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*A Review*

# From 2D to 3D: decoding tuberculosis pathobiology and drug development with *ex vivo* disease models

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Tuberculosis (TB) remains a global health challenge, requiring advanced models to understand its complex pathobiology and develop effective treatments. Despite extensive research, TB evades full understanding and control due to its complex interaction with the human immune system and latent state. *Ex vivo* models are essential for capturing these complexities and advancing TB research. Historically, TB research relied on two-dimensional (2D) cell cultures and animal models, which fell short of replicating the intricate lung environment. The advent of three-dimensional (3D) *ex vivo* models marks a significant leap forward, offering more physiologically relevant systems. These models, including spheroid cultures, organoid cultures, and lab-on-a-chip technologies, accurately represent human lung tissue and its interaction with *Mycobacterium tuberculosis*. 3D *ex vivo* models replicate the cellular diversity, architecture, and microenvironment of lung tissue, enabling detailed studies of TB pathogenesis, immune response, and granuloma formation. They also offer superior platforms for drug screening for efficacy and toxicity. Integrating microfluidics, advanced imaging techniques, and omics-based analytical platforms enhances these models' ability to simulate dynamic infection and treatment processes. This review highlights the development and transformative impact of *ex vivo* models on TB research, promising accelerated discovery of new therapeutic strategies.

**Keywords**: *Ex vivo* model, Granuloma, *Mycobacterium tuberculosis*, Organoid, Pulmosphere, Spheroid, Tuberculosis

## Introduction

Tuberculosis (TB) remains a global health crisis despite significant advancements in medical science. With roots tracing back to ancient civilizations, TB persists as one of humanity's oldest known afflictions, affecting millions worldwide with an estimated 10 million new cases and 1.4 million deaths annually[1]. The complex interplay between the pathogen, *Mycobacterium tuberculosis*, and the host immune response presents formidable obstacles to effective diagnosis, treatment, and prevention[2]. Developing and refining disease models is pivotal in understanding TB pathogenesis and improving therapeutic interventions.

Traditional *in vitro* models for macrophage-based TB studies have significant limitations. These models typically use 2D cultures of macrophages, which fail to capture the complex three-dimensional (3D) architecture and diverse cell interactions present in human tissues[3]. Consequently, they do not fully replicate the microenvironment within infected tissues or the dynamic immune responses seen in vivo. The lack of cellular diversity and spatial organization in 2D cultures impairs the accurate simulation of granuloma formation, a hallmark of TB infection[4]. Furthermore, these models often overlook the influence of other immune cells, stromal cells, and extracellular matrix components, leading to an incomplete understanding of host-pathogen interactions. This simplification can result in misleading conclusions about the efficacy and toxicity of potential TB drugs. Therefore, the development of more advanced, physiologically relevant models is necessary to improve our understanding of TB immunology and enhance the drug discovery process[5].

Similarly, *in vivo* animal models, although indispensable for preclinical testing, are limited by species-specific differences in immune physiology and the inability to faithfully mimic the spectrum of disease manifestations observed in humans. Given these limitations, there is a critical need for better disease models that bridge the gap between *in vitro* and in vivo systems, providing researchers with a more physiologically relevant platform for studying TB pathogenesis and evaluating potential interventions[6].

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In this context, *ex vivo* models offer several possibilities for addressing these limitations. For instance, 3D models, such as lung organoids, lung tissue explants, and tissue-engineered constructs, lung cells spheroids, better mimic the complex architecture and microenvironment of in vivo lung tissues[7]. This enhanced physiological relevance can provide more accurate insights into how *M. tuberculosis* interacts with host cells and the immune system. The ability to replicate the formation of granulomas, a hallmark of TB infection, allows for a deeper understanding of their development, maintenance, and role in containing the bacteria. Furthermore, multicellular *ex vivo* models facilitate the study of interactions between different immune cells, such as macrophages, T cells, and dendritic cells, within a more realistic physiological context. This can help identify key cellular and molecular mechanisms that contribute to the immune response to *M. tuberculosis* infection. Additionally, these models can be used to evaluate the efficacy of new drugs and therapies in conditions that closely resemble *in vivo* host tissue physiology, improving the prediction of clinical performance.

By using patient-derived cells to create 3D models, researchers can study individual responses to TB infection and treatment. This approach supports the development of personalized therapies and enhances the understanding of variability in immune responses[8]. Long-term studies in 3D models compared to traditional *in vitro* macrophage 2D models also enable the investigation of chronic aspects of TB infection, providing insights into the persistence of *M. tuberculosis* and the factors that trigger reactivation.

In summary, incorporating *ex vivo* models into TB research can overcome several limitations of traditional 2D cell cultures and the complexity and inadequate species specificity of animal models. By providing a more comprehensive and physiologically relevant platform for studying TB pathogenesis and immunity, *ex vivo* models hold great potential for advancing our understanding of the disease and accelerating the development of new therapeutic interventions.

## Traditional models used in TB research

Robert Koch's identification of *M. tuberculosis* in 1882 was a monumental breakthrough, establishing the bacterial cause of TB. This discovery laid the foundation for subsequent research into TB's pathology and immune response. Early animal experiments, including those involving guinea pigs and rabbits, demonstrated the pathogenicity and severity of TB, helping researchers understand the disease's progression and the host's response. The development of cell lines in the mid-20th century marked a significant advancement. Researchers could culture immune cells, such as macrophages and dendritic cells, and study their interactions with *M. tuberculosis*. By observing how these cells phagocytose bacteria, present antigens, and activate T cells, researchers gained insights into the mechanisms of the immune response[9].

Experimental models for TB is broadly divided into two categories Human based models and Animal based models. Human-based models encompass cell lines, whole blood, peripheral blood mononuclear cell (PBMC), bronchoalveolar lavage (BAL), and humanized mouse models. Human cell lines, such as macrophages and alveolar epithelial cells, offer a controlled environment to study TB infection and immune responses. Whole blood, PBMCs, and alveolar lavage cells offer significant advantages for TB research, particularly in the context of human studies. Figure 1 summarizes the various traditional experimental TB research models.

Using these human-derived cells provides a direct insight into the human immune response to *M. tuberculosis*, enhancing the relevance and applicability of findings. Whole blood assays are straightforward and minimally invasive, allowing for high-throughput screening and real-time monitoring of immune responses. PBMCs, isolated from blood, provide a rich source of immune cells for detailed mechanistic studies, including T-cell and macrophage function, Alveolar lavage cells, obtained from the

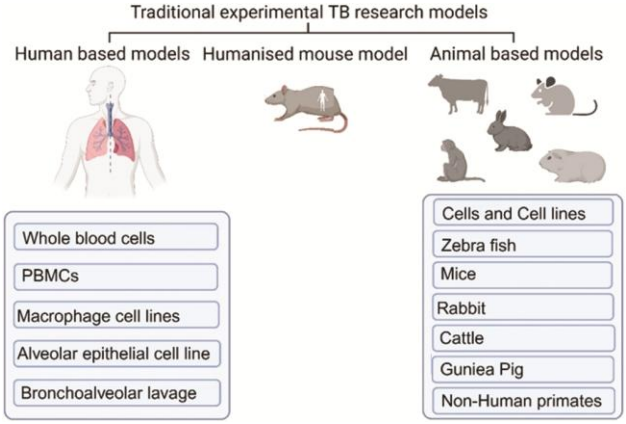



Fig. 1 — An overview of the various traditional TB research models

lungs, offer a unique perspective on the local immune environment in the respiratory tract, the primary site of TB infection[9]. However, these approaches have limitations. Whole blood and PBMC assays may not fully capture the complexity of the immune response occurring in the lungs. The procedures to obtain alveolar lavage samples are invasive and carry risks for the subjects. Additionally, variability in cell yield and function due to donor differences can affect the consistency and reproducibility of results. Despite these challenges, these human cell-based methods remain invaluable for understanding TB immunology and developing targeted therapies.

Humanized mouse models, created by engrafting immune compromised mice with human immune cells or tissues, provide a platform to study human-specific immune responses to TB[10]. Humanized mouse models of *M. tuberculosis* infection successfully recapitulate key pathological hallmarks of human tuberculosis, including bronchial obstruction, granuloma formation, and caseous necrosis. However, these models also exhibit atypical T-cell responses and impaired bacterial control, highlighting the limitations of this model in fully mirroring human disease progression. These models bridge the gap between human and animal studies, enabling researchers to assess vaccine candidates, study host-pathogen interactions, and evaluate immune-based therapies *in vivo*.

Animal based models have been invaluable in tuberculosis (TB) research, offering insights into disease pathology, immune responses, and potential treatments[6]. Figure 2 depicts the percentage of published studies corresponding to various animal models used in TB research.

The guinea pig model, for instance, closely mirrors human TB in terms of granuloma formation and caseous necrosis, providing relevant data on disease progression and host-pathogen interactions. Similarly, Kramnik's mouse model, which can develop granulomas and caseous necrosis, helps study TB's progression and immune responses. Rabbits, which develop pulmonary granulomatous lesions and latency, and non-human primates (NHPs), which exhibit human-like TB pathology including latency and immune response, also play crucial roles. Additionally, zebrafish embryos offer unique advantages for studying granuloma formation due to their optical transparency, despite their lack of a fully developed immune system. By combining human-based and animal-based models, researchers enhanced our understanding of TB and accelerate the development of effective therapies.

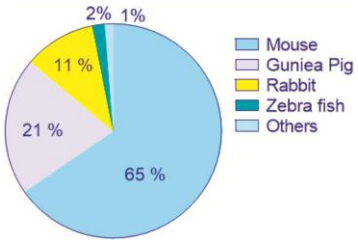



Fig. 2 — Relative distribution of TB research publications utilizing various animal models. Data were compiled from a PubMed search conducted on June 18, 2024, using keywords 'mouse AND tuberculosis,' 'guinea pig AND tuberculosis,' 'rabbit AND tuberculosis,' 'non-human primate AND tuberculosis,' and 'zebrafish AND tuberculosis.' Percentages indicate the proportion of publications attributed to each model based on the total number of publications across all animal models

Despite their utility, animal models have significant limitations. Mice and guinea pigs, while useful, differ significantly in immune system and lung structure from humans, affecting the extrapolation of findings[5]. Rabbits and NHPs, though more similar to humans in terms of pathology, are expensive and require specialized facilities and expertise. Zebrafish embryos, despite their advantages, lack the complexity of a fully developed immune system. Further significant concerns of animal models include their high costs, logistical challenges, and ethical concerns. This has led to the adoption of the "3Rs principle" in scientific research: replace, reduce, and refine animal use. Alternatives like *ex vivo* cell/organ culture models and computer simulations are encouraged to minimize animal testing.

## Evolution of *ex vivo* models in TB research

Given the limitations and ethical concerns surrounding animal usage, cell culture models have significantly evolved in TB research. A variety of *in vitro* cell culture models, including macrophage, epithelial, dendritic, T cell, natural killer (NK) cell lines, and *ex vivo* primary culture of human and animal whole blood or PBMCs are essential for understanding the complex interactions between *M. tuberculosis* and host cells. Macrophage models, such as immortalized cell lines like THP-1 and RAW 264.7, J774 are frequently used to investigate the intracellular lifestyle of *M. tuberculosis*, including its mechanisms of immune evasion and persistence. Epithelial cell lines, such as A549, BEAS-2B, Calu-3, H292, and 16HBE14o, model the lung environment and study pathogen interactions with alveolar and bronchial epithelial cells. These models help explore

bacterial adherence, invasion, immune responses, and the impact on epithelial barrier function. Dendritic cell (DC) lines, like the human-derived MUTZ-3 and the murine JAWS II, are crucial for studying the immune response, particularly how *M. tuberculosis* affects dendritic cell maturation, antigen presentation, and T cell activation. T cell lines such as Jurkat, MOLT-4, and SUP-T1, along with primary T cells, are pivotal for understanding T cell-mediated immunity, including T cell activation, differentiation, and the development of memory T cells in response to. Additionally, NK cell lines like NK-92, derived from a patient with non-Hodgkin's lymphoma, are used to study the role of NK cells in the immune response against *M. tuberculosis*, focusing on their cytotoxic activity and cytokine production. Cell culture methods also enabled the exploration of cytokines, the signaling molecules that play a critical role in the immune response. Researchers identified and studied important cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-12 (IL-12)[11]. These cytokines were found to be essential in activating macrophages and coordinating the overall immune response against TB. Understanding the roles of these cytokines helped clarify how the immune system mounts an effective defense against TB infection. Additionally, cultures of human monocyte-derived macrophages (hMDMs), BAL cells, murine bone marrow-derived macrophages (BMDMs), murine splenocytes, lung cells, peritoneal macrophages are some of the traditional *ex vivo* cell culture models used in TB research. Further advancements in cell culture techniques, such as autologous mixed lymphocyte cultures, allowed for more sophisticated studies of T-cell activation. Researchers discovered the pivotal roles of CD4+ and CD8+ T cells in controlling TB infection by using such experimental models. These studies also highlighted the development of memory T cells, which are crucial for long-term immunity. By elucidating the functions of these T cell subsets, scientists gained valuable insights into how the immune system remembers and combats TB upon subsequent exposures[12].

Advancements in cell culture models, such as co-culture methods, reveal complex interactions between different cell types. By culturing multiple cell types together, researchers observed direct cell-cell interactions and signaling pathways crucial for understanding TB pathogenesis and immune responses. Co-culturing macrophages with epithelial cells mimics the alveolar environment, helping study *M. tuberculosis* infection, immune response, and macrophage-epithelial cell interactions[13,14]. Another model involves DCs and T cells to investigate antigen processing, presentation, and T cell activation, aiding vaccine development[15]. However, these co-culture models do not fully replicate TB disease, leading to the development of more physiologically relevant models that better mimic TB granulomas in the lungs.

Granuloma is a hallmark of TB infection, which are organized structures aimed at containing the infection[16]. Granulomas consist of a core of infected macrophages, some of which differentiate into multinucleated giant cells and epithelioid cells. Surrounding this core are T cells, primarily CD4+ and CD8+ T cells, which provide cytokine signals essential for maintaining the structure and function of the granuloma. This layer also consists of various other immune cells, B cells, neutrophils, and additional macrophages. The outermost layer consists of a fibrotic capsule that helps contain the infection. Within the granuloma, the architecture facilitates a microenvironment where the bacteria can be both contained and persist. The formation of caseous necrosis, a hallmark of TB granulomas, occurs when the central core of cells undergoes necrosis, creating a cheese-like appearance. The overall structure of TB granulomas reflects a dynamic balance between the host's attempt to contain the infection and the pathogen's strategies to evade immune responses, often leading to a state of latent infection where the bacteria remain dormant but can reactivate under certain conditions[17]. Figure 3 depicts cartoons of two

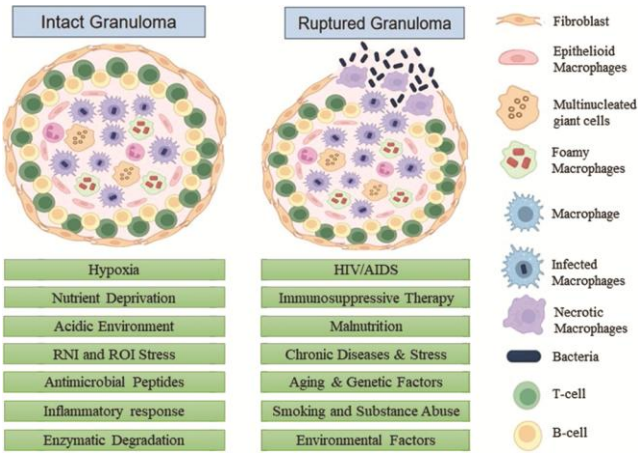



Fig. 3 — Two stages of a typical TB granuloma with distinct cellular organization, stress conditions within the granuloma, and factors responsible for compromised immunity and granuloma breakdown

stages of a typical TB granuloma with distinct cellular organisation.

The granuloma structure in TB creates an environment that contributes to the development of hypoxia, nutrient starvation, and other stress conditions, which in turn induce dormancy and latency in *M. tuberculosis*. As granulomas form, the dense aggregation of immune cells limits blood flow to the central core, reducing oxygen availability and creating hypoxic conditions[18]. The tightly packed cells and fibrotic capsule further restrict the diffusion of nutrients, leading to nutrient starvation[19]. Additionally, the immune response generates reactive nitrogen and oxygen species, contributing to oxidative and nitrosative stress. These harsh conditions within the granuloma force the tubercle bacilli to enter a non-replicative, dormant state, characterized by metabolic downregulation and resistance to host defenses. This dormancy allows the bacteria to persist within the host for long periods, potentially reactivating when the host immune system is weakened, thus leading to latent TB infection[20]. Furthermore, the granuloma structure significantly impacts the penetration of anti-TB drugs, making it difficult for therapeutic agents to reach the bacteria effectively. The dense cellular layers and fibrotic capsule act as physical barriers, impeding drug diffusion and leading to suboptimal drug concentrations within the granuloma's core. This inadequate drug delivery can result in improper drug concentrations, insufficient bacterial killing, and the development of antimicrobial resistance (AMR). Consequently, the complex architecture of granulomas not only aids in bacterial persistence but also poses a significant challenge for TB treatment and the eradication of the infection.

In the process of creating more physiologically relevant environment further complicated *ex vivo* models were emerged. *Ex vivo* models which preserve the architectural and cellular complexity of the host microenvironment while allowing for experimental manipulation and observation, hold great potential for deciphering the immune landscape of TB and uncovering novel insights into disease mechanisms. These models include multicellular setups that incorporate various immune cells, such as macrophages, dendritic cells, T cells (CD4+ and CD8+), regulatory T cells, B cells, natural killer (NK) cells, and neutrophils, to replicate the *in vivo* environment more accurately. Multicellular *ex vivo* models allow for the study of complex cellular interactions and cytokine networks, crucial for understanding granuloma formation and coordinated immune responses. These models provide a better platform not only for drug target identification during comparative virulence studies of targeted gene mutants of the tubercle bacilli, but also for testing new therapies, providing a comprehensive platform for evaluating the efficacy and safety of drugs, vaccines, and immunotherapies. By simulating the complex immune landscape of TB infection, these models help identify promising candidates and refine therapeutic strategies, ultimately accelerating the development of effective treatments and improving our understanding of TB pathogenesis and immune defense mechanisms.

## Types of *ex vivo* models

### Tissue explant culture

Lung tissue explant models use freshly isolated human tissue biopsies cultured *ex vivo* to study TB in a physiologically relevant context. These models retain the structural integrity and cellular complexity of human lung tissues, enabling the investigation of tissue-specific responses to TB infection and evaluation of therapeutic interventions. In a human lung tissue explant model, when lung tissue is exposed to *M. tuberculosis*, innate lymphoid cells type 3 (ILC3s) significantly increase the production of cytokines IL-22 and GM-CSF, with IL-22 playing a crucial role in defending against infection by promoting the fusion of phagosomes with lysosomes[21]. This model showed that although gamma/delta T cells, MAIT cells, and NK cells also contribute to the immune response, their activation is weaker and less consistent compared to ILC3s. This response variation is more pronounced between different donors, indicating potential differences in individual immune capabilities.

The Myco-GEM 'mycobacterial granuloma explant model' allows culturing mycobacterial granulomas outside a living organism for a week, enabling long-term, high-resolution imaging of cellular dynamics[22]. Fully organized granulomas are micro-dissected from zebrafish embryos and maintained in three-dimensional culture, showing significant macrophage movement and cellular reorganization. Myco-GEM facilitates genetic manipulation, enabling studies on gene function and host-pathogen interactions, and aiding in drug target and genetic factor identification for improved TB treatments. These cultured granulomas closely resemble human TB granulomas in immune cell diversity, inflammation levels,

epithelioid cell structure, and protein expression for cell connections.

In pursuit of understanding the early innate immune mechanisms in mycobacterial infections, the *ex vivo* 'short-term stimulation of tissues' (STST) model has emerged as a valuable tool. This model, utilizing human lung tissue, maintains the intact lung microenvironment with its diverse cell populations, natural organization, and structural integrity. Leveraging the STST model, researchers have successfully gleaned insights into the initial stages of pathogenesis for various lung diseases, including those caused by *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, and *Haemophilus influenzae*[23]. When applied to mycobacterial infections, the STST model demonstrated successful mycobacterial invasion into lung tissue without causing immediate histological damage, a distinct observation compared to other bacterial pathogens. Furthermore, the model revealed relatively low levels of apoptosis in *M. tuberculosis*-infected tissue, suggesting potential immune evasion strategies employed by the bacterium[24]. Despite its strengths in providing a human-relevant platform and preserving the lung microenvironment, the STST model has limitations. The study's high bacterial dose and short infection duration may not fully recapitulate natural infection dynamics. Additionally, the model's inability to maintain tissue viability beyond 16 hours restricts the investigation of long-term infection processes.

Precision-cut lung slices (PCLS) are thin slices of lung tissue that retain the structural and functional characteristics of the whole lung[25]. These slices are typically 200-500 micrometres thick and harbor a diverse array of resident cells, including fibroblasts, alveolar epithelial cells, macrophages, monocytes, NK cells, and T cells. While neutrophils are initially present, their numbers decline in culture due to the lack of ongoing recruitment from the bloodstream, allowing researchers to specifically dissect the roles of resident lung cells in the early stages of infection[25]. Studies utilizing murine PCLS (mPCLS) infected with *M. abscessus* demonstrate progressive tissue damage within 48 hours post-infection, including alveolar edema, vascular congestion, and the extravasation of lymphocytes and erythrocytes into septa and alveolar spaces[26]. Additionally, rupture and thickening of the alveolar septa, infiltration of histiocytes, aggregation of foamy macrophages, and fragmentation of polymorphonuclear cells are observed. PCLS have been used to study early host-pathogen interactions in the lungs of cattle, maintaining the natural lung environment and cell interactions[27]. This model also helped in understanding how different mycobacterial strains and cattle breeds respond to infection, providing insights into the innate immune response and the potential for new therapeutic targets. The ability of PCLS to faithfully recapitulate the structural and cellular complexity of the lung microenvironment makes them an invaluable tool for investigating host-pathogen interactions and therapeutic responses. Figure 4 depicts an outline of the methodology of PCLS model of TB infection.

**3-Dimensional (3D) static models**

3D static models of *ex vivo* cell culture are advanced experimental systems used to study cellular behavior and interactions in a three-dimensional context, closely mimicking the natural tissue environment. Unlike traditional 2D cultures, where cells grow in a flat monolayer, 3D models allow cells to grow in all directions, providing a more physiologically relevant setting. These models are created by embedding cells in a scaffold or matrix made of biocompatible materials such as collagen, gelatine, or synthetic polymers, which support cell attachment and growth. In 3D static models, cells can form more complex structures, such as spheroids or organoids, which better replicate the architecture and

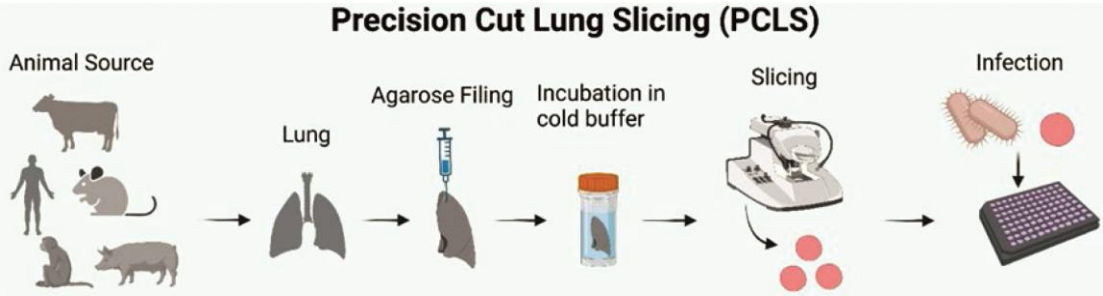



Fig. 4 — An outline of the methodology of the PCLS model of TB infection

function of actual tissues. This allows researchers to study cellular processes like differentiation, proliferation, and migration in a more realistic manner. Additionally, these models enable the investigation of cell-cell and cell-matrix interactions, which are critical for understanding tissue development, disease progression, and the effects of therapeutic interventions[28]. Figure 5 depicts an outline of lung pulmosphere/spheroid model of TB.

Belogorodtsev and colleagues developed a 3D model of granuloma formation- called '3D granulomatosis' model and elucidated the significant role of *M. tuberculosis*-specific proteins ESAT-6 and CFP-10 in mycobacterial infection[29]. In this model, BALB/c mice were infected with *M. tuberculosis* strain H37Rv. After one-month, peritoneal macrophages and splenocytes were collected and mixed with autoplasma containing sodium citrate. BCG was added to the cell suspension at a 1:1 ratio. Calcium chloride was then added to induce coagulation, forming a plasma clot containing the cells and mycobacteria. Using this model the authors demonstrated the strong necrosis-inducing effects of ESAT-6 and CFP-10 and suggested the roles of these proteins in creating necrotic cavities in tuberculosis lesions *in vivo*.

Mukundan and colleagues developed a 'miniaturized TB spheroid model' using THP-1 human monocyte/macrophage[30]. These macrophage spheroids exhibited hypoxic core with dead cells co-localized with mycobacteria and showed higher levels of pro-inflammatory factor TNF-α and growth factors GM-CSF and VEGF when compared to non-infected control. Additionally, this model also recapitulates the phenomenon of lipid deposition inside the granuloma like structures. They also adapt the spheroid model to form a co-culture of PBMCs and lung fibroblasts.

Kapoor and colleagues reported a laboratory model of human TB granulomas using PBMC cells[31]. They infected human PBMCs placed in a collagen matrix with *M. tuberculosis* and incubated for 8 days, and they showed that infected PBMCs tended to form microscopic granulomas (micro-granuloma) as an aggregation of lymphocytes surrounding infected macrophages. Within this model, *M. Tuberculosis* displays characteristics of dormancy, including a loss of acid-fast staining, an accumulation of lipid bodies, a tolerance to the antibiotic rifampicin, and distinct changes in gene expression. Additionally, treating these micro-granulomas with anti-TNF-α mAbs reactivated dormant *M. tuberculosis*, mimicking a phenomenon observed in human TB patients. This model offers a valuable tool for studying *M. tuberculosis* dormancy and resuscitation.

In a similar line, Guirado and colleagues showed granuloma-like structure formation by infecting PBMCs with *M. tuberculosis* in the presence of 10% autologous serum and they could maintain the cell cultures up to 12 days post-infection[32]. Cellular aggregation started around day 4 to day 6 post-infection, depending on inter-individual variability. They showed individuals with latent tuberculosis infection (LTBI) form stronger granulomas compared to individuals without prior exposure to the bacterium.

Lerm and colleagues created a model of early TB granulomas using human lung tissue derived cell lines and primary macrophages from peripheral blood, and the model displays characteristics of human lung tissue, including evenly integrated macrophages throughout the epithelium, production of extracellular matrix, stratified epithelia and mucus secretion[33]. Establishment of experimental infection in the model tissue with *M. tuberculosis*, resulted in clustering of macrophages at the site of infection, reminiscent of early TB granuloma formation. This model confirmed the importance of bacterial factors like ESX-1 system and the ESAT-6 protein in the early stages of TB infection, aligned with findings from previous animal studies.

Rapidly replicating microorganisms pose a significant challenge for static cell culture models.

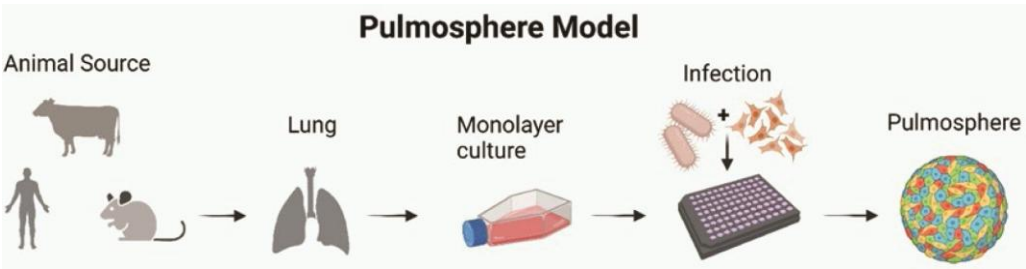



Fig. 5 — An outline of the lung pulmosphere/spheroid model of TB

The excessive growth of microbes can overwhelm host cells, deplete nutrients, and ultimately lead to cell death, obscuring the true nature of host-pathogen interactions observed in living organisms. To mitigate this issue, researchers often resort to measures such as using specialized media, frequent media changes, shortened experiments, or antibiotics, all of which can introduce biases and limitations.

**Organotypic models**

Organotypic models represent a cutting-edge approach to studying TB, allowing for real-time visualization of *M. tuberculosis* infection dynamics under physiological conditions that include airflow and fluid flow. These models popularly known as 'organ-on-a-chip' (OoC) models enable the investigation of how mechanical forces, such as those involved in breathing, affect *M. tuberculosis* infection and the host immune response. By modeling immune cell recruitment and activation in a dynamic lung environment, OoC models provide valuable insights into the interactions between *M. tuberculosis* and the host immune system. They also facilitate the evaluation of drug efficacy under physiological conditions, including aspects like drug penetration and distribution. Importantly, these models allow for personalized medicine approaches by using patient-derived cells, offering a platform to test individual responses to TB treatments. In addition, the continuous flow within OoC models effectively washes away non-adherent microorganisms, preventing their overgrowth and the associated adverse effects. This not only enables the study of pathogenic mechanisms in rapidly proliferating microbes but also allows for the integration of stable commensal microbial communities. One of the challenges in modeling mycobacterial infections is the bacterium's slow growth rate and ability to persist in a dormant state within granulomas. OoC models with continuous flow can overcome this limitation by preventing microbial overgrowth and maintaining a stable environment for studying both active and dormant mycobacterial infections[34]. Figure 6 depicts an outline of organotypic model of TB.

Accurately modelling biological barriers in OoC systems is crucial for understanding pathogen dissemination and immune cell recruitment. By replicating barrier-forming cells, intercellular junctions, and extracellular matrix connections, researchers can study how these barriers protect against pathogens. Integrating vascularization and circulating immune cells in models allows for realistic replication of human tissue environments, including blood flow and nutrient exchange in the lungs, essential for studying *M. tuberculosis* infections. These models create realistic oxygen gradients to study bacterial dormancy and reactivation, and improve drug testing by simulating human pharmacokinetics and pharmacodynamics. They replicate the blood-air barrier in the lungs, aiding drug delivery system development, and facilitate immune cell trafficking studies crucial for understanding TB's immune responses. By tailoring treatments based on patient-specific *ex vivo* organotypic models using iPSCs or primary cells allow personalized studies of TB infection and treatment, leading to tailored therapies. Figure 7 depicts an outline of *ex vivo* models for personalized medicine.

## Advantages of *ex vivo* models

*Ex vivo* models on one hand reduce reliance on animal studies by providing a more human-relevant platform for TB research, on the other hand augments the physiological relevance of an *in vitro* model. For instance, organoid models derived from human lung cells can be infected with *M. tuberculosis* to study host-pathogen interactions and drug responses, minimizing the need for animal experimentation while generating clinically relevant data. This approach accelerates the development of TB treatments while adhering to ethical considerations. Moreover, incorporating advanced technologies like OoC platforms and 3D tissue models can enhance the

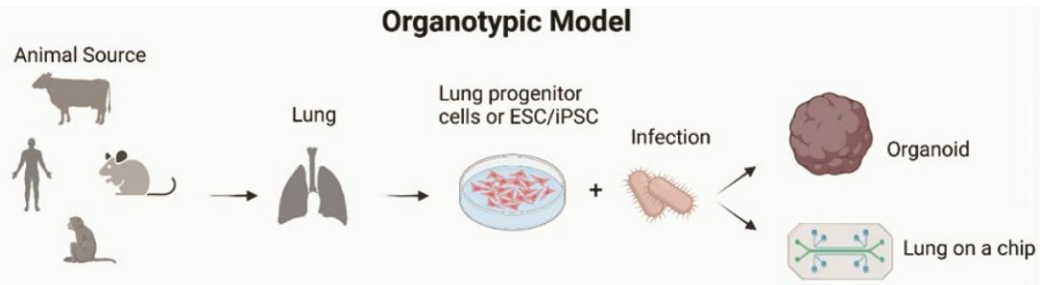



Fig. 6 — An outline of the organotypic model of TB

complexity and physiological relevance of these species-specific models. These technologies allow for the recreation of the lung's intricate microenvironment, facilitating the study of *M. tuberculosis*'s interactions with different cell types under dynamic conditions. Furthermore, integrating omics approaches, such as genomics, transcriptomics, and proteomics, can provide valuable insights into the molecular mechanisms underlying species-specific susceptibility and resistance to TB. *ex vivo* models using patient-derived cells, such as lung organoids from TB patients, offer a personalized approach to studying disease variability and treatment responses. These models allow for testing individualized drug regimens and understanding genetic factors influencing TB susceptibility and drug resistance. Table 1 depicts an overview of the comparative features of different TB disease model.

## Applications of *ex vivo* models in TB drug discovery and development

The use of *ex vivo* models in drug testing and regulatory science offers several benefits, including the ability to select more effective and less toxic agents while reducing the reliance on animal testing[35]. *Ex vivo* models support high-throughput screening, which is often not possible with animal models, and they can be used when suitable animal models for a specific infectious disease are unavailable or resources are limited. This is particularly advantageous for preparing for pandemics and dealing with infectious diseases that require Biosafety Level 3 (BSL-3) facilities, which are costly and resource-intensive.

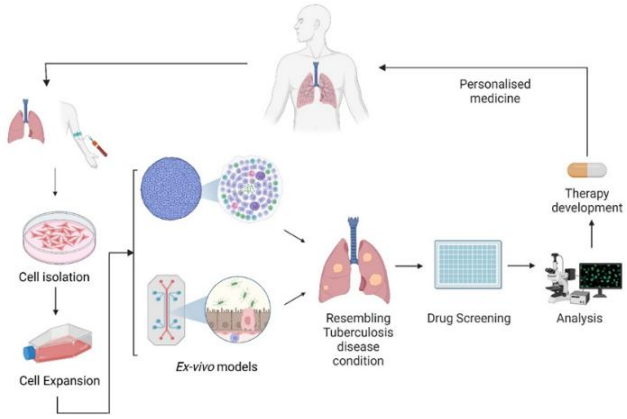



Fig. 7 — Overview of *ex vivo* models for personalized medicine

The use of 3D lung culture extends its application to drug delivery. Magalhaes *et al*. address the potential toxicity and biodistribution of nanostructured lipid carriers (NLCs)[36]. These lipid nanoparticles are appealing for pulmonary drug delivery due to their biocompatibility, high drug loading capacity, and stability. However, their development has been challenged by concerns regarding their safety and distribution within the lungs. To tackle these issues, Magalhaes *et al.* developed mannose-functionalized NLCs and assessed their interaction and biocompatibility using a three-dimensional (3D) co-culture model. This model, which includes epithelial cells and immune cells (monocyte-derived macrophages and dendritic cells), effectively mimics the human alveolar epithelial tissue barrier, providing a more accurate evaluation of the NLCs' safety and efficacy for potential therapeutic use.

Riechman *et al.* made an attempt to identify potential therapeutic targets using laser captured micro dissection of 3D granuloma through whole transcriptome analysis[37]. In their study they explained the importance of sphingosine kinase 1 inhibition in controlling *M. tuberculosis* growth. In addition to the lung, these models also extend their applicability to studying liver functions in TB infection. For instance, Srivastava and colleagues developed a 3D liver hollow

Table 1 — Comparative features of different TB disease model

| Criteria | Animals | 2D-cell culture | 3D-organoid | Tissue explant |
|---|---|---|---|---|
| Ease of establishment | - | ++ | + | + |
| Ease of maintenance | - | ++ | + | + |
| Cellular complexity | +++ | - | ++ | ++ |
| Long term maintenance | + | + | + | + |
| Genetic manipulations | - | ++ | + | - |
| unravelling disease complexity | ++ | - | ++ | + |
| Physiological complexity | +++ | - | ++ | ++ |
| Relative cost | - | + | ++ | + |
| Drug screening | - | ++ | ++ | + |
| Ethical approval | - | +++ | +++ | ++ |
| Patient specific | - | - | ++ | ++ |
| Consistency | - | + | + | + |

fibre model to evaluate the efficacy and toxicity of anti-tuberculosis drugs for children's[38]. Such models overcome the concerns related to drug toxicity that often prevent children from participating in clinical trials, resulting in suboptimal treatment regimens.

Several studies reported use of spheroid model for TB drug screening. One such study, wherein Mukundan and colleagues employed spheroids infected with BCG mCherry and treated with variable doses of INH and RIF, and showed a time-dependent decrease in bacterial CFU[30]. Additionally, by including *M. tuberculosis* strains with differential virulence such as HN878 and CDC1551, the study highlights the TB spheroid model's effectiveness in differentiating drug responses based on bacterial strain virulence, proving useful for initial drug screening.

INH and RIF are key first-line anti-TB drugs. Studies using *in vitro* and *in vivo* models have shown their varied levels of effectiveness. Tasneen *et al*. found increased bactericidal activity but higher relapse rates with INH, RIF, and PZA combined[39]. Conversely, Genestet *et al*. demonstrated synergy between INH and RIF[40]. In an *ex vivo* granuloma model, Ashley *et al*. showed that Everolimus (EVR), an mTOR inhibitor, enhances the effects of INH and PZA[41]. Further demonstration of increased host-cell autophagy with EVR treatment supports its role in enhancing host protection against *M. Tuberculosis*[42].

Furthermore, growing resistance is exacerbated by *M. tuberculosis*'s ability to persist in a dormant state within host tissues, evading the immune system and standard antibiotic treatments. The phenomenon of phenotypic heterogeneity, where subpopulations of bacteria exhibit different traits such as non-replicating persistence, further complicates treatment efforts and promotes infection relapse[43]. *Ex vivo* models were employed to mitigate the inaccuracy of drug response through its high physiological relevance.

### *Ex vivo* models in host-pathogen interactions and biomarker discovery

Gupta *et al*. highlighted the critical role of ECM components in experimental TB systems, emphasizing their significance in shaping host-pathogen interactions[44]. By entrapping macrophages within a collagen matrix, they generated a gel like 3D structure in which human macrophages remain viable for 2-3 weeks allowing tracing of infection much longer than traditional 2D macrophage cultures. Their findings revealed that monocytes in conventional 2D cultures exhibit reduced TB bacterial uptake compared to macrophages cultured in 3D environments, illustrating the heightened phagocytic capability of macrophages in 3D settings. Moreover, they observed markedly improved cell viability among both THP-1 monocytes and primary human monocyte-derived macrophages when grown in their 3D collagen gels, contrasting with outcomes from 2D cultures. Furthermore, their studies demonstrated the effectiveness of Pyrazinamide (PZA) against *M. Tuberculosis* infection in primary human monocytes. Their 3D collagen cell culture system facilitated a prolonged observation period for studying host immune cell-pathogen interactions, reflecting characteristics observed *in vivo* including immune cells exhibiting lipid accumulation, cord formation, and gene expression profiles akin to those observed in infected tissues.

Tezera *et al*. introduced an innovative 3D culture system using microspheres, where *M. tuberculosis*-infected human blood cells were embedded in a collagen and alginate mix, refined through bioelectrospray technology[45]. This approach facilitated enhanced cell growth, aggregation formation within a week, and the maturation of macrophages, alongside the presence of multinucleate giant cells akin to those observed in human TB granulomas. Their model allowed for prolonged culture of primary human cells, revealing significant experimental differences that typically emerge after more than 7 days—contrasting with the shorter durations of standard 2D cultures lasting 3-4 days. Furthermore, their findings indicated that boosting PGE2 levels enhances host control over mycobacterial proliferation. Additionally, their exploration of IFN-β in TB immune responses, informed by genomic studies, suggested a predominantly protective role against TB infection.

Kotze and colleagues validated that within their 3D spheroid granulomas, the central core consists of both necrotic and non-necrotic alveolar macrophages (AM), encircled by a perimeter of non-necrotic T cells[4]. This configuration faithfully replicates the structural and functional characteristics observed in human TB granulomas. They further demonstrated that these 3D adaptive spheroid granulomas closely resemble the spatial organization found in non-human primate (NHP) TB granulomas. They conducted a comparative analysis, contrasting the gene expression profiles of their 3D spheroid granulomas with those derived from conventional cell cultures of human cells, meticulously matching cell origins, types, ratios, and quantities. This approach aims to uncover how

the unique three-dimensional architecture impacts immune cell behavior and genetic activity, offering critical insights into TB pathogenesis and therapeutic development.

Given the zoonotic and anthropozoonotic significance of tuberculosis (TB), studying the pathobiology of animal lineage *Mycobacterium* species like *M. bovis* has been a critical research focus for centuries[46]. Our current knowledge of immune responses to TB in cattle largely stems from *in vitro* models using bovine peripheral blood mononuclear cells (PBMCs) and a limited number of *in vivo* infection studies in cattle[47]. Additionally, researchers have used bovine primary alveolar macrophages and macrophage cell lines from mice and humans to study early host-pathogen interactions in bovine TB[48]. However, the differences in immune responses across various host species and organs highlight the need for developing bovine lung-based models to better understand the complexities of bovine pulmonary TB. We introduced a bovine lung cell based spheroid model 'bovine 3D pulmosphere' to study the host pathogen interactions and to identify the potential biomarkers of early infection in the bovines[49]. We isolated total lung cells from cattle lung tissues and assembled them into a spheroid containing more than 30 different cell types, which remains viable up to 4 weeks in the culture. We have identified significant difference in cellular composition and functional behaviour between primary monolayer and 3D pulmospheres systems. The ECM related proteins in the 3D pulmospheres are significantly higher compared with monolayer mixed population of lung cells. Furthermore, we showed distinct interconnected signaling pathways in response to avirulent (BCG) and virulent (*M. tuberculosis*) mycobacterial infection of bovine 3D pulmospheres *via* integrated transcriptomics and proteomics analysis.

**Limitations of *ex vivo* TB models**

Despite their growing potential, *ex vivo* models face several hurdles before becoming fully integrated into pre-clinical research. Standardization and validation are critical for ensuring reliable results, but remain ongoing efforts. The materials used in fabrication can absorb molecules and proteins, affecting experimental accuracy. Additionally, artificial membranes and scaffolds can hinder natural diffusion and exchange of substances. Compared to traditional models, *Ex-vivo* models tend to have lower throughput at the current stage of development, limiting their use in large-scale drug screening[50]. Their complexity, while beneficial for mimicking biological systems, can be a drawback for studying specific interactions due to the increased number of variables. Furthermore, specialized equipment and expertise are required, potentially increasing experimental costs. While these models show promise, they cannot fully replace animal models at the current stage of development, which are still necessary for evaluating systemic effects and complex biological processes not captured by *ex vivo* models.

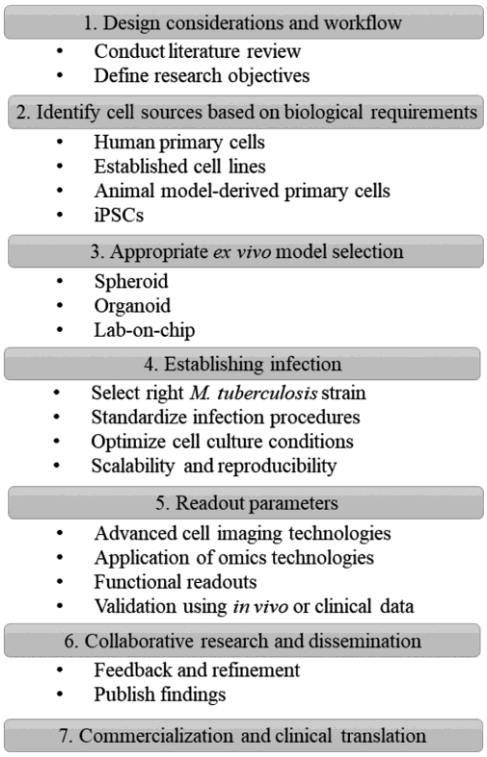
**A roadmap for developing appropriate *ex vivo* TB disease models**

Tuberculosis (TB) is a complex, multi species (both host and pathogen) and multi-stage disease characterized by a wide range of symptoms and requiring a combination of several drugs for effective treatment. Despite extensive research, the mechanisms of host protective immune responses against TB remain largely elusive. This complexity makes it challenging to model TB accurately *in vitro* or *ex vivo*. Developing reliable *ex vivo* models that closely mimic the human lung environment is crucial for understanding TB pathobiology and facilitating drug discovery. Box-1 provides an outline of the roadmap to develop an appropriate *ex vivo* model of TB.

Developing an *ex vivo* model for TB research may involve a multi-phase approach integrating advanced techniques such as spheroid culture, organoid culture, lab-on-a-chip technology, and considerations for species and host specificity. The process can begin with a thorough literature review and defining specific research objectives, followed by identifying key biological requirements. In the model design and development phase, researchers may select appropriate cell sources, including human primary cells, animal models, or induced pluripotent stem cells (iPSCs), to create 3D cell culture spheroids or lung organoids. Lab-on-a-chip technology can be employed to replicate the lung environment through microfluidic systems, while genetic engineering tools like CRISPR/Cas9 may ensure host and species-specific studies. The introduction of *M. tuberculosis* strains into the model may be standardized for infection, focusing on clinically relevant and drug-resistant variants.

Functional characterization can involve studying immune responses, granuloma formation, and *M. tuberculosis* dynamics, along with drug screening for efficacy and toxicity using high-throughput

**Box-1**




Box-1 — Outline of the roadmap to develop an appropriate *ex vivo* model of TB

techniques. Advanced imaging and omics technologies can be utilized for comprehensive data analysis. Continuous refinement of cell culture conditions and scalability may ensure reproducibility and broader application in drug discovery. Stringent validation against established animal models and clinical data ensures the model's accuracy and predictive power, paving the way for experimental design. Subsequent data analysis and interpretation, employing robust statistical methods, inform model refinement in an iterative process, enhancing its precision and utility. Additionally, ethical considerations, especially pertinent to usage of animal and human derived cells, and adherence to stringent biosafety protocols for handling *M. tuberculosis* are non-negotiable.

Collaborative research and dissemination of findings through publications and conferences can be crucial for validation and expansion of the model's use. Opportunities for commercialization and clinical translation may be explored to enhance the impact of the research. By following this detailed roadmap, researchers can create sophisticated *ex vivo* TB models that closely mimic *in vivo* environments, offering valuable insights into TB pathobiology and facilitating effective drug screening.

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## Conflict of interest

Both the authors declare no conflicts of interest.

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