.

Flynn, J. L., Chan, J., & Lin, P. L. (2011). Macrophages and control of granulomatous inflammation in tuberculosis. *Mucosal Immunology*, *4*(3), 271–278.

Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schrelber, R., Mak, T. W., & Bloom, B. R. (1995). Tumor necrosis factor-α is required in the protective immune response against mycobacterium tuberculosis in mice. *Immunity*, *2*(6), 561–572.

Flynn, J. L., Goldstein, M. M., Triebold, K. J., Sypek, J., Wolf, S., & Bloom, B. R. (1995). IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection. *Journal of Immunology (Baltimore, Md. : 1950)*, *155*(5), 2515–2524.

Fremond, C. M., Doz, E., Rose, S., Vasseur, V., Maillet, I., Jacobs, M., Ryffel, B., & Quesniaux, V. F. J. (2007). IL-1 Receptor-Mediated Signal Is an Essential Component tuberculosis Infection. *The Journal of Immunology*, *18*(31), 1178.

Fremond, C. M., Togbe, D., Doz, E., Rose, S., Vasseur, V., Maillet, I., Jacobs, M., Ryffel, B., Valerie, F. J., Fremond, C. M., Doz, E., Rose, S., Vasseur, V., Maillet, I., Jacobs, M., Ryffel, B., & Quesniaux, V. F. J. (2007). IL-1 receptor mediated signal is an essential component of MyD88-Dependent Innate response to Mycobacterium tuberculosis infection. *The Journal of Immunology*, *179*, 1178–1189.

Friedman, N., Cai, L., & Xie, X. S. (2006). Linking stochastic dynamics to population distribution: An analytical framework of gene expression. *Physical Review Letters*, *97*(16), 1–4.

Gaffney, E., Murphy, D., Walsh, A., Connolly, S., Basdeo, S. A., Keane, J., & Phelan, J. J. (2022). Defining the role of neutrophils in the lung during infection: Implications for tuberculosis disease. *Frontiers in Immunology*, *13*(September), 1–14.

Gandotra, S., Jang, S., Murray, P. J., Salgame, P., & Ehrt, S. (2007). Nucleotide-binding oligomerization domain protein 2-deficient mice control infection with Mycobacterium tuberculosis. *Infection and Immunity*, *75*(11), 5127–5134.

Garces, A., Atmakuri, K., Chase, M. R., Woodworth, J. S., Krastins, B., Rothchild, A. C., Ramsdell, T.

L., Lopez, M. F., Behar, S. M., Sarracino, D. A., & Fortune, S. M. (2010). EspA acts as a critical mediator of ESX1-dependent virulence in Mycobacterium tuberculosis by affecting bacterial cell wall integrity. *PLoS Pathogens*, *6*(6), 15–16.

Gibbings, S. L., Thomas, S. M., Atif, S. M., McCubbrey, A. L., Desch, A. N., Danhorn, T., Leach, S. M., Bratton, D. L., Henson, P. M., Janssen, W. J., & Jakubzick, C. V. (2017). Three unique interstitial macrophages in the murine lung at steady state. *American Journal of Respiratory Cell and Molecular Biology*, *57*(1), 66–76.

Gideon, H. P., Hughes, T. K., Tzouanas, C. N., Wadsworth, M. H., Tu, A. A., Gierahn, T. M., Peters, J. M., Hopkins, F. F., Wei, J. R., Kummerlowe, C., Grant, N. L., Nargan, K., Phuah, J. Y., Borish, H. J., Maiello, P., White, A. G., Winchell, C. G., Nyquist, S. K., Ganchua, S. K. C., … Shalek, A. K. (2022). Multimodal profiling of lung granulomas in macaques reveals cellular correlates of tuberculosis control. *Immunity*, *55*(5), 827-846.e10.

Gideon, H. P., Skinner, J. A., Baldwin, N., Flynn, J. L., & Lin, P. L. (2016). Early Whole Blood Transcriptional Signatures Are Associated with Severity of Lung Inflammation in Cynomolgus Macaques with Mycobacterium tuberculosis Infection . *The Journal of Immunology*, *197*(12), 4817–4828.

Gierahn, T. M., Wadsworth, M. H., Hughes, T. K., Bryson, B. D., Butler, A., Satija, R., Fortune, S., Christopher Love, J., & Shalek, A. K. (2017). Seq-Well: Portable, low-cost rna sequencing of single cells at high throughput. *Nature Methods*, *14*(4), 395–398.

Gijsbers, A., Eymery, M., Gao, Y., Menart, I., Vinciauskaite, V., Siliqi, D., Peters, P. J., McCarthy, A., & Ravelli, R. B. G. (2023). The crystal structure of the EspB-EspK virulence factor-chaperone complex suggests an additional type VII secretion mechanism in Mycobacterium tuberculosis. *Journal of Biological Chemistry*, *299*(1), 102761.

Giosuè, S., Casarini, M., Alemanno, L., Galluccio, G., Mattia, P., Pedicelli, G., Rebek, L., Bisetti, A., & Ameglio, F. (1998). Effects of aerosolized interferon-α in patients with pulmonary tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, *158*(4), 1156–1162.

Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D. J., & Sansonetti, P. J. (2003). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *Journal of Biological Chemistry*, *278*(11), 8869–8872.

Gonzalez-Juarrero, M., Hattle, J. M., Izzo, A., Junqueira-Kipnis, A. P., Shim, T. S., Trapnell, B. C., Cooper, A. M., & Orme, I. M. (2005). Disruption of granulocyte macrophage-colony stimulating factor production in the lungs severely affects the ability of mice to control Mycobacterium tuberculosis infection . *Journal of Leukocyte Biology*, *77*(6), 914–922.

Goren, M. B., Brokl, O., & Schaefer, W. B. (1974). Lipids of Putative Relevance to Virulence in Mycobacterium tuberculosis: Phthiocerol Dimycocerosate and the Attenuation Indicator Lipid. *Infection and Immunity*, *9*(1), 150–158.

Goude, R., Amin, A. G., Chatterjee, D., & Parish, T. (2009). The arabinosyltransferase EmbC is inhibited by ethambutol in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, *53*(10), 4138–4146.

Greenwood, D. J., Dos Santos, M. S., Huang, S., Russell, M. R. G., Collinson, L. M., MacRae, J. I., West, A., Jiang, H., & Gutierrez, M. G. (2019). Subcellular antibiotic visualization reveals a dynamic drug reservoir in infected macrophages. *Science*, *364*(6447), 1279–1282.

Gröschel, M. I., Sayes, F., Simeone, R., Majlessi, L., & Brosch, R. (2016). ESX secretion systems: Mycobacterial evolution to counter host immunity. *Nature Reviews Microbiology*, *14*(11), 677–691.

Guenin-Macé, L., Siméone, R., & Demangel, C. (2009). Lipids of pathogenic mycobacteria: Contributions to virulence and host immune suppression. *Transboundary and Emerging Diseases*, *56*(6–7), 255–268.

Guo, Q., Bi, J., Wang, H., & Zhang, X. (2021). Mycobacterium tuberculosis ESX-1-secreted substrate protein EspC promotes mycobacterial survival through endoplasmic reticulum stress-mediated apoptosis. *Emerging Microbes and Infections*, *10*(1), 19–36.

Gupta, A., Ahmad, F. J., Ahmad, F., Gupta, U. D., Natarajan, M., Katoch, V. M., & Bhaskar, S. (2012). Protective efficacy of Mycobacterium indicus pranii against tuberculosis and underlying local lung immune responses in guinea pig model. *Vaccine*, *30*(43), 6198–6209.

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I., & Deretic, V. (2004).

Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell*, *119*(6), 753–766.

He, D., Bai, F., Zhang, S., Jiang, T., Shen, J., Zhu, Q., Yue, T., Shao, L., Gao, Y., Feng, Y., Weng, X., Zou, H., Zhang, Y., & Zhang, W. (2013). High incidence of tuberculosis infection in rheumatic diseases and impact for chemoprophylactic prevention of tuberculosis activation during biologics therapy. *Clinical and Vaccine Immunology*, *20*(6), 892–899.

Heitmann, L., Schoenen, H., Ehlers, S., Lang, R., & Hölscher, C. (2013). Mincle is not essential for controlling Mycobacterium tuberculosis infection. *Immunobiology*, *218*(4), 506–516.

Herbst, S., Schaible, U. E., & Schneider, B. E. (2011). Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *Plos One*, *6*(5), 1–8.

Herrtwich, L., Nanda, I., Evangelou, K., Nikolova, T., Horn, V., Sagar, Erny, D., Stefanowski, J., Rogell, L., Klein, C., Gharun, K., Follo, M., Seidl, M., Kremer, B., Münke, N., Senges, J., Fliegauf, M., Aschman, T., Pfeifer, D., … Triantafyllopoulou, A. (2016). DNA Damage Signaling Instructs Polyploid Macrophage Fate in Granulomas. *Cell*, *167*(5), 1264-1280.e18.

Hirsch, C. S., Ellner, J. J., Russell, D. G., & Rich, E. A. (1994). Complement receptor-mediated uptake and tumor necrosis factor-alpha-mediated growth inhibition of Mycobacterium tuberculosis by human alveolar macrophages. *Journal of Immunology (Baltimore, Md. : 1950)*, *152*(2), 743–753.

Hou, B., Reizis, B., & DeFranco, A. L. (2008). Toll-like Receptors Activate Innate and Adaptive Immunity by using Dendritic Cell-Intrinsic and -Extrinsic Mechanisms. *Immunity*, *29*(2), 272–282.

Houben, D., Demangel, C., van Ingen, J., Perez, J., Baldeón, L., Abdallah, A. M., Caleechurn, L., Bottai, D., van Zon, M., de Punder, K., van der Laan, T., Kant, A., Bossers-de Vries, R., Willemsen, P., Bitter, W., van Soolingen, D., Brosch, R., van der Wel, N., & Peters, P. J. (2012). ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. *Cellular Microbiology*, *14*(8), 1287–1298.

Houk, V. N., Baker, J. H., Sorensen, K., & Kent, D. C. (1968). The epidemiology of tuberculosis infection in a closed environment. *Archives of Environmental Health*, *16*(1), 26–35.

Huang, D. D., & Bao, L. (2016). Mycobacterium tuberculosis EspB protein suppresses interferon-γ-induced autophagy in murine macrophages. *Journal of Microbiology, Immunology and Infection*, *49*(6), 859–865.

Huang, L., Nazarova, E. V., Tan, S., Liu, Y., & Russell, D. G. (2018). Growth of Mycobacterium tuberculosis in vivo segregates with host macrophage metabolism and ontogeny. *Journal of Experimental Medicine*, *215*(4), 1135–1152.

Huber, A., Killy, B., Grummel, N., Bodendorfer, B., Paul, S., Wiesmann, V., Naschberger, E., Zimmer, J., Wirtz, S., Schleicher, U., Vera, J., Ekici, A. B., Dalpke, A., & Lang, R. (2020). Mycobacterial Cord Factor Reprograms the Macrophage Response to IFN-γ towards Enhanced Inflammation yet Impaired Antigen Presentation and Expression of GBP1. *The Journal of Immunology*, *205*(6), 1580–1592.

Intemann, C. D., Thye, T., Niemann, S., Browne, E. N. L., Chinbuah, M. A., Enimil, A., Gyapong, J., Osei, I., Owusu-Dabo, E., Helm, S., Rüsch-Gerdes, S., Horstmann, R. D., & Meyer, C. G. (2009). Autophagy gene variant IRGM -261T contributes to protection from tuberculosis caused by Mycobacterium tuberculosis but not by M. africanum strains. *PLoS Pathogens*, *5*(9).

Ishikawa, E., Ishikawa, T., Morita, Y. S., Toyonaga, K., Yamada, H., Takeuchi, O., Kinoshita, T., Akira, S., Yoshikai, Y., & Yamasaki, S. (2009). Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *Journal of Experimental Medicine*, *206*(13), 2879–2888.

Jagannath, C., Actor, J. K., & Hunter, R. L. (1998). Induction of nitric oxide in human monocytes and monocyte cell lines by Mycobacterium tuberculosis. *Nitric Oxide - Biology and Chemistry*, *2*(3), 174–186.

Jayaraman, P., Sada-Ovalle, I., Nishimura, T., Anderson, A. C., Kuchroo, V. K., Remold, H. G., & Behar, S. M. (2013). IL-1 beta promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *The Journal of Immunology*, *190*(8), 4196–4204.

Józefowski, S., Sobota, A., Pawłowski, A., & Kwiatkowska, K. (2011). Mycobacterium tuberculosis lipoarabinomannan enhances LPS-induced TNF-α production and inhibits NO secretion by engaging scavenger receptors. *Microbial Pathogenesis*, *50*(6), 350–359.

Juárez, E., Carranza, C., Hernández-Sánchez, F., León-Contreras, J. C., Hernández-Pando, R., Escobedo, D., Torres, M., & Sada, E. (2012). NOD2 enhances the innate response of alveolar macrophages to Mycobacterium tuberculosis in humans. *European Journal of Immunology*, *42*(4), 880–889.

Juffermans, N. P., Florquin, S., Camoglio, L., Verbon, A., Kolk, A. H., Speelman, P., Deventer, S. J. H. Van, & van der Poll, T. (2000). Interleukin-1 Signaling Is Essential for Host Defense during Murine Pulmonary Tuberculosis. *The Journal of Infectious Diseases*, *182*, 902–908.

Jung, J., Madan-lala, R., Georgieva, M., Rengarajan, J., Sohaskey, C. D., Bange, F., & Robinson, C. M. (2013). The intracellular environment of human macrophages that produce nitric oxide promotes growth of Mycobacteria. *Infection and Immunity*, *81*(9), 3198–3209.

Kang, P. B., Azad, A. K., Torrelles, J. B., Kaufman, T. M., Beharka, A., Tibesar, E., DesJardin, L. E., & Schlesinger, L. S. (2005). The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. *Journal of Experimental Medicine*, *202*(7), 987–999.

Kaveh, D. A., Garcia-Pelayo, M. C., Bull, N. C., Sanchez-Cordon, P. J., Spiropoulos, J., & Hogarth, P. J. (2020). Airway delivery of both a BCG prime and adenoviral boost drives CD4 and CD8 T cells into the lung tissue parenchyma. *Scientific Reports*, *10*(1), 1–13.

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., Cuellar, T., Haley, B., Roose-Girma, M., Phung, Q. T., Liu, P. S., Lill, J. R., Li, H., Wu, J., Kummerfeld, S., Zhang, J., Lee, W. P., Snipas, S. J., Salvesen, G. S., … Dixit, V. M. (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*, *526*(7575), 666–671.

Kayagaki, N., Warming, S., Lamkanfi, M., Walle, L. Vande, Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., Zhang, J., Lee, W. P., Roose-Girma, M., & Dixit, V. M. (2011). Non-canonical inflammasome activation targets caspase-11. *Nature*, *479*(7371), 117–121.

Keane, J., Gershon, S., Wise, R. P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W. D., Siegel, J. N., & Braun, M. M. (2001). Tuberculosis associated with infliximab, a tumor necrosis factor alpha neutralizing agent. *The New England Journal of Medicine*, *345*(15).

Keller, M., Mazuch, J., Abraham, U., Eom, G. D., Herzog, E. D., Volk, H. D., Kramer, A., & Maier, B. (2009). A circadian clock in macrophages controls inflammatory immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(50), 21407–21412.

Khoshnood, S., Goudarzi, M., Taki, E., Darbandi, A., Kouhsari, E., Heidary, M., Motahar, M., Moradi, M., & Bazyar, H. (2021). Bedaquiline: Current status and future perspectives. *Journal of Global Antimicrobial Resistance*, *25*, 48–59.

Kim, B.-H., Shenoy, A. R., Kumar, P., Das, R., Tiwari, S., & MacMicking, J. D. (2011). A Family of IFN-g–Inducible 65-kD GTPases Protects Against Bacterial Infection. *Science*, *332*.

Kim, J. J., Lee, H. M., Shin, D. M., Kim, W., Yuk, J. M., Jin, H. S., Lee, S. H., Cha, G. H., Kim, J. M., Lee, Z. W., Shin, S. J., Yoo, H., Park, Y. K., Park, J. B., Chung, J., Yoshimori, T., & Jo, E. K. (2012). Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. © 2012 Elsevier Inc. *Cell Host and Microbe*, *11*(5), 457–468.

King, K. Y., Lew, J. D., Ha, N. P., Lin, J. S., Ma, X., Graviss, E. A., & Goodell, M. A. (2011). Polymorphic allele of human IRGM1 is associated with susceptibility to tuberculosis in African Americans. *PLoS ONE*, *6*(1), 4–6.

Kisich, K. O., Higgins, M., Diamond, G., & Heifets, L. (2002). Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophils. *Infection and Immunity*, *70*(8), 4591–4599.

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

Kurenuma, T., Kawamura, I., Hara, H., Uchiyama, R., Daim, S., Dewamitta, S. R., Sakai, S., Tsuchiya, K., Nomura, T., & Mitsuyama, M. (2009). The RD1 Locus in the Mycobacterium tuberculosis Genome Contributes to Activation of Caspase-1 via Induction of Potassium Ion Efflux in Infected Macrophages. *Infection and Immunity*, *77*(9), 3992–4001.

Kusuhara, K., Yamamoto, K., Okada, K., Mizuno, Y., & Hara, T. (2007). Association of IL12RB1 polymorphisms with susceptibility to and severity of tuberculosis in Japanese: A gene-based association analysis of 21 candidate genes. *International Journal of Immunogenetics*, *34*(1), 35–

44.

Kyei, G. B., Vergne, I., Chua, J., Roberts, E., Harris, J., Junutula, J. R., & Deretic, V. (2006). Rab14 is critical for maintenance of Mycobacterium tuberculosis phagosome maturation arrest. *EMBO Journal*, *25*(22), 5250–5259.

Ladel, C. H., Blum, C., Dreher, A., Reifenberg, K., Kopf, M., & Kaufmann, S. H. E. (1997). Lethal tuberculosis in interleukin-6-deficient mutant mice. *Infection and Immunity*, *65*(11), 4843–4849.

Ladel, C. H., Szalay, G., Riedel, D., & Kaufmann, S. H. E. (1997). Interleukin-12 secretion by Mycobacterium tuberculosis-infected macrophages. *Infection and Immunity*, *65*(5), 1936–1938.

Lafuse, W. P., Rajaram, M. V. S., Wu, Q., Moliva, J. I., Torrelles, J. B., Turner, J., & Schlesinger, L. S. (2019). Identification of an Increased Alveolar Macrophage Subpopulation in Old Mice That Displays Unique Inflammatory Characteristics and Is Permissive to Mycobacterium tuberculosis Infection . *The Journal of Immunology*, *203*(8), 2252–2264.

Lai, Y. F., Lin, T. M., Wang, C. H., Su, P. Y., Wu, J. T., Lin, M. C., & Eng, H. L. (2016). Functional polymorphisms of the TLR7 and TLR8 genes contribute to Mycobacterium tuberculosis infection. *Tuberculosis*, *98*, 125–131.

Lalvani, A., & Pareek, M. (2010). A 100 year update on diagnosis of tuberculosis infection. *British Medical Bulletin*, *93*(1), 69–84.

Landes, M. B., Rajaram, M. V. S., Nguyen, H., & Schlesinger, L. S. (2015). Role for NOD2 in Mycobacterium tuberculosis-induced iNOS expression and NO production in human macrophages. *Journal of Leukocyte Biology*, *97*, 1111–1119.

Lannan, R., Maity, A., & Wollman, R. (2022). Epigenetic fluctuations underlie gene expression timescales and variability. *Physiological Genomics*, *54*(6), 220–229.

Lee, B. L., Stowe, I. B., Gupta, A., Kornfeld, O. S., Roose-Girma, M., Anderson, K., Warming, S., Zhang, J., Lee, W. P., & Kayagaki, N. (2018). Caspase-11 auto-proteolysis is crucial for noncanonical inflammasome activation. *Journal of Experimental Medicine*, *215*(9), 2279–2288.

Lee, W. L., Gold, B., Darby, C., Brot, N., Jian, X., de Carvalho, L. P. S., Wellner, D., St John, G., Jacobs Jr, W. R., & Nathan, C. (2009). Mycobacterium tuberculosis expresses methionine sulfoxide reductases A and B that protect from killing by nitrite and hypochlorite. *Molecular Microbiology*, *71*(3).

Lerner, T. R., Borel, S., Greenwood, D. J., Repnik, U., Russell, M. R. G., Herbst, S., Jones, M. L., Collinson, L. M., Griffiths, G., & Gutierrez, M. G. (2017). Mycobacterium tuberculosis replicates within necrotic human macrophages. *The Journal of Cell Biology*, *216*(3), 583–594.

Lerner, T. R., Queval, C. J., Lai, R. P., Russell, M. R. G., Fearns, A., Greenwood, D. J., Collinson, L., Wilkinson, R. J., & Gutierrez, M. G. (2020). Mycobacterium tuberculosis cords within lymphatic endothelial cells to evade host immunity. *JCI Insight*, *5*(10).

Lewis, K. N., Liao, R., Guinn, K. M., Hickey, M. J., Smith, S., Behr, M. A., & Sherman, D. R. (2003). Deletion of RD1 from Mycobacterium tuberculosis mimics bacille Calmette-Guérin attenuation. *Journal of Infectious Diseases*, *187*(1), 117–123.

Li, Q., Liu, C., Yue, R., El-Ashram, S., Wang, J., He, X., Zhao, D., Zhou, X., & Xu, L. (2019). Cgas/sting/tbk1/irf3 signaling pathway activates bmdcs maturation following mycobacterium bovis infection. *International Journal of Molecular Sciences*, *20*(4), 1–13.

Li, Y., & Jiang, Q. (2023). Uncoupled pyroptosis and IL-1β secretion downstream of inflammasome signaling. *Frontiers in Immunology*, *14*(April).

Lienard, J., Nobs, E., Lovins, V., Movert, E., Valfridsson, C., & Carlsson, F. (2020). The Mycobacterium marinum ESX-1 system mediates phagosomal permeabilization and type I interferon production via separable mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(2), 1160–1166.

Lim, Z. L., Drever, K., Dhar, N., Cole, S. T., & Chen, J. M. (2022). Mycobacterium tuberculosis EspK Has Active but Distinct Roles in the Secretion of EsxA and EspB. *Journal of Bacteriology*, *204*(4).

Lin, P. L., Ford, C. B., Coleman, M. T., Myers, A. J., Gawande, R., Ioerger, T., Sacchettini, J., Fortune, S. M., & Flynn, J. L. (2014). Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nature Medicine*, *20*(1), 75–79.

Liu, C. H., Liu, H., & Ge, B. (2017). Innate immunity in tuberculosis: Host defense vs pathogen

evasion. *Cellular and Molecular Immunology*, *14*(12), 963–975.

Liu, L., Zhai, K., Chen, Y., Chen, X., Wang, G., & Wu, L. (2021). Effect and Mechanism of Mycobacterium tuberculosis Lipoprotein LpqH in NLRP3 Inflammasome Activation in Mouse Ana-1 Macrophage. *BioMed Research International*, *2021*.

Liu, Q., Wang, J., Sandford, A. J., Wu, J., Wang, Y., Wu, S., Ji, G., Chen, G., Feng, Y., Tao, C., & He, J. Q. (2015). Association of CYBB polymorphisms with tuberculosis susceptibility in the Chinese Han population. *Infection, Genetics and Evolution*, *33*, 169–175.

Lúcia Moreira-Teixeira, Mayer-Barber, K., Sher, A., & O'Garra, A. (2018). Type I interferons in tuberculosis: Foe and occasionally friend. *Journal of Experimental Medicine*, *215*(5), 1273–1285.

Ly, A., & Liu, J. (2020). Mycobacterial virulence factors: Surface-exposed lipids and secreted proteins. *International Journal of Molecular Sciences*, *21*(11).

MacGilvary, N. J., & Tan, S. (2018). Fluorescent Mycobacterium tuberculosis reporters: Illuminating host-pathogen interactions. *Pathogens and Disease*, *76*(3), 1–12.

MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K., & Nathan, C. F. (1997). Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proceedings of the National Academy of Sciences*, *94*, 5243–5248.

MacMicking, J. D., Taylor, G. A., & McKinney, J. D. (2003). Immune Control of Tuberculosis by IFN-γ-inducible LRG-47. *Science*, *302*(5645), 654–659.

Madan-Lala, R., Peixoto, K. V., Re, F., & Rengarajan, J. (2011). Mycobacterium tuberculosis hip1 dampens macrophage proinflammatory responses by limiting toll-like receptor 2 activation. *Infection and Immunity*, *79*(12), 4828–4838.

Madan-Lala, R., Sia, J. K., King, R., Adekambi, T., Monin, L., Khader, S. A., Pulendran, B., & Rengarajan, J. (2014). Mycobacterium tuberculosis Impairs Dendritic Cell Functions through the Serine Hydrolase Hip1 . *The Journal of Immunology*, *192*(9), 4263–4272.

Mahamed, D., Boulle, M., Ganga, Y., Mc Arthur, C., Skroch, S., Oom, L., Catinas, O., Pillay, K., Naicker, M., Rampersad, S., Mathonsi, C., Hunter, J., Sreejit, G., Pym, A. S., Lustig, G., & Sigal, A. (2017). Intracellular growth of Mycobacterium tuberculosis after macrophage cell death leads to serial killing of host cells. *ELife*, *6*, 1–26.

Manca, C., Tsenova, L., Bergtold, A., Freeman, S., Tovey, M., Musser, J. M., Barry, C. E., Freedman, V. H., & Kaplan, G. (2001). Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α/β. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(10), 5752–5757.

Manina, G., Dhar, N., & Mckinney, J. D. (2015). Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host and Microbe*, *17*, 32–46.

Manina, G., Griego, A., Singh, L. K., McKinney, J. D., & Dhar, N. (2019). Preexisting variation in DNA damage response predicts the fate of single mycobacteria under stress. *The EMBO Journal*, *38*(22), 1–19.

Mansoori, D., Tavana, S., Mirsaeidi, M., Yazdanpanah, M., & Sohrabpour, H. (2002). The Efficacy of Interferon-α in the Treatment of Multidrug Resistant Tuberculosis. *Tanaffos*, *1*(3), 29–34.

Manzanillo, P. S., Shiloh, M. U., Portnoy, D. A., & Cox, J. S. (2012). Mycobacterium tuberculosis activates the DNA-dependent cytosolic surveillance pathway within macrophages. *Cell Host and Microbe*, *11*(5), 469–480.

Marakalala, M. J., Raju, R. M., Sharma, K., Zhang, Y. J., Eugenin, E. A., Prideaux, B., Daudelin, I. B., Chen, P. Y., Booty, M. G., Kim, J. H., Eum, S. Y., Via, L. E., Behar, S. M., Barry, C. E., Mann, M., Dartois, V., & Rubin, E. J. (2016). Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nature Medicine*, *22*(5), 531–538.

Marinho, F. V., Benmerzoug, S., Rose, S., Campos, P. C., Marques, J. T., Báfica, A., Barber, G., Ryffel, B., Oliveira, S. C., & Quesniaux, V. F. J. (2018). The cGAS/STING Pathway Is Important for Dendritic Cell Activation but Is Not Essential to Induce Protective Immunity against Mycobacterium tuberculosis Infection. *Journal of Innate Immunity*, *10*(3), 239–252.

Marino, S., Cilfone, N. A., Mattila, J. T., Linderman, J. J., Flynn, J. L., & Kirschner, D. E. (2015). Macrophage polarization drives granuloma outcome during Mycobacterium tuberculosis

infection. *Infection and Immunity*, *83*(1), 324–338.

Marino, S., Pawar, S., Fuller, C. L., Reinhart, T. A., Flynn, J. L., & Kirschner, D. E. (2004). Dendritic Cell Trafficking and Antigen Presentation in the Human Immune Response to Mycobacterium tuberculosis . *The Journal of Immunology*, *173*(1), 494–506.

Martin, C. J., Cadena, A. M., Leung, V. W., Lin, L., Maiello, P., Hicks, N., Chase, M. R., Flynn, J. L., & Fortune, S. M. (2017). Digitally Barcoding Mycobacterium tuberculosis Reveals In Vivo infection Dynamics in the Macaque Model of Tuberculosis. *MBio*, *8*(3).

Martineau, A. R., Newton, S. M., Wilkinson, K. A., Kampmann, B., Hall, B. M., Nawroly, N., Packe, G. E., Davidson, R. N., Griffiths, C. J., & Wilkinson, R. J. (2007). Neutrophil-mediated innate immune resistance to mycobacteria. *Journal of Clinical Investigation*, *117*(7), 1988–1994.

Martinon, F., Burns, K., Boveresses, C., & Epalinges, C.-. (2002). The Inflammasome : A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-beta. *Molecular Cell*, *10*, 417–426.

Mayer-Barber, K. D., Barber, D. L., Shenderov, K., White, S. D., Wilson, M. S., Cheever, A., Kugler, D., Hieny, S., Caspar, P., Wilson, S., Cheever, A., Kugler, D., Hieny, S., Caspar, P., Nunez, G., Schlueter, D., Flavell, R. A., Sutterwala, F. S., & Sher, A. (2010). Cutting Edge: Caspase-1 Independent IL-1 β Production Is Critical for Host Resistance to Mycobacterium tuberculosis and Does Not Require TLR Signaling In Vivo. *The Journal of Immunology*, *184*, 3326–3330.

Mayo, R. E. P., & Stanford, J. L. (2000). Double-blind placebo-controlled trial of Mycobacterium vaccae immunotherapy for tuberculosis in KwaZulu, South Africa, 1991-97. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *94*(5), 563–568.

Mcadams, H. H., & Arkin, A. (1997). Stochastic mechanisms in gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(3), 814–819.

McCaffrey, E. F., Donato, M., Keren, L., Chen, Z., Delmastro, A., Fitzpatrick, M. B., Gupta, S., Greenwald, N. F., Baranski, A., Graf, W., Kumar, R., Bosse, M., Fullaway, C. C., Ramdial, P. K., Forgó, E., Jojic, V., Van Valen, D., Mehra, S., Khader, S. A., … Angelo, M. (2022). The immunoregulatory landscape of human tuberculosis granulomas. *Nature Immunology*, *23*(2), 318–329.

McClean, C. M., & Tobin, D. M. (2016). Macrophage form, function, and phenotype in mycobacterial infection: Lessons from tuberculosis and other diseases. *Pathogens and Disease*, *74*(7), 1–15.

Melo, E. M., Oliveira, V. L. S., Boff, D., & Galvão, I. (2021). Pulmonary macrophages and their different roles in health and disease. *International Journal of Biochemistry and Cell Biology*, *141*(October).

Meunier, E., & Broz, P. (2015). Interferon-induced guanylate-binding proteins promote cytosolic lipopolysaccharide detection by caspase-11. *DNA and Cell Biology*, *34*(1), 1–5.

Meunier, E., Dick, M. S., Dreier, R. F., Schürmann, N., Broz, D. K., Warming, S., Roose-Girma, M., Bumann, D., Kayagaki, N., Takeda, K., Yamamoto, M., & Broz, P. (2014). Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature*, *509*(7500), 366–370.

Miller, B. H., Fratti, R. A., Poschet, J. F., Timmins, G. S., Master, S. S., Burgos, M., Marletta, M. A., & Deretic, V. (2004). Mycobacteria Inhibit Nitric Oxide Synthase Recruitment to Phagosomes during Macrophage Infection. *Infection and Immunity*, *72*(5), 2872–2878.

Mishra, A., Singh, V. K., Actor, J. K., Hunter, R. L., Jagannath, C., Subbian, S., & Khan, A. (2020). GM-CSF Dependent Differential Control of Mycobacterium tuberculosis Infection in Human and Mouse Macrophages: Is Macrophage Source of GM-CSF Critical to Tuberculosis Immunity? *Frontiers in Immunology*, *11*(July).

Mishra, B. B., Moura-alves, P., Sonawane, A., Hacohen, N., Griffiths, G., Moita, L. F., & Anes, E. (2010). Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3 / ASC inflammasome. *Cellular Microbiology*, *12*(8), 1046–1063.

Mishra, B. B., Moura-Alves, P., Sonawane, A., Hacohen, N., Griffiths, G., Moita, L. F., & Anes, E. (2010). Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cellular Microbiology*, *12*(8), 1046–1063.

Mishra, B. B., Rathinam, V. A. K., Martens, G. W., Martinot, A. J., Kornfeld, H., Fitzgerald, K. A., & Sassetti, C. M. (2013). Nitric oxide controls the immunopathology of tuberculosis by inhibiting

NLRP3 inflammasome – dependent processing of IL-1 b. *Nature Immunology*, *14*(1), 52–61.

Moreira-Teixeira, L., Redford, P. S., Stavropoulos, E., Ghilardi, N., Maynard, C. L., Weaver, C. T., Freitas do Rosário, A. P., Wu, X., Langhorne, J., & O'Garra, A. (2017). T Cell–Derived IL-10 Impairs Host Resistance to Mycobacterium tuberculosis Infection . *The Journal of Immunology*, *199*(2), 613–623.

Moxon, R., & Kussell, E. (2017). The impact of bottlenecks on microbial survival, adaptation, and phenotypic switching in host–pathogen interactions. *Evolution*, *71*(12), 2803–2816.

Najmi, N., Kaur, G., Sharma, S. K., & Mehra, N. K. (2010). Human Toll-like receptor 4 polymorphisms TLR4 Asp299Gly and Thr399Ile influence susceptibility and severity of pulmonary tuberculosis in the Asian Indian population. *Tissue Antigens*, *76*(2), 102–109.

Naqvi, K. F., & Endsley, J. J. (2020). Myeloid C-Type Lectin Receptors in Tuberculosis and HIV Immunity: Insights Into Co-infection? *Frontiers in Cellular and Infection Microbiology*, *10*(June), 1–13.

Nathan, C. (2006). Neutrophils and immunity: Challenges and opportunities. *Nature Reviews Immunology*, *6*(3), 173–182.

Ng, V. H., Cox, J. S., Sousa, A. O., MacMicking, J. D., & McKinney, J. D. (2004). Role of KatG catalase-peroxidase in mycobacterial pathogenisis: Countering the phagocyte oxidative burst. *Molecular Microbiology*, *52*(5), 1291–1302.

Nguyen, T. K. T., d'Aigle, J., Chinea, L., Niaz, Z., Hunter, R. L., Hwang, S. A., & Actor, J. K. (2020). Mycobacterial Trehalose 6,6′-Dimycolate–Induced M1-Type Inflammation. *American Journal of Pathology*, *190*(2), 286–294.

Nicholson, S., Bonecini-Almeida, M. D. G., Lapa E Silva, J. R., Nathan, C., Xie, Q. W., Mumford, R., Weidner, J. R., Calaycay, J., Geng, J., Boechat, N., Linhares, C., Rom, W., & Ho, J. L. (1996). Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *Journal of Experimental Medicine*, *183*(5), 2293–2302.

Novick, A., & Weiner, M. (1957). Enzyme induction as an all or none phenomenon. *Proceedings of the National Academy of Sciences*, *1*, 553–566.

O'Leary, S., O'Sullivan, M. P., & Keane, J. (2011). IL-10 blocks phagosome maturation in Mycobacterium tuberculosis-infected human macrophages. *American Journal of Respiratory Cell and Molecular Biology*, *45*(1), 172–180.

Ohol, Y. M., Goetz, D. H., Chan, K., Shiloh, M. U., Craik, C. S., & Cox, J. S. (2010). Mycobacterium tuberculosis MycP1 Protease Plays a Dual Role in Regulation of ESX-1 Secretion and Virulence. *Cell Host and Microbe*, *7*(3), 210–220.

Olive, A. J., Smith, C. M., Baer, C. E., Coers, J., & Sassetti, C. M. (2023). Mycobacterium tuberculosis Evasion of Guanylate Binding Protein-Mediated Host Defense in Mice Requires the ESX1 Secretion System. *International Journal of Molecular Sciences*, *24*(3), 1–13.

Onyebujoh, P. C., Levin, J. B., Fourie, P. B., & Gathiram, V. (1999). Immunotherapy with Mycobacterium vaccae in patients with newly diagnosed pulmonary tuberculosis: A randomised controlled trial. *Lancet*, *354*(9173), 116–119.

Ordonez, A. A., Wang, H., Magombedze, G., Ruiz-Bedoya, C. A., Srivastava, S., Chen, A., Tucker, E. W., Urbanowski, M. E., Pieterse, L., Fabian Cardozo, E., Lodge, M. A., Shah, M. R., Holt, D. P., Mathews, W. B., Dannals, R. F., Gobburu, J. V. S., Peloquin, C. A., Rowe, S. P., Gumbo, T., … Jain, S. K. (2020). Dynamic imaging in patients with tuberculosis reveals heterogeneous drug exposures in pulmonary lesions. *Nature Medicine*, *26*(4), 529–534.

Osman, M. M., Pagan, A. J., Shanahan, J. K., & Ramakrishnan, L. (2020). Mycobacterium marinum phthiocerol dimycocerosates enhance macrophage phagosomal permeabilization and membrane damage. *PLoS ONE*, *15*(7 July), 1–10.

Pan, H., Dai, Y., Tang, S., & Wang, J. (2012). Polymorphisms of NOD2 and the risk of tuberculosis: A validation study in the Chinese population. *International Journal of Immunogenetics*, *39*(3), 233–240.

Pandey, A. K., Yang, Y., Jiang, Z., Fortune, S. M., Coulombe, F., Behr, M. A., Fitzgerald, K. A., Sassetti, C. M., & Kelliher, M. A. (2009). Nod2, Rip2 and Irf5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. *PLoS Pathogens*, *5*(7).

Pathak, S. K., Basu, S., Basu, K. K., Banerjee, A., Pathak, S., Bhattacharyya, A., Kaisho, T., Kundu,

M., & Basu, J. (2007). Direct extracellular interaction between the early secreted antigen ESAT-6 of Mycobacterium tuberculosis and TLR2 inhibits TLR signaling in macrophages. *Nature Immunology*, *8*(6), 610–618.

Patin, E. C., Geffken, A. C., Willcocks, S., Leschczyk, C., Haas, A., Nimmerjahn, F., Lang, R., Ward, T. H., & Schaible, U. E. (2017). Trehalose dimycolate interferes with FcγRmediated phagosome maturation through Mincle, SHP-1 and FcγRIIB signalling. *PLoS ONE*, *12*(4), 1–11.

Pecora, N. D., Gehring, A. J., Canaday, D. H., Boom, H., Harding, C. V, & Boom, W. H. (2012). Mycobacterium tuberculosis LprA Is a Lipoprotein Agonist of TLR2 That Regulates Innate Immunity and APC Function. *The Journal of Immunology*, *4*, 41–45.

Peters, W., Scott, H. M., Chambers, H. F., Flynn, J. L., Charo, I. F., & Ernst, J. D. (2001). Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(14), 7958–7963.

Piddington, D. L., Fang, F. C., Laessig, T., Cooper, A. M., Orme, I. M., & Buchmeier, N. A. (2001). Cu,Zn superoxide dismutase of Mycobacterium tuberculosis contributes to survival in activated macrophages that are generating an oxidative burst. *Infection and Immunity*, *69*(8), 4980–4987.

Pilli, M., Arko-Mensah, J., Ponpuak, M., Roberts, E., Master, S., Mandell, M. A., Dupont, N., Ornatowski, W., Jiang, S., Bradfute, S. B., Bruun, J. A., Hansen, T. E., Johansen, T., & Deretic, V. (2012). TBK-1 Promotes Autophagy-Mediated Antimicrobial Defense by Controlling Autophagosome Maturation. *Immunity*, *37*(2), 223–234.

Pilli, M., Arko-mensah, J., Ponpuak, M., Roberts, E., Master, S., Mandell, M., Dupont, N., Ornatowski, W., Jiang, S., Bruun, J., Hansen, T. E., Johansen, T., & Deretic, V. (2012). TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. *Immunity*, *37*(2), 223–234.

Pisu, D., Huang, L., Grenier, J. K., & Russell, D. G. (2020). Dual RNA-Seq of Mtb-Infected Macrophages In Vivo Reveals Ontologically Distinct Host-Pathogen Interactions. *Cell Reports*, *30*(2), 335-350.e4.

Pisu, D., Huang, L., Narang, V., Theriault, M., Lê-Bury, G., Lee, B., Lakudzala, A. E., Mzinza, D. T., Mhango, D. V., Mitini-Nkhoma, S. C., Jambo, K. C., Singhal, A., Mwandumba, H. C., & Russell, D. G. (2021). Single cell analysis of M. tuberculosis phenotype and macrophage lineages in the infected lung. *Journal of Experimental Medicine*, *218*(9).

Ponpuak, M., Davis, A. S., Roberts, E. A., Delgado, M. A., Dinkins, C., Zhao, Z., Virgin, H. W., Kyei, G. B., Johansen, T., Vergne, I., & Deretic, V. (2010). Delivery of Cytosolic Components by Autophagic Adaptor Protein p62 Endows Autophagosomes with Unique Antimicrobial Properties. *Immunity*, *32*(3), 329–341.

Prideaux, B., Via, L. E., Zimmerman, M. D., Eum, S., Sarathy, J., O'Brien, P., Chen, C., Kaya, F., Weiner, D. M., Chen, P. Y., Song, T., Lee, M., Shim, T. S., Cho, J. S., Kim, W., Cho, S. N., Olivier, K. N., Barry, C. E., & Dartois, V. (2015). The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nature Medicine*, *21*(10), 1223–1227.

Purwantini, E., & Mukhopadhyay, B. (2009). Conversion of NO2 to NO by reduced coenzyme F420 protects mycobacteria from nitrosative damage. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(15), 6333–6338.

Qian, J., Hu, Y., Zhang, X., Chi, M., Xu, S., Wang, H., & Zhang, X. (2022). Mycobacterium tuberculosis PE_PGRS19 Induces Pyroptosis through a Non-Classical Caspase-11/GSDMD Pathway in Macrophages. *Microorganisms*, *10*(12).

Qu, Z., Zhou, J., Zhou, Y., Xie, Y., Jiang, Y., Wu, J., Luo, Z., Liu, G., Yin, L., & Zhang, X. L. (2020). Mycobacterial EST12 activates a RACK1-NLRP3-gasdermin D pyroptosis-IL-1β immune pathway. *Science Advances*, *6*(43), 1–19.

Quigley, J., Hughitt, V. K., Velikovsky, C. A., Mariuzza, R. A., El-Sayed, N. M., & Briken, V. (2017). The cell wall lipid PDIM contributes to phagosomal escape and host cell exit of Mycobacterium tuberculosis. *MBio*, *8*(2).

Raffetseder, J., Iakobachvili, N., Loitto, V., Peters, P. J., & Lerm, M. (2019). Retention of EsxA in the capsule-like layer of Mycobacterium tuberculosis is associated with cytotoxicity and is counteracted by Lung surfactant. *Infection and Immunity*, *87*(3).

Raj, A., Peskin, C. S., Tranchina, D., Vargas, D. Y., & Tyagi, S. (2006). Stochastic mRNA synthesis in

mammalian cells. *PLoS Biology*, *4*(10), 1707–1719.

Rajaram, M. V. S., Arnett, E., Azad, A. K., Guirado, E., Ni, B., Gerberick, A. D., He, L. Z., Keler, T., Thomas, L. J., Lafuse, W. P., & Schlesinger, L. S. (2017). M. tuberculosis-Initiated Human Mannose Receptor Signaling Regulates Macrophage Recognition and Vesicle Trafficking by FcRγ-Chain, Grb2, and SHP-1. *Cell Reports*, *21*(1), 126–140.

Ramakrishnan, L. (2012). Revisiting the role of the granuloma in tuberculosis. *Nature Reviews Immunology*, *12*(5), 352–366.

Ramos-Kichik, V., Mondragón-Flores, R., Mondragón-Castelán, M., Gonzalez-Pozos, S., Muñiz-Hernandez, S., Rojas-Espinosa, O., Chacón-Salinas, R., Estrada-Parra, S., & Estrada-García, I. (2009). Neutrophil extracellular traps are induced by Mycobacterium tuberculosis. *Tuberculosis*, *89*(1), 29–37.

Rastogi, S., & Briken, V. (2022). Interaction of Mycobacteria With Host Cell Inflammasomes. *Frontiers in Immunology*, *13*, 791136.

Rastogi, S., Ellinwood, S., Augenstreich, J., Mayer-Barber, K. D., & Briken, V. (2021). Mycobacterium tuberculosis inhibits the NLRP3 inflammasome activation via its phosphokinase PknF. *PLoS Pathogens*, *17*(7), 1–30.

Ravesloot-Chavez, M. M., Van Dis, E., & Stanley, S. A. (2021). The Innate Immune Response to Mycobacterium tuberculosis Infection. *Annual Review of Immunology*, *39*, 611–637.

Redford, P. S., Boonstra, A., Read, S., Pitt, J., Graham, C., Stavropoulos, E., Bancroft, G. J., & O'Garra, A. (2010). Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *European Journal of Immunology*, *40*(8), 2200–2210.

Rens, C., Chao, J. D., Sexton, D. L., Tocheva, E. I., & Av-Gay, Y. (2021). Roles for phthiocerol dimycocerosate lipids in mycobacterium tuberculosis pathogenesis. *Microbiology*, *167*(3).

Renshaw, P. S., Panagiotidou, P., Whelan, A., Gordon, S. V., Glyn Hewinson, R., Williamson, R. A., & Carr, M. D. (2002). Conclusive evidence that the major T-cell antigens of the Mycobacterium tuberculosis complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6, CFP-10, and the ESAT-6·CFP-10 complex. Implications for p. *Journal of Biological Chemistry*, *277*(24), 21598–21603.

Riley, R. L., Mills, C. C., Nyka, W., Weinstock, N., Storey, P. B., Sultan, L. U., Riley, M. C., & Wells, W. F. (1995). Aerial dissemination of pulmonary tuberculosis. A two-year study of contagion in a tuberculosis ward. 1959. *American Journal of Epidemiology*, *142*(1), 3–14.

Rivers-Auty, J., & Brough, D. (2015). Potassium efflux fires the canon: Potassium efflux as a common trigger for canonical and noncanonical NLRP3 pathways. *European Journal of Immunology*, *45*(10), 2758–2761.

Rohde, K., Yates, R. M., Purdy, G. E., & Russell, D. G. (2007). Mycobacterium tuberculosis and the environment within the phagosome. *Immunological Reviews*, *219*(1), 37–54.

Romagnoli, A., Etna, M. P., Giacomini, E., Pardini, M., Remoli, M. E., Corazzari, M., Falasca, L., Goletti, D., Gafa, V., Simeone, R., Delogu, G., Piacentini, M., Brosch, R., Fimia, G. M., & Coccia, E. M. (2012). ESX-1 dependent impairment of autophagic flux by Mycobacterium tuberculosis in human dendritic cells. *Autophagy*, *8*(9), 1357–1370.

Rothchild, A. C., Olson, G. S., Nemeth, J., Amon, L. M., Mai, D., Gold, E. S., Diercks, A. H., & Aderem, A. (2019). Alveolar macrophages generate a noncanonical NRF2-driven transcriptional response to Mycobacterium tuberculosis in vivo. *Science Immunology*, *4*(37).

Rovetta, A. I., Peña, D., Hernández Del Pino, R. E., Recalde, G. M., Pellegrini, J., Bigi, F., Musella, R. M., Palmero, D. J., Gutierrez, M., Colombo, M. I., & García, V. E. (2014). IFNG-mediated immune responses enhance autophagy against Mycobacterium tuberculosis antigens in patients with active tuberculosis. *Autophagy*, *10*(12), 2109–2121.

Rühl, S., & Broz, P. (2015). Caspase-11 activates a canonical NLRP3 inflammasome by promoting K+ efflux. *European Journal of Immunology*, *45*(10), 2927–2936.

Rutschmann, O., Toniolo, C., & McKinney, J. D. (2022). Preexisting Heterogeneity of Inducible Nitric Oxide Synthase Expression Drives Differential Growth of Mycobacterium tuberculosis in Macrophages. *MBio*, *13*(5).

Sachdeva, K., Goel, M., Sudhakar, M., Mehta, M., Raju, R., Raman, K., Singh, A., & Sundaramurthy,

V. (2020). Mycobacterium tuberculosis (Mtb) lipid mediated lysosomal rewiring in infected macrophages modulates intracellular Mtb trafficking and survival. *Journal of Biological Chemistry*, *295*(27), 9192–9210.

Saiga, H., Kitada, S., Shimada, Y., Kamiyama, N., Okuyama, M., Makino, M., Yamamoto, M., & Takeda, K. (2012). Critical role of AIM2 in Mycobacterium tuberculosis infection. *International Immunology*, *24*(10), 637–644.

Saini, D., Hopkins, G. W., Seay, S. A., Chen, C., Perley, C. C., Click, E. M., & Frothingham, R. (2012). Ultra-low Dose of Mtb Aerosol Creates Partial Infection in Mice. *Tuberculosis*, *92*(2), 160–165.

Sala, C., Odermatt, N. T., Soler-Arnedo, P., Gülen, M. F., von Schultz, S., Benjak, A., & Cole, S. T. (2018). EspL is essential for virulence and stabilizes EspE, EspF and EspH levels in Mycobacterium tuberculosis. *PLoS Pathogens*, *14*(12), 1–27.

Salari, N., Kanjoori, A. H., Hosseinian-Far, A., Hasheminezhad, R., Mansouri, K., & Mohammadi, M. (2023). Global prevalence of drug-resistant tuberculosis: a systematic review and meta-analysis. *Infectious Diseases of Poverty*, *12*(1), 1–12.

Samstein, M., Schreiber, H. A., Leiner, I. M., Sušac, B., Glickman, M. S., & Pamer, E. G. (2013). Essential yet limited role for CCR2+ inflammatory monocytes during Mycobacterium tuberculosis-specific T cell priming. *ELife*, *2013*(2), 1–10.

Sanjuan, M. A., Dillon, C. P., Tait, S. W. G., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J. L., Withoff, S., & Green, D. R. (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature*, *450*(7173), 1253–1257.

Santos, J. C., Boucher, D., Schneider, L. K., Demarco, B., Dilucca, M., Shkarina, K., Heilig, R., Chen, K. W., Lim, R. Y. H., & Broz, P. (2020). Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. *Nature Communications*, *11*(1).

Scanga, C. A., Bafica, A., Feng, C. G., Cheever, A. W., Hieny, S., & Sher, A. (2004). MyD88-Deficient Mice Display A Profound Loss in Resistance to Mycobacterium tuberculosis Associated with Partially Impaired Th1 Cytokine and Nitric Oxide Synthase 2 Expression. *Infection and Immunity*, *72*(4), 2400–2404.

Schauvliege, R., Vanrobaeys, J., Schotte, P., & Beyaert, R. (2002). Caspase-11 gene expression in response to lipopolysaccharide and interferon-γ requires nuclear factor-κB and signal transducer and activator of transcription (STAT) 1. *Journal of Biological Chemistry*, *277*(44), 41624–41630.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682.

Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S., & Dinarello, C. (1990). Correlations and interactions in the production of interleukin-6 (IL- 6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood*, *75*(1), 40–47.

Schmid-Burgk, J. L., Gaidt, M. M., Schmidt, T., Ebert, T. S., Bartok, E., & Hornung, V. (2015). Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *European Journal of Immunology*, *45*(10), 2911–2917.

Schnettger, L., Rodgers, A., Repnik, U., Lai, R. P., Pei, G., Verdoes, M., Wilkinson, R. J., Young, D. B., & Gutierrez, M. G. (2017). A Rab20-Dependent Membrane Trafficking Pathway Controls M. tuberculosis Replication by Regulating Phagosome Spaciousness and Integrity. *Cell Host and Microbe*, *21*(5), 619-628.e5.

Schön, T., Elmberger, G., Negesse, Y., Hernandez Pando, R., Sundqvist, T., & Britton, S. (2004). Local production of nitric oxide in patients with tuberculosis. *International Journal of Tuberculosis and Lung Disease*, *8*(9), 1134–1137.

Schweneker, K., Gorka, O., Schweneker, M., Poeck, H., Tschopp, J., Peschel, C., Ruland, J., & Groß, O. (2013). The mycobacterial cord factor adjuvant analogue trehalose-6,6'-dibehenate (TDB) activates the Nlrp3 inflammasome. *Immunobiology*, *218*(4), 664–673.

Scott, H. M., & Flynn, J. A. L. (2002). Mycobacterium tuberculosis in chemokine receptor 2-deficient mice: Influence of dose on disease progression. *Infection and Immunity*, *70*(11), 5946–5954.

Serbina, N. V., & Pamer, E. G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nature Immunology*, *7*(3),

311–317.

Seto, S., Morimoto, K., Yoshida, T., Hiramatsu, M., Hijikata, M., Nagata, T., Kikuchi, F., Shiraishi, Y., Kurashima, A., & Keicho, N. (2020). Proteomic Profiling Reveals the Architecture of Granulomatous Lesions Caused by Tuberculosis and Mycobacterium avium Complex Lung Disease. *Frontiers in Microbiology*, *10*(January), 1–15.

Shah, S., Bohsali, A., Ahlbrand, S. E., Srinivasan, L., Rathinam, V. A. K., Vogel, S. N., Fitzgerald, K. A., Sutterwala, F. S., & Briken, V. (2013). Cutting Edge: Mycobacterium tuberculosis but Not Nonvirulent Mycobacteria Inhibits IFN-β and AIM2 Inflammasome–Dependent IL-1β Production via Its ESX-1 Secretion System . *The Journal of Immunology*, *191*(7), 3514–3518.

Sharma, S. K., Katoch, K., Sarin, R., Balambal, R., Kumar Jain, N., Patel, N., Murthy, K. J. R., Singla, N., Saha, P. K., Khanna, A., Singh, U., Kumar, S., Sengupta, A., Banavaliker, J. N., Chauhan, D. S., Sachan, S., Wasim, M., Tripathi, S., Dutt, N., … Rani, R. (2017). Efficacy and Safety of Mycobacterium indicus pranii as an adjunct therapy in Category II pulmonary tuberculosis in a randomized trial. *Scientific Reports*, *7*(1), 1–12.

Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., & Shao, F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature*, *526*(7575), 660–665.

Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., Hu, L., & Shao, F. (2014). Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature*, *514*(7521), 187–192.

Shi, S., & Ehrt, S. (2006). Dihydrolipoamide acyltransferase is critical for Mycobacterium tuberculosis pathogenesis. *Infection and Immunity*, *74*(1), 56–63.

Shim, D., Kim, H., & Shin, S. J. (2020). Mycobacterium tuberculosis Infection-Driven Foamy Macrophages and Their Implications in Tuberculosis Control as Targets for Host-Directed Therapy. *Frontiers in Immunology*, *11*(May), 1–10.

Shin, D. M., Jeon, B. Y., Lee, H. M., Jin, H. S., Yuk, J. M., Song, C. H., Lee, S. H., Lee, Z. W., Cho, S. N., Kim, J. M., Friedman, R. L., & Jo, E. K. (2010). Mycobacterium tuberculosis eis regulates autophagy, inflammation, and cell death through redox-dependent signaling. *PLoS Pathogens*, *6*(12).

Shin, D. M., Yuk, J. M., Lee, H. M., Lee, S. H., Son, J. W., Harding, C. V., Kim, J. M., Modlin, R. L., & Jo, E. K. (2010). Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling. *Cellular Microbiology*, *12*(11), 1648–1665.

Shukla, S., Richardson, E. T., Drage, M. G., Boom, H. W., & Harding, C. V. (2018). Mycobacterium tuberculosis Lipoprotein and Lipoglycan Binding to Toll-Like Receptor 2 Correlates with Agonist Activity and Functional Outcomes. *Infection and Immunity*, *86*(10), 1–12.

Sia, J. K., & Rengarajan, J. (2019). Immunology of mycobacterium tuberculosis infections. *Gram-Positive Pathogens*, 1056–1086.

Silvério, D., Gonçalves, R., Appelberg, R., & Saraiva, M. (2021). Advances on the Role and Applications of Interleukin-1 in Tuberculosis. *MBio*, *12*(6), 1–13.

Simeone, R., Bobard, A., Lippmann, J., Bitter, W., Majlessi, L., Brosch, R., & Enninga, J. (2012). Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. *PLoS Pathogens*, *8*(2).

Simmons, J. D., Stein, C. M., Seshadri, C., Campo, M., Alter, G., Fortune, S., Schurr, E., Wallis, R. S., Churchyard, G., Mayanja-Kizza, H., Boom, W. H., & Hawn, T. R. (2018). Immunological mechanisms of human resistance to persistent Mycobacterium tuberculosis infection. *Nature Reviews Immunology*, *18*(9), 575–589.

Singh, B., Cocker, D., Ryan, H., & Sloan, D. J. (2019). Linezolid for drug-resistant pulmonary tuberculosis. *Cochrane Database of Systematic Reviews*, *2019*(3).

Sköld, M., & Behar, S. M. (2008). Tuberculosis Triggers a Tissue-Dependent Program of Differentiation and Acquisition of Effector Functions by Circulating Monocytes. *The Journal of Immunology*, *181*(9), 6349–6360.

Smith, N. H., Hewinson, R. G., Kremer, K., Brosch, R., & Gordon, S. V. (2009). Myths and misconceptions: The origin and evolution of Mycobacterium tuberculosis. *Nature Reviews Microbiology*, *7*(7), 537–544.

Songane, M., Kleinnijenhuis, J., Alisjahbana, B., Sahiratmadja, E., Parwati, I., Oosting, M., Plantinga,

T. S., Joosten, L. A. B., Netea, M. G., Ottenhoff, T. H. M., van de Vosse, E., & van Crevel, R. (2012). Polymorphisms in autophagy genes and susceptibility to tuberculosis. *PloS One*, *7*(8), e41618.

Spertini, F., Audran, R., Chakour, R., Karoui, O., Steiner-Monard, V., Thierry, A. C., Mayor, C. E., Rettby, N., Jaton, K., Vallotton, L., Lazor-Blanchet, C., Doce, J., Puentes, E., Marinova, D., Aguilo, N., & Martin, C. (2015). Safety of human immunisation with a live-attenuated Mycobacterium tuberculosis vaccine: A randomised, double-blind, controlled phase I trial. *The Lancet Respiratory Medicine*, *3*(12), 953–962.

Srivastava, S., & Ernst, J. D. (2014). Cell-to-cell transfer of M. tuberculosis antigens optimizes CD4 T cell priming. *Cell Host and Microbe*, *15*(6), 741–752.

Strong, E. J., & Lee, S. (2021). Targeting Autophagy as a Strategy for Developing New Vaccines and Host-Directed Therapeutics Against Mycobacteria. *Frontiers in Microbiology*, *11*(January).

Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., Hao, Y., Stoeckius, M., Smibert, P., & Satija, R. (2019). Comprehensive Integration of Single-Cell Data. *Cell*, *177*(7), 1888-1902.e21.

Subbian, S., Tsenova, L., Kim, M., Wainwright, H. C., Visser, A., Bandyopadhyay, N., Bader, J. S., Karakousis, P. C., Murrmann, G. B., Bekker, L.-G., Russel, D. G., & Kaplan, G. (2015). Lesion-Specific Immune Response in Granulomas of Patients with Pulmonary Tuberculosis : A Pilot Study. *Plos One*, 1–21.

Talbot, E. A., & Raffa, B. J. (2014). Mycobacterium tuberculosis. In *Molecular Medical Microbiology*. Elsevier Ltd.

Tallieux, L., Schwartz, O., Herrmann, J. L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L. P., Gluckman, J. C., Lagrange, P. H., Gicquel, B., & Neyrolles, O. (2003). DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. *Journal of Experimental Medicine*, *197*(1), 121–127.

Tan, S. Y. S., & Krasnow, M. A. (2016). Developmental origin of lung macrophage diversity. *Development*, *143*, 1318–1327.

Tanne, A., & Neyrolles, O. (2010). C-type lectins in immune defense against pathogens the murine DC-SIGN homologue SIGNR3 confers early protection against mycobacterium tuberculosis infection. *Virulence*, *1*(4), 285–290.

Thacker, V. V., Dhar, N., Sharma, K., Barrile, R., Karalis, K., & McKinney, J. D. (2020). A lung-on-chip model of early m. Tuberculosis infection reveals an essential role for alveolar epithelial cells in controlling bacterial growth. *ELife*, *9*, 1–73.

Thomas, S. M., & Olive, A. J. (2023). High Lethality of Mycobacterium tuberculosis Infection in Mice Lacking the Phagocyte Oxidase and Caspase1/11. *Infection and Immunity*.

Thuong, N. T. T., Tram, T. T. B., Dinh, T. D., Thai, P. V. K., Heemskerk, D., Bang, N. D., Chau, T. T. H., Russell, D. G., Thwaites, G. E., Hawn, T. R., Caws, M., & Dunstan, S. J. (2016). MARCO variants are associated with phagocytosis, pulmonary tuberculosis susceptibility and Beijing lineage. *Genes and Immunity*, *17*(7), 419–425.

Thurston, T. L. M., Matthews, S. A., Jennings, E., Alix, E., Shao, F., Shenoy, A. R., Birrell, M. A., & Holden, D. W. (2016). Growth inhibition of cytosolic Salmonella by caspase-1 and caspase-11 precedes host cell death. *Nature Communications*, *7*, 1–15.

Tian, T., Woodworth, J., Sköld, M., Behar, M., Sko, M., & Behar, S. M. (2010). In Vivo Depletion of CD11c+ Cells Delays the CD4+ T Cell Response to Mycobacterium tuberculosis and Exacerbates the Outcome of Infection. *The Journal of Immunology*, 3–8.

Tiberi, S., du Plessis, N., Walzl, G., Vjecha, M. J., Rao, M., Ntoumi, F., Mfinanga, S., Kapata, N., Mwaba, P., McHugh, T. D., Ippolito, G., Migliori, G. B., Maeurer, M. J., & Zumla, A. (2018). Tuberculosis: progress and advances in development of new drugs, treatment regimens, and host-directed therapies. *The Lancet Infectious Diseases*, *18*(7), e183–e198.

Timmins, G. S., & Deretic, V. (2006). Mechanisms of action of isoniazid. *Molecular Microbiology*, *62*(5), 1220–1227.

Toniolo, C., Dhar, N., & McKinney, J. D. (2023). Uptake-independent killing of macrophages by extracellular Mycobacterium tuberculosis aggregates . *The EMBO Journal*, *42*(9), 1–20.

Toniolo, C., Rutschmann, O., & McKinney, J. D. (2021). Do chance encounters between

heterogeneous cells shape the outcome of tuberculosis infections? *Current Opinion in Microbiology*, *59*(Figure 1), 72–78.

Toossi, Z., Gogate, P., Shiratsuchi, H., Young, T., & Ellner, J. J. (1995). Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. *Journal of Immunology (Baltimore, Md. : 1950)*, *154*(1), 465–473.

Tsao, T. C. Y., Hong, J. H., Li, L. F., Hsieh, M. J., Liao, S. K., & Chang, K. S. S. (2000). Imbalances between tumor necrosis factor-α and its soluble receptor forms, and interleukin-1β and interleukin-1 receptor antagonist in BAL fluid of cavitary pulmonary tuberculosis. *Chest*, *117*(1), 103–109.

Turner, J., & Torrelles, J. B. (2018). Mannose-capped lipoarabinomannan in Mycobacterium tuberculosis pathogenesis. *Pathogens and Disease*, *76*(4), 1–15.

Ufimtseva, E. G., Eremeeva, N. I., Umpeleva, T. V, Vakhrusheva, D. V, & Skornyakov, S. N. (2021). Mycobacterium tuberculosis Load in Host Cells and the Antibacterial Activity of Alveolar Macrophages Are Linked and Differentially Regulated in Various Lung Lesions of Patients with Pulmonary Tuberculosis. *International Journal of Molecular Sciences*, *21*.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., & Peters, P. J. (2007). M. tuberculosis and M. leprae Translocate from the Phagolysosome to the Cytosol in Myeloid Cells. *Cell*, *129*(7), 1287–1298.

VanHeyningen, T. K., Collins, H. L., & Russell, D. G. (1997). IL-6 produced by macrophages infected with Mycobacterium species suppresses T cell responses. *Journal of Immunology (Baltimore, Md. : 1950)*, *158*(1), 330–337.

Vankayalapati, R., Garg, A., Porgador, A., Griffith, D. E., Klucar, P., Safi, H., Girard, W. M., Cosman, D., Spies, T., & Barnes, P. F. (2005). Role of NK Cell-Activating Receptors and Their Ligands in the Lysis of Mononuclear Phagocytes Infected with an Intracellular Bacterium. *The Journal of Immunology*, *175*(7), 4611–4617.

Vergne, I., Fratti, R. A., Hill, P. J., Chua, J., Belisle, J., & Deretic, V. (2004). Mycobacterium tuberculosis phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. *Molecular Biology of the Cell*, *15*.

Von Reyn, C. F., Arbeit, R. D., Yeaman, G., Waddell, R. D., Marsh, B. J., Morin, P., Modlin, J. F., & Remold, H. G. (1997). Immunization of healthy adult subjects in the United States with inactivated Mycobacterium vaccae administered in a three-dose series. *Clinical Infectious Diseases*, *24*(5), 843–848.

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., & McKinney, J. D. (2013). Dynamic Persistence of Antibiotic-Stressed Mycobacteria. *Science*, *339*.

Walburger, A., Koul, A., Ferrari, G., Nguyen, L., Prescianotto-Baschong, C., Huygen, K., Klebl, B., Thompson, C., Bacher, G., & Pieters, J. (2004). Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science*, *304*(5678), 1800–1804.

Wand, M. P., Kim, B.-H., Park, E.-S., Boyle, K. B., Nayak, K., Lagrange, B., Herod, A., Henry, T., Zilbauer, M., Rohde, J., MacMicking, J. D., & Randow, F. (2020). Guanylate-Binding Proteins (GBPs) convert cytosolic bacteria into caspase-4 signaling platforms. *Nature Immunology*, *21*(8), 880–891.

Wang, C., Chen, Z. L., Pan, Z. F., Wei, L. L., Xu, D. D., Jiang, T. T., Zhang, X., Ping, Z. P., Li, Z. J., & Li, J. C. (2013). NOD2 polymorphisms and pulmonary tuberculosis susceptibility: A systematic review and meta-analysis. *International Journal of Biological Sciences*, *10*(1), 103–108.

Wang, C. H., & Kuo, H. P. (2001). Nitric oxide modulates interleukin-1β and tumour necrosis factor-α synthesis, and disease regression by alveolar macrophages in pulmonary tuberculosis. *Respirology*, *6*(1), 79–84.

Wang, J., Jin, T., Mo, F., Dong, H., Wang, Y., Kang, L., & Hu, Q. (2018). Association between IL-1 gene polymorphisms and tuberculosis susceptibility in the Chinese Tibetan population. *International Journal of Clinical and Experimental Pathology*, *11*(11), 5441–5449.

Wang, W., Shao, Y., Li, S., Xin, N., Ma, T., Zhao, C., & Song, M. (2017). Caspase-11 plays a protective role in pulmonary Acinetobacter baumannii infection. *Infection and Immunity*, *85*(10).

Wassermann, R., Gulen, M. F., Sala, C., Hornung, V., Cole, S. T., Wassermann, R., Gulen, M. F., Sala, C., Perin, S. G., Lou, Y., & Rybniker, J. (2015). Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1. *Cell Host and Microbe*, *17*(6), 799–810.

Watson, R. O., Bell, S. L., MacDuff, D. A., Kimmey, J. M., Diner, E. J., Olivas, J., Vance, R. E., Stallings, C. L., Virgin, H. W., & Cox, J. S. (2015). The cytosolic sensor cGAS detects Mycobacterium tuberculosis DNA to induce type I interferons and activate autophagy. *Cell Host and Microbe*, *17*(6).

Watson, R. O., Manzanillo, P. S., & Cox, J. S. (2012). Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell*, *150*(4), 803–815.

Wehrli, W. (1983). Rifampin: Mechanisms of Action and Resistance. *Reviews of Infectious Diseases*, *5*(3).

Weiss, G., & Schaible, U. E. (2015). Macrophage defense mechanisms against intracellular bacteria. *Immunological Reviews*, *264*(1), 182–203.

Welin, A., & Lerm, M. (2012). Inside or outside the phagosome? the controversy of the intracellular localization of Mycobacterium tuberculosis. *Tuberculosis*, *92*, 113–120.

Welin, A., Raffetseder, J., Daniel, E., Stendhal, O., & Lerm, M. (2011). Importance of Phagosomal Functionality for Growth Restriction of Mycobacterium tuberculosis in Primary Human Macrophages. *Journal of Innate Immunity*, *3*, 508–518.

Welin, A., Winberg, M. E., Abdalla, H., Särndahl, E., Rasmusson, B., Stendahl, O., & Lerm, M. (2008). Incorporation of Mycobacterium tuberculosis lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. *Infection and Immunity*, *76*(7), 2882–2887.

Westman, J., & Grinstein, S. (2021). Determinants of Phagosomal pH During Host-Pathogen Interactions. *Frontiers in Cell and Developmental Biology*, *8*(January), 1–9.

WHO. (2010). *Treatment of tuberculosis Guidelines*.

WHO. (2022). WHO consolidated guidelines on tuberculosis. In *WHO Press*.

Wojtas, B., Fijalkowska, B., Wlodarczyk, A., Schollenberger, A., Niemialtowski, M., Hamasur, B., Pawlowski, A., & Krzyzowska, M. (2011). Mannosylated lipoarabinomannan balances apoptosis and in flammatory state in mycobacteria-infected and uninfected bystander macrophages. *Microbial Pathogenesis*, *51*, 9–21.

Wolf, A. J., Linas, B., Trevejo-Nuñez, G. J., Kincaid, E., Tamura, T., Takatsu, K., & Ernst, J. D. (2007). Mycobacterium tuberculosis Infects Dendritic Cells with High Frequency and Impairs Their Function In Vivo . *The Journal of Immunology*, *179*(4), 2509–2519.

Wong, D., Bach, H., Sun, J., Hmama, Z., & Av-Gay, Y. (2011). Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar-H +-ATPase to inhibit phagosome acidification. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(48), 19371–19376.

Wong, E. A., Evans, S., Kraus, C. R., Engelman, K. D., Maiello, P., Flores, W. J., Cadena, A. M., Klein, E., Thomas, K., White, A. G., Causgrove, C., Stein, B., Tomko, J., Mattila, J. T., Gideon, H., Lin, P. L., Reimann, K. A., Kirschner, D. E., & Flynn, J. L. (2020). IL-10 Impairs Local Immune Response in Lung Granulomas and Lymph Nodes during Early Mycobacterium tuberculosis Infection . *The Journal of Immunology*, *204*(3), 644–659.

Wong, K. W., & Jacobs, W. R. (2011). Critical role for NLRP3 in necrotic death triggered by Mycobacterium tuberculosis. *Cellular Microbiology*, *13*(9), 1371–1384.

Wu, S., Liu, X., Chen, L., Wang, Y., Zhang, M., & Wang, M. (2020). *Polymorphism of TLR2, TLR4 and TOLLIP and Tuberculosis in Two Independent Studies*. *0*(July), 1–10.

Xu, Y., Yang, E., Huang, Q., Ni, W., Kong, C., Liu, G., Li, G., Su, H., & Wang, H. (2015). PPE57 induces activation of macrophages and drives Th1-type immune responses through TLR2. *Journal of Molecular Medicine*, *93*(6), 645–662.

Yamada, H., Mizumo, S., Horai, R., Iwakura, Y., & Sugawara, I. (2000). Protective role of interleukin-1 in mycobacterial infection in IL-1 α/β double-knockout mice. *Laboratory Investigation*, *80*(5), 759–767.

Yang, Y., Xu, P., He, P., Shi, F., Tang, Y., Guan, C., Zen, H., Zhou, Y., Song, Q., Zhou, B., Jiang, S., Shao, C., Sun, J., Yang, Y., Wang, X., & Song, H. (2020). Mycobacterial PPE13 activates inflammasome by interacting with the NATCH and LRR domains of NLRP3. *The FASEB Journal*, *34*(9).

Yang, Y., Yin, C., Pandey, A., Abbott, D., Sassetti, C., & Kelliher, M. A. (2007). NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2. *Journal of Biological Chemistry*, *282*(50), 36223–36229.

Yonekawa, A., Saijo, S., Hoshino, Y., Miyake, Y., Ishikawa, E., Suzukawa, M., Inoue, H., Tanaka, M., Yoneyama, M., Oh-hora, M., Akashi, K., & Yamasaki, S. (2014). Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. *Immunity*, *41*(3), 402–413.

Yoshida, A. Y. A., Inagawa, H., Kohchi, C., Nishizawa, T., & Soma, G. I. (2009). The role of toll-like receptor 2 in survival strategies of Mycobacterium tuberculosis in macrophage phagosomes. *Anticancer Research*, *29*(3), 907–910.

Yu, Q., Zhu, X., Di, W., & Jiang, Y. (2016). High Beclin-1 Expression in Human Alveolar Macrophage Significantly Correlate with the Bacteriologic Sterilization in Pulmonary Tuberculosis Patients. *Clinical Laboratory*, *62*(6), 1003–1007.

Zanoni, I., Tan, Y., Gioia, M. Di, Broggi, A., Ruan, J., Shi, J., Donado, C. A., Shao, F., Wu, H., Springstead, J. R., & Kagan, J. C. (2016). An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. *Science*, *352*(6290), 1232–1236.

Zhang, Y., Shi, W., Zhang, W., & Mitchison, D. (2015). Mechanisms of pyrazinamide action and resistance. *Molecular Genetics of Mycobacteria*, 479–491.

Ophélie Rutschmann

PhD Student in Bioengineering (EPFL)

Avenue de Sévery 4
CH-1004 Lausanne

ophelie.rutschmann@gmail.com

Technical skills

- Molecular & cellular biology
- Microbiology (BSL2 & BSL3)
- Fluorescence microscopy
- Flow cytometry
- Primary cell isolation
- Crispr/Cas9 mutagenesis
- Bacterial genetics
- Data analysis
- Microfluidic chip fabrication
- Project management
- Student supervision (Bachelor & Master level)
- Strong presentation skills

Computer skills

- R
- Matlab
- Python
- ImageJ
- MsOffice

Languages

- French *(mother tongue)*
- English *(C2)*
- German *(B2)*
- Swiss German *(B2)*
- Italian *(A2)*

Hobbies

- Mountaineering
- Skiing
- Climbing
- Piano

Profile

I am an enthusiastic biologist with a passion for finding solutions to complex problems. I have solid experience in molecular and cellular biology, infectious diseases, and innate immunity.

Professional Experience

Since 2019
PhD thesis – McKinney Laboratory – EPFL
- Independent planning and execution of research projects investigating the interactions between *M. tuberculosis* and the innate immune system.
- Utilization of computational tools for the analysis of complex datasets.
- Written and oral presentation of scientific results in research articles and at international conferences.

August 2017 – February 2018
Internship in R&D – Geneva Biotech SA
- Development of a method for multigene delivery in primary T lymphocytes using viral vectors.

Academic projects

February 2018 – October 2018
Master thesis – Bioanalytics group – ETH Zürich
- Design of a microfluidic chip for the capture & analysis of circulating tumor cells at the single cell level. Grade: 6/6.

Spring 2016
Bachelor thesis – Laboratory of Psychophysics – EPFL
- Collection and analysis of eyetracking and behavioral data on the differences in memory between elderly and young adults. Grade: 6/6.

Awards

SwissTB Award 2023 – Swiss foundation for TB research
This award honors outstanding works in the field of tuberculosis research done predominantly in Switzerland.

Best Oral Presentation – Swiss Society for Microbiology Meeting 2019

Education

2019 – 2023
PhD in Biotechnology and Bioengineering – EPFL

2016 – 2018
Master in Life Sciences and Technology – EPFL
Average grade: 5.7/6. Mention of excellency

2013 – 2016
Bachelor in Life Sciences and Technology – EPFL
Average grade: 5.3/6

Personal situation

- Swiss nationality
- 30 years old
- Single

Volunteering

2019 – 2021
PhD Student Representative – EDBB doctoral school – EPFL

2019-2021
Board member – Doctoral Student Association – EPFL

Since 2019
Board member – SV Gender Committee – EPFL

Publications

Rutschmann, Toniolo, McKinney. "Preexisting Heterogeneity of iNOS Expression Drives Differential Growth of *Mycobacterium tuberculosis* in Macrophages". *mBio*, 2022.

Zhang, Reymond, **Rutschmann,** Meyer, Denereaz, Qiao, Ryckebusch, Griffié, Stepp, Manley. "Fluorescent d-Amino Acids for Super-resolution Microscopy of the Bacterial Cell Wall". *ACS Chemical Biology*, 2022.

Toniolo*, **Rutschmann***, McKinney. "Do chance encounters between heterogeneous cells shape the outcome of tuberculosis infections?". *Current Opinion in Microbiology*, 2021. *These authors contributed equally.

Armbrecht, **Rutschmann**, Szczerba, Nikoloff, Aceto, Dittrich. "Quantification of Protein Secretion from Circulating Tumor Cells in Microfluidic Chambers". *Advanced Science*, 2020.

Conference contributions

2022
Oral Presentation – EMBO Tuberculosis Workshop – Pasteur Institute (Paris, France)

2019, 2021 and 2022
Oral Presentations – Swiss Society for Microbiology Meetings

2020
Oral Presentation – LS^2 Annual Meeting – Zürich