TUBERCULOSIS 2022

EMBO Workshop on Tuberculosis 2022

From innovation to intervention

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LabEx IBEID

September 12-16, 2022 - CIS auditorium, Institut Pasteur

EMBO

EMBO Workshop

INSTITUT PASTEUR

WELCOME TO INSTITUT PASTEUR

The EMBO Workshop on Tuberculosis 2022 will be the 3rd congress on tuberculosis (TB) and pathogenic mycobacteria in the CIS of the Institut Pasteur in the 21st century, following the previous two very successful EMBO-sponsored congresses held in 2012 and 2016 at the same location, which included more than 500 scientists at each meeting.

Despite some major scientific advances in recent years, the global situation with TB is unfortunately still very far from being solved and TB remains a key infectious disease that globally still claims many human lives each day. Thus, the search for novel, scientifically challenging, solutions to this human tragedy is of urgent and continued interest. The objectives of this meeting are to unite a critical mass of scientists involved in research to communicate and exchange novel information on the disease and the causative agent, as well as related mycobacteria that are of medical interest.

According to the current conditions, the number of participants, physically in the conference centre is limited to maximal 480 people, including invited speakers and chairs. The conference organizers thus reserve the right to close inscription once the maximal number of attendees is reached. In this case, additional possibilities to follow the meeting via videoconference might be proposed and such information would be announced on the website, closer to the date of the workshop.

Local Organization Committee

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Institute of Tropical Medicine, Antwerp, Belgium

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Sarah Fortune,
Harvard University, Boston, United States

Stephen Gordon,
University College Dublin, Ireland

Maximiliano G. Gutierrez,
The Francis Crick Institute, London, United Kingdom

Jean-Louis Herrmann,
Université Paris-Saclay, Saint-Quentin-en-Yvelines, France

Candie Joly,
CEA, Fontenay Aux Roses, France

Simone Joosten,
Leiden University, Leiden, The Netherlands

Andreas Kupz,
James Cook University, Cairns, Australia

Margo Maex,
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Giulia Manina,
Institut Pasteur, Paris, France

Thomas Marlovits,
Institute of Structural and Systems Biology, Hamburg, Germany

Hedia Marrakchi,
IPBS, Toulouse, France

Carlos Martin,
University of Zargossa, Zaragossa, Spain

Helen McShane,
University of Oxford, Oxford, United Kingdom

Olivier Neyrolles,
Institut for Pharmacology and Structural Biology, Toulouse, France

Stefan Niemann,
Research Center Borstel, Germany

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The Francis Crick Institute, London, United Kingdom

Lalita Ramakrishnan,
University of Cambridge, United Kingdom

Santiago Ramón García,
ARAID Foundation, Zaragoza, Spain

Jeremy Rock,
Rockefeller University, New York, United States

Eric Rubin,
Harvard University, Boston, United States

David Russell,
Cornell University, Ithaca, United States

Jan Rybniker,
University of Cologne, Germany

Ludovic Tailleux,
Institut Pasteur, Paris, France

Laura Via,
NIH NIAID, Bethesda, United States

Gerhard Walzl,
Stellenbosch University, Stellenbosch, South Africa

Annemarie Wehenkel,
Institut Pasteur, Paris, France

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INSTITUT PASTEUR

On the Institut Pasteur campus, the "Plan Vigipirate Attentats" is on, so please make sure to have an official ID or passport to enter the campus.

If your registration is fully settled, you will be given a badge that you should wear at all times. The certificate of attendance will be sent after the conference by email.

ACCESS TO THE CONFERENCE CENTER

The only access to the Conference Center is located at

205, rue de Vaugirard, Paris 15th

Pasteur or Volontaires metro Line 12

The welcome desk opens at 2:00 pm on Monday, September 12th, 2022.

OPENING HOURS OF THE CONFERENCE CENTER

- Monday September 12th, 2022 — 2:00 pm - 10:00 pm
- Tuesday September 13th, 2022 — 7:30 am - 9:00 pm
- Wednesday September 14th, 2022 — 7:30 am - 7:30 pm
- Thursday September 15th, 2022 — 7:30 am - 6:30 pm
- Friday September 16th, 2022 — 7:30 am - 3:00 pm

The "Vaugirard" access closes at the indicated times.
Afterwards the exit will be through the main campus exit.

- A cloakroom is available at the auditorium level during all the conference. We ask you not to leave any personal belongings unattended in the auditorium.
- Congress staff assistance is also available during coffee breaks, lunches and cocktails.

PLENARY SESSIONS

Scientific sessions take place in the auditorium
of the CIS (Scientific Information Center).

LUNCHES & COFFEE BREAKS

All Coffee breaks and lunches will be served in the hall of CIS. Access to lunches is limited to participants who are registered.

All attendees are invited to the Welcome Reception which will be held on Monday 20th, at 6:35 pm in the hall of the CIS.

COVID-19 RECOMMENDATIONS

The Covid-19 epidemic has been circulating in our territory for several weeks, driven by the BA.4 and BA.5 sub-variants of Omicron. The **BCP/PBRP committee therefore calls on Pasteurians and external guests to be vigilant and recommends :**

- wearing a mask, particularly in enclosed spaces including auditorium, shared work areas and during the posters sessions
- regular ventilation of the premises, as much as possible

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CODE : 490113

Congres Gala dinner at the Museum of fairground arts for participants who have registered for the congres dinner, including invited speakers and chairs.

You must have your badge and present the digital invitation received by email

This millstone building, designed at the end of the 19th century by one of Gustave Eiffel's apprentices now houses the replica of an **ancient funfair** with merry-go-rounds and attractions.

This extraordinary site, also known as the **Musée des Arts Forains** (Museum of fairground arts), takes you off the beaten track.

Discover a **unique collection** of objects from the performing arts of the 19th and 20th centuries. It is a **timeless getaway** into the themes of curiosity cabinets, carnivals, incredible gardens and Belle Epoque funfairs.

EVENING SCHEDULE

7:30 pm	**Welcome in the golden age of the funfairs!**
8:00 pm	Cocktail and fairies attractions
8:45 pm	Seated dinner
10:30 pm	Coffee and last opportunity to ride
Midnight	the centenary merry-go-rounds !

Address:

Les Pavillons de Bercy - Musée des Arts Forains
53 av. Des Terroirs de France - 75012 Paris

Metro access:

Line 6 from Pasteur to Bercy Station + Line 14 from Bercy to Cour St Emilion Station.
Line 14 will be closed from 10 :00 pm. Return only by Bercy Station, Line 6

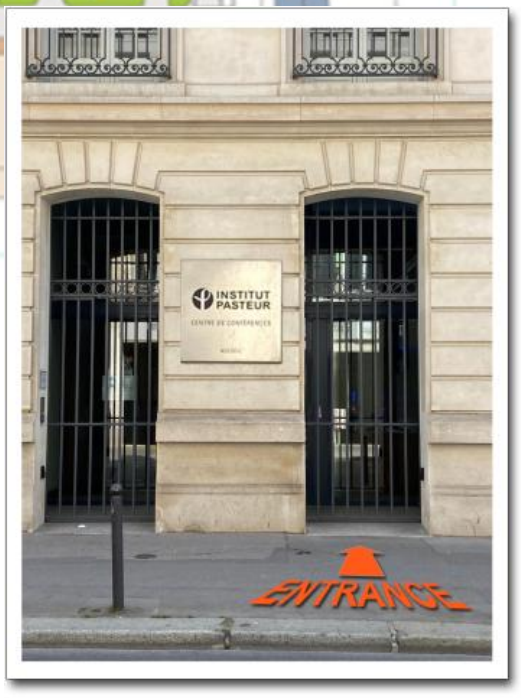
MAP OF THE CAMPUS

The entrance during the five days of the congress is located at

205, rue de Vaugirard, 75015 Paris

Auditorium CIS

Welcome Desk, Lecture hall, Posters,
Cocktail, Lunches and Coffee breaks



SUMMARY PAGE

PROGRAMME

Tuesday, September 13th-16th, 2022

Institut Pasteur - CIS Auditorium

EMBO | EMBO Workshop

INSTITUT PASTEUR

MONDAY SEPTEMBER 12TH, 2022

Registration	12/09/2022 2 pm - 5.30 pm

Welcome addresses & Opening Keynote lecture	12/09/2022 5.45 pm - 7 pm

Chair: Roland Brosch, Institut Pasteur, France

5.45 pm **Welcome addresses,**
the Director of the Institut Pasteur Stewart COLE and members of the organizing committee
Institut Pasteur, Paris, France

1 **Developing a controlled human infection model for TB**
6.15 pm Helen McShane
University of Oxford, United Kingdom

Welcome Reception (buffet dinner) at CIS Institut Pasteur	12/09/2022 7 pm - 9 pm

TUESDAY SEPTEMBER 13TH, 2022

1	From Mycobacterial genomics to global epidemiology & resistance prediction	13/09/2022 8.30 am - 10.15 am

Chairs: Howard Takiff, Institut Pasteur, Paris, France
Philip Supply, CIIL - Center for Infection and Immunity of Lille, France

2 **Transmission and evolution of MDR *Mycobacterium tuberculosis* complex strains**
8.30 am Stefan Niemann
Research Center Borstel, Borstel, Germany

3 **Cis-regulatory effects of constitutive and context-specific intercellular mosaic methylation in *M. tuberculosis***
8.55 am Samuel Modlin
San Diego State University, San Diego, United States

4 **Mycobacterial diversity's lens on public health**
9.10 am Bouke De Jong
Institute of Tropical Medicine, Antwerp, Belgium

5 9.35 am | **Epistasis defines the transmission fitness of multidrug-resistant *Mycobacterium tuberculosis***
Etthel Windels
Department of Biosystems Science and Engineering, ETH Zurich, Basel, Switzerland

6 9.50 am | **Fast RNA-based drug susceptibility testing fills the gap between slow phenotypic and ultra-fast DNA-based resistance detection**
Margo Maex
Sciensano, Ixelles, Belgium

Coffee break — 13/09/2022 10.15 am - 10.45 am

2 Mycobacterial Biology & Physiology I

13/09/2022 10.45 am - 12.30 pm

Chairs: Sabine Ehrt, Weill Cornell Medical College, United States
Jeremy Rock, Rockefeller University, United States

7 10.45 am | **Finding the most "essential" essential genes in *M. tuberculosis***
Jeremy Rock
Rockefeller University, New York, United States

8 11.10 am | **ADP-ribosylation of DNA in *Mycobacterium tuberculosis* – from "How" to "Why?"**
Graham Stewart
Dept of Microbial Sciences, University of Surrey, Guildford, United Kingdom

9 11.25 am | **TbD1 deletion in *Mycobacterium tuberculosis* adaptation to the human host**
Daria Bottai
University of Pisa, Italy

10 11.50 am | **Phase variation as a major mechanism of adaptation in *Mycobacterium tuberculosis* complex**
Maha Farhat
Biomedical Informatics, Harvard Medical School, Boston, United States

11 12.05 pm | **Mycobacterial determinants of intrinsic multidrug tolerance and resistance**
Sabine Ehrt
Weill Cornell Medical College, New York, United States

Lunch — 13/09/2022 12.30 pm - 1.45 pm

Poster session 1

13/09/2022
1.45 pm - 3.30 pm

3 Mycobacterial Biology & Physiology II

13/09/2022
3.30 pm - 4.55 pm

Chairs: Giulia Manina, Institut Pasteur, France
Sarah Fortune, Harvard University, United States

12 3.30 pm
It's about time: Antibiotic resilience as a driver of treatment failure in tuberculosis
Sarah Fortune
Harvard University, Boston, United States

13 3.55 pm
Exploring horizontal gene transfer in predominantly clonal tuberculosis-causing mycobacteria
Jan Madacki
Institut Pasteur, Paris, France

14 4.10 pm
The *Mycobacterium tuberculosis* protein *O*-phosphorylation landscape
Christoph Grundner
Seattle Children's Research Institute, University of Washington, Seattle, United States

15 4.25 pm
Pheno-tuning: A strategy to undermine mycobacterial cells
Giulia Manina
Cell Biology and Infection Dept., Microbial Individuality and Infection Lab, Institut Pasteur, Paris, France

Coffee break

13/09/2022
4.50 pm - 5.20 pm

4 Drug development & resistance/persistence management

13/09/2022
5.20 pm - 6.50 pm

Chairs: Bree Aldridge, Tufts University, United States
Santiago Ramón García, ARAID Foundation, Spain

16 5.20 pm
OPTIKA, a new high content *in vitro* kill-kinetic assay to evaluate the efficacy of novel anti-TB drug combinations
Santiago Ramón García
ARAID Foundation, Zaragoza, Spain

17 5.45 pm
Targeted protein self-degradation as a novel therapeutic strategy for tuberculosis
Harim Won
Immunology and Infectious Diseases, Eric Rubin Lab, Harvard University, Boston, United States

18 6 pm
Sanfetrinem Cilexetil - From (re-) discovery to clinical trial
Robert Bates
GSK Medicines Development Centre, Tres Cantos, Spain

19 6.25 pm
Design Principles of Combination Therapies for TB
Bree Aldridge
Tufts University, Boston, United States

Wine cheese reception at CIS Institut Pasteur — 13/09/2022 7 pm - 9 pm

WEDNESDAY SEPTEMBER 14TH, 2022

5 Biomarkers and host responses — 14/09/2022 8.30 am - 10.15 am

Chairs: Lalita Ramakrishnan, University of Cambridge, United Kingdom
Simone Joosten, Leiden University, The Netherlands

20 8.30 am
Mycobacterial growth control in natural vs vaccine induced protection
Simone Joosten
Leiden University, Leiden, The Netherlands

21 8.55 am
A *Mycobacterium tuberculosis* fingerprint in human breath allows tuberculosis diagnosis
Jérôme Nigou
Institut de Pharmacologie et de Biologie Structurale, CNRS - University of Toulouse, France

22 9.10 am
Mycobacterial virulence and macrophage mitochondrial metabolism face off
Lalita Ramakrishnan
University of Cambridge, Cambridge, United Kingdom

23 9.35 am
Human Antibodies Targeting a Transporter Mediate Protection Against Tuberculosis
Natalia Freund
Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

24 9.50 am
Roles of antibody characteristics in the protection against TB - from epitope to Fc and isotype significance
Jacqueline Achkar
Albert Einstein College, New York, United States

Coffee break — 14/09/2022 10.25 am - 10.55 am

6 Live attenuated vaccines & mechanisms of attenuation

14/09/2022
10.55 am - 12.25 pm

Chairs: Carlos Martin, University of Zargossa, Spain
Andreas Kupz, James Cook University, Australia

25 10.55 am
BCG::ESAT6-PE25SS - a new recombinant BCG strain - insights into delivery and protective mechanisms
Andreas Kupz
James Cook University, Cairns, Australia

26 11.20 am
MTBVAC from the lab to clinical efficacy trials, the moment of truth 100 years after BCG
Carlos Martin
University of Zargossa, Spain

27 11.45 am
Mind the Gap: Functional Characterisation of the Mycobacterial Virulence Protein Erp
Jonathan Shanahan
Medicine, Molecular Immunity Unit, Ramakrishnan, University of Cambridge, Cambridge, United Kingdom

28 12 pm
ESX-1-proficient BCG vaccines: challenges and opportunities
Roland Brosch
Unit for Integrated Mycobacterial Pathogenomics, Institut Pasteur, Paris, France

Lunch — 14/09/2022 12.25 pm - 1.45 pm

Poster session 2 — 14/09/2022 1.45 pm - 3.15 pm

7 Mycobacterial Host-Pathogen interactions

14/09/2022
3.15 pm - 5 pm

Chairs: Maximiliano G. Gutierrez, The Francis Crick Institute, United Kingdom
Olivier Neyrolles, Institut for Pharmacology and Structural Biology, France

29 3.15 pm
Mycobacterial resistance to metal stress during infection
Olivier Neyrolles
Institut for Pharmacology and Structural Biology, Toulouse, France

30 3.40 pm
Pre-existing heterogeneity of inducible nitric oxide synthase expression drives differential growth of *Mycobacterium tuberculosis* in macrophages
Ophélie Rutschmann
UPKIN, EPFL, Lausanne, Switzerland

31 3.55 pm
Is *Mycobacterium tuberculosis* the greatest cell biologist in the world?
Maximiliano G. Gutierrez
The Francis Crick Institute, London, United Kingdom

32 4.20 pm
Making mistakes in the goldilocks zone: mistranslation as a novel form of mycobacterial virulence
Babak Javid
UCSF, San Francisco, United States

33 4.35 pm
Drivers of diversity in tuberculosis *in vivo*
David Russell
Microbiology and Immunology, Cornell University, Ithaca, United States

Coffee break — 14/09/2022 5 pm - 5.30 pm

8 Non-rodent animal models for TB — 14/09/2022 5.30 pm - 7 pm

Chairs: Laura Via, NIH NIAID, United States
Stephen Gordon, University College Dublin, Ireland

34 5.30 pm
One Health analysis of *Mycobacterium bovis*
Stephen Gordon
University College Dublin, Dublin, Ireland

35 5.55 pm
Drosophila melanogaster as a host model to study mycobacterial host-pathogen interactions
Marte Singsås Dragset
Centre of Molecular Inflammation Research (CEMIR), Norwegian University of Science and Technology, Trondheim, Norway

36 6.10 pm
Implementation of a cynomolgus macaque model of tuberculosis
Candie Joly
IMVA-HB/IDMIT, CEA, Fontenay Aux Roses, France

37 6.35 pm
Efficacy of new TB regimens in an NHP model: focusing on pathology
Laura Via
NIH NIAID, Bethesda, United States

On your own for dinner — 14/09/2022 7 pm

THURSDAY SEPTEMBER 15TH, 2022

9 Emerging mycobacterial pathogens

15/09/2022
8.30 am - 10.15 am

Chairs: Marcel Behr, McGill University, Canada
Jean-Louis Herrmann, Université Paris-Saclay, France

38 8.30 am
***Mycobacterium abscessus* fitness modulation *in vivo*: a bacterial trait or ... a bacterial trait**
Jean-Louis Herrmann
Université Paris-Saclay, Saint-Quentin-en-Yvelines, France

39 8.55 am
The small RNA B11 is a master regulator of ESX-4 secretion and *M. abscessus* pathogenesis
Daniel Barkan
Hebrew University, Rehovot, Israel

40 9.10 am
A drug candidate against *Mycobacterium abscessus* and other cystic fibrosis pathogens
Giulia Degiacomi
Department of Biology and Biotechnologies, Università di Pavia, Pavia, Italy

41 9.25 am
Unpacking the molecular determinants of *Mycobacterium abscessus* infections
Virginia Pichler
Microbiology & Immunology, University of British Columbia, Vancouver, Canada

42 9.40 am
***M. orygis:* zTB's missing link**
Marcel Behr
McGill University, Montreal, Canada

Coffee break

15/09/2022
10.05 am - 10.35 am

10 Host-responses and host-directed therapies

15/09/2022
10.35 am - 12.35 pm

Chairs: Priscille Brodin, INSERM, Institut Pasteur de Lille, France
Jan Rybniker, University of Cologne, Germany

43 10.35 am
Exploiting MAP-kinase signaling as a host directed target in tuberculosis
Jan Rybniker
University of Cologne, Cologne, Germany

44 11 am
WNT6/ACC2-induced storage of triacylglycerols in macrophages is exploited by *Mycobacterium tuberculosis*
Julius Brandenburg
Research Center Borstel, Germany

45 11.15 am
Targeting human macrophages to improve the efficacy of anti-TB drugs
Ludovic Tailleux
Institut Pasteur, Paris, France

46 11.40 am
Host regulators of phagosomal membrane integrity in TB infection
Charul Jani
Dr. Amy Barczak, Ragon Institute of MGH, MIT and Harvard, Cambridge, United States

47 11.55 am
Neutrophil-driven immunosuppressive feedback loops underlying susceptibility to *M. tuberculosis*
Dmitri Kotov
Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, United States

48 12.10 pm
Is intracellular activity a potent driver for novel TB drug development?
Priscille Brodin
INSERM, Institut Pasteur de Lille, Lille, France

Lunch — 15/09/2022 12.35 pm - 1.45 pm

Poster session 3 — 15/09/2022 1.45 pm - 3.15 pm

11 Biochemistry & chemical biology / drugs I

15/09/2022 3.15 pm - 4.35 pm

Chairs: Hedia Marrakchi, IPBS, France
Clifton Barry, NIH NIAID, United States

49 3.15 pm
Exploiting a Bacterial Enzyme for Selective Prodrug Activation: *Mycobacterium tuberculosis* N-acetylates 5-aminomethyl oxazolidinones
Clifton E. Barry
NIH NIAID, Bethesda, United States

50 3.40 pm
Expression of a novel mycobacterial phosphodiesterase successfully lowers cAMP levels resulting in reduced tolerance to cell-wall targeting antimicrobials
Gerald Larrouy-Maumus
MRC-CMBI Imperial College London, London, United Kingdom

51 3.55 pm
Targeting mycolic condensation enzymes: from screens to insights
Hedia Marrakchi
IPBS, Toulouse, France

52 4.20 pm | **Solution structure of the type I polyketide synthase Pks13 from *Mycobacterium tuberculosis***
Cécile Bon
Structural Biophysics, IPBS/Faculty of pharmacy, Toulouse, France

Coffee break — 15/09/2022 4.35 pm - 5 pm

12 Biochemistry & chemical biology / drugs II — 15/09/2022 5 pm - 6.05 pm

Chairs: Thomas Marlovits, Institute of Structural and Systems Biology, Germany
Pedro Alzari, Structural Microbiology, Institut Pasteur, France

53 5 pm | **Novel insights into mycobacterial cell division mechanisms using *C. glutamicum* as a model organism**
Annemarie Wehenkel
Department of Structural Biology and Chemistry, Structural Microbiology Unit, Alzari Lab, Institut Pasteur, Paris, France

54 5.25 pm | **Compartment-specific proximity labeling uncovers the exposure of Type VII ESX secretion system substrates to the mycobacterial periplasm**
Jessica Seeliger
Department of Pharmacological Sciences, Stony Brook University, Stony Brook, United States

55 5.40 pm | **Structure and dynamics of a mycobacterial type VII secretion system**
Thomas Marlovits
Institute of Structural and Systems Biology, Hamburg, Germany

Congress dinner (Musée des Arts Forains) (with reservation) — 15/09/2022 8 pm - 11 pm

FRIDAY SEPTEMBER 16TH, 2022

13 Human susceptibility & Clinical trials — 16/09/2022 9 am - 10.30 am

Chairs: Anne O'Garra, The Francis Crick Institute, United Kingdom
Gerhard Walzl, Stellenbosch University, South Africa

56 9 am | **Human Genetics of tuberculosis : the TYK2 story**
Laurent Abel
Inserm, Necker, Paris, France

57 9.25 am	**Systems immunomonitoring to support development of new treatments and vaccines for TB** Darragh Duffy *Institut Pasteur, Paris, France*
58 9.40 am	**Transcriptional signatures reveal the immune response underlying progression and pathogenesis in tuberculosis** Anne O'Garra *The Francis Crick Institute, London, United Kingdom*
59 10.05 am	**Using Biomarkers to Predict TB Treatment Duration** Gerhard Walzl *Stellenbosch University, Stellenbosch, South Africa*

Coffee break — 16/09/2022 10.30 am - 11 am

14 Closing Keynote lecture and outlook

16/09/2022 11 am - 12 pm

Chairs: Roland Brosch, Institut Pasteur, France
Pedro Alzari, Structural Microbiology, Institut Pasteur, France

60 11 am	**Drugs hug, bugs chug - genetic-chemical synergy in mycobacteria** Eric Rubin *Harvard University, Boston, United States*
11.50 am	**Closing comments / End of congress**

ORAL COMMUNICATIONS

Tuesday, September 13th-16th, 2022

Institut Pasteur - CIS Auditorium

Developing a controlled human infection model for TB

H. McShane
University of Oxford, Oxford, United Kingdom

A more effective vaccine against *Mycobacterium tuberculosis* would significantly reduce the burden of disease and mortality associated with this highly successful pathogen. Challenges to successful vaccine research and development include the uncertain predictive value of animal models and the lack of defined and validated immune correlates of protection.

Despite encouraging advances in recent years, we need more effective ways of selecting which vaccine candidates should be progressed into large, expensive field efficacy studies. In other fields where vaccine development is complex, e.g. malaria, controlled human infection models have facilitated vaccine R&D. We have used BCG, an attenuated strain of *Mycobacterium bovis*, to establish a skin and mucosal mycobacterial human challenge model. Such models can be used for vaccine development and selection, for the identification of potential immune correlates, and to study the immunobiology of mycobacterial infection after defined time point infection in the host target species. I will present data from published and unpublished studies demonstrating the utility of this approach.

Transmission and evolution of MDR *Mycobacterium tuberculosis* complex strains

S. Niemann

Molecular and Experimental Mycobacteriology, Research Center Borstel, Germany German Center for Infection Research, Borstel Site, Germany

Multidrug resistant (MDR), Pre-extensively drug resistant (Pre-XDR), and XDR *Mycobacterium tuberculosis* complex (MTBC) strains have emerged worldwide and represent a serious challenge for global tuberculosis (TB) control. In several areas of the world, high rates of MDR TB have been associated with strains of particular phylogenetic MTBC lineages such as L2 (Beijing). Detailed data on the genetic diversity of particular MDR-TB outbreak strains, possible transmission networks and on their evolution are urgently needed esp. in light of implementation of the new WHO MDR TB treatment regimens.

Here, whole genome sequencing (WGS) allows for high-resolution strain typing e.g. studying the evolution of resistant MTBC strains in particular settings, for outbreak analysis or even for comprehensive molecular epidemiological studies in combination with information on virtually all target genes involved in resistance development (resistome analysis).

We applied WGS to investigate population structure, transmission dynamics and evolution of MDR/XDR MTBC strains in different settings of the world. Besides previous assumptions that suggested a lower virulence of MDR strains, person to person transmission, clonal expansion of particular outbreak strains and step-wise fixation of resistance mediating mutations appear to be an important factor driving the MDR TB epidemic in different settings of the world such as Eastern Europe, and India. In the latter, epidemic success analysis based on the time scaled haplotypic density method showed that L2 MDR/pre-XDR/XDR strains outperformed strains of other lineages e.g. of L1, L3, and L4 in short and long-term time scales. This was linked to high rates of compensatory mutation and positive selection in genes associated with drug tolerance (*prpB* and *ppsA*), and virulence (Rv2828c). Compensatory mutations in L2 strains were associated with a three-fold increase of THD indices, suggesting improved transmissibility. Particular MDR outbreak clones have gained high resistance levels even to new WHO group A MDR TB treatment drugs in line with compensatory mutations.

The data demonstrate a massive transmission of MDR/pre-XDR/XDR strains particularly in different parts of the world. The genetic background, the acquisition of compensatory mutations and in other genes associated with drug tolerance, and virulence are fostering higher transmission rates esp. of L2 strains. The implementation of rapid molecular diagnostics linked to individualized MDR TB treatment to ensure effective MDR detection, MDR TB therapy and breaking transmission chains. Prospective genome-based surveillance is essential to detect emergence of new MTBC MDR clones and to closely monitor resistance levels.

Cis-regulatory effects of constitutive and context-specific intercellular mosaic methylation in M. tuberculosis

S. Modlin*[1], D. Conkle-Gutierrez[1], C. Kim[1], C. Morrissey[1], S. Mitchell[1], B. Weinrick[2], W. Jacobs[3], F. Valafar[1]

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DNA methylation (DNAm) is a semi-heritable epigenetic phenomenon that mediates important roles in evolution, physiology, pathogenesis for many bacterial species. This study investigated how DNAm pattern changes in *M. tuberculosis* might engender phenotypic consequences and how this varies between strains. Heterogeneity analysis of partially active methyltransferase alleles revealed that intracellular stochastic methylation generates a mosaic of methylomes within isogenic cultures, termed 'Intercellular Mosaic Methylation' (IMM). Mutation-driven IMM was nearly ubiquitous in the Beijing sublineage yet rare in other lineages. Combining 93 fully-annotated, finished, de novo assembled genomes of MTBC clinical isolates and methionine-auxotrophic H37Rv mutants ($H37Rv_{\Delta metA}$) with DNA adenine methylomes, RNA expression, and transcription factor binding data identified putative interactions between DNAm and regulatory effectors. We find that promoter methylation is widespread and associated with differential expression in the *ΔhsdM* (now known as *mamC*) transcriptome, suggesting promoter MamC-methylation directly influences transcription. We also find that methionine-starved $H37Rv_{\Delta metA}$ mutants methylation preference under limited methyl group donor availability associate with the local transcription factor binding site landscape. The effect of DNAm on gene expression and the differential capacity for IMM between clinical isolates implicates a differential capacity for semi-heritable phenotypic plasticity. This has important ramifications for within-host evolution and could impact the accuracy of diagnostics and efficacy of therapeutics in a strain-specific manner. The numerous putative regulatory interactions identified provide a basis for developing specific functional hypotheses for DNA adenine methylation in *M. tuberculosis*. We exemplify this through subsequent integrative analysis of cell-cycle stage specific transcriptomic data, which found methylated promoters enriched among genes overexpressed at particular cell cycle stages, informing specific hypotheses of processes under epigenetic influence in *M. tuberculosis*.

Mycobacterial diversity's lens on public health

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For communicable diseases like tuberculosis and leprosy, accurate extrapolation of the incidence from case notifications is challenging. When case notifications decline, does that reflect that control is effective, transmission was interrupted, new infections averted? Or did we stop looking for patients, who continue to transmit, and incidence increases? Advances in bioinformatics approaches allow for independent estimates of the total pathogen population- a welcome tool in public health.

Epistasis defines the transmission fitness of multidrug-resistant Mycobacterium tuberculosis

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Experimental studies have shown that antibiotic resistance causes replicative fitness costs that are modulated by the bacterial genetic background and compensatory evolution. How such epistatic interactions affect transmission of bacterial pathogens in human populations, however, is unknown. In this study, we aimed to quantify the relative transmission fitness of multidrug-resistant tuberculosis (MDR-TB) strains. To this end, a nation-wide genomic epidemiological study was conducted in Georgia, a country characterized by a high burden of MDR-TB. We applied Bayesian phylodynamic inference methods accounting for population structure to the genomic data, showing that MDR-TB strains of lineage 4 transmit less than their drug-susceptible counterparts, whereas most MDR-TB strains of lineage 2 suffer no such defect. Our findings further indicate that the high transmission fitness of these lineage 2 strains results from epistatic interactions between the rifampicin resistance-conferring mutation RpoB S450L, compensatory mutations in the RNA polymerase and particular lineage 2 genetic backgrounds. We conclude that the transmission of multidrug-resistant bacteria can be as efficient as drug-susceptible strains, which has important implications for the control of bacterial diseases.

Fast RNA-based drug susceptibility testing fills the gap between slow phenotypic and ultra-fast DNA-based resistance detection

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Over the last years, DNA-based drug resistance detection has strongly increased the capacity of TB diagnostics worldwide. The fast turnaround time and the clinical significance of an increasing number of characterized causative mutations have contributed to the success of targeted amplification diagnostics like GeneXpert MTB/RIF. However, the need for phenotypic drug susceptibility testing (pDST) will never disappear, in light of drug discovery, synergy testing, and emerging drug resistance mechanisms.

Over the last years, our group developed a fast pDST method based on antibiotic-specific RNA biomarkers. The basic principle is that an antibiotic exposure triggers transcriptional stress responses in susceptible but not in resistant microbes, enabling the distinction between resistant and susceptible strains after 6 hours of drug exposure. I will show that this quantitative method outcompetes traditional MGIT in case of disputed mutations, while resistance caused by rare or unknown mutations, which are missed by DNA-based testing, is equally identifiable. The independence of the method of resistance makes this method a valuable tool for research, which will be illustrated by our application in the investigation of new molecules as booster molecules, studying the in vivo effect of drug resistance in a macrophage model and comparing the stress response produced by strains carrying the same and/or different mutations.

Finding the most "essential" essential genes in *M. tuberculosis*

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Antibacterial agents target the products of essential genes but rarely achieve complete target inhibition. Thus, the all-or-none definition of essentiality afforded by traditional genetic approaches fails to discern some of the most attractive bacterial targets: those whose incomplete inhibition results in major fitness costs. In contrast, gene "vulnerability" is a continuous, quantifiable trait that relates the magnitude of gene inhibition to the effect on bacterial fitness. We developed a CRISPR interference-based functional genomics method to systematically titrate gene expression in Mycobacterium tuberculosis (Mtb) and monitor fitness outcomes. We identified highly vulnerable genes in various processes, including novel targets unexplored for drug discovery. Equally important, we identified invulnerable essential genes, potentially explaining failed drug discovery efforts. Comparison of vulnerability between the reference and a hypervirulent Mtb isolate revealed incomplete conservation of vulnerability and that differential vulnerability can predict differential antibacterial susceptibility. Our results quantitatively redefine essential bacterial processes and identify high-value targets for drug development.

ADP-ribosylation of DNA in Mycobacterium tuberculosis – from "How" to "Why?"

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The bacterial DarTG toxin-antitoxin system is present in a number of important pathogens including the *Mycobacterium tuberculosis* complex, enteropathogenic *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The DarT toxin is able to add ADP-ribose from nicotinamide adenine dinucleotide (NAD+) onto thymidine in a sequence-specific manner in ssDNA. Unregulated expression of DarT results in arrest of DNA replication, induction of the mutagenic SOS-response, and ultimately death of the bacterium. The DarG anti-toxin is able to both inhibit the activity of the DarT toxin and reverse the ADP-ribosylation of thymidine. Structural study of DarT from *Thermus sp*, reveals the highly coordinated interactions between DarT and its DNA target which enable the sequence specific addition of ADP-ribose to the thymidine base. We recently demonstrated the sequence specificity of *M. tuberculosis* DarT to be the motif TTTW and we identified ADP-ribosylation of the AT-rich origin of replication (oriC) in mycobacteria. Together with an elevated growth rate of DarTG mutant *M. tuberculosis*, this suggests a fundamental physiological role for the DarTG system as a molecular switch coordinating replication. The potential wider role of the DarTG DNA-ADP-ribosylation system in bacterial physiology will be discussed.

TbD1 deletion in *Mycobacterium tuberculosis* adaptation to the human host

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The deletion of the *Mycobacterium tuberculosis*-specific deletion 1 region (TbD1), encoding for the MmpS6/MmpL6 operon, is the hallmark of the globally-spread *Mycobacterium tuberculosis* (*Mtb*) strains belonging to phylogenetic lineages (L) L2, L3 and L4. The characterization of a panel of recombinant TbD1-knock-in and knock-out strains and comparison with clinical isolates showed that deletion of TbD1 confers to *Mtb* an enhanced virulence in selected cellular, guinea pig and C3HeB/FeJ mouse infection models, the latter two mirroring in part the development of hypoxic granulomas in human disease progression. TbD1-deletion also correlates with a significant increase in the *Mtb* resistance to oxidative stress and survival in the Wayne model of hypoxia-induced in vitro dormancy. RNAseq studies revealed TbD1-deletion-dependent gene expression profiles in different *in vitro* conditions, including exposure to oxidative stress and hypoxia. These results suggest that the loss of the TbD1 region was a key event in the evolutionary adaptation of L2, L3 and L4 *Mtb* lineages to the host.

Phase variation as a major mechanism of adaptation in Mycobacterium tuberculosis complex

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Phase variation induced by insertions and deletions (INDELs) in genomic homopolymeric tracts (HT) have been recently implicated in MTBC antibiotic tolerance. Phase variation can also potentiate virulence across an array of pathogenic bacteria, but it is not well characterized in MTBC. We leverage a sample of 31,428 diverse clinical isolates to identify genomic regions under positive selection and quantify the contribution of phase variation to MTBC adaptation. Over 90,000 unique single nucleotide variants (SNV) and INDELS emerged repeatedly across the phylogeny (10.6% of all variants). Of 87,651 INDEL events, 12.4% occur within HTs (0.02% of the genome by length). We estimated the *in-vitro* frameshift rate in a neutral HT at 1.1 x 10^-5 frameshifts/HT/year, ~100x the average genomic rate of SNVs. Using simulations, we identified 4,098 SNVs and 45 HTs to have a higher rate of parallel evolution than expected by chance based on the neutral evolutionary rate (P<0.002). We experimentally confirm that a positively-selected INDEL near the transcriptional start site of the *espA* gene alters the expression of this critical mediator of ESX-1 dependent virulence in MTBC. We present evidence that MTBC genomes are strongly and regionally shaped by positive selection not only to modulate the resistance phenotype but likely also virulence mechanisms, and hypothesize that phase variation in ESX-1 system of MTBC can act as a toggle between antigenicity and survival in the host.

Mycobacterial determinants of intrinsic multidrug tolerance and resistance

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The ability of *Mycobacterium tuberculosis* (Mtb) to resist and tolerate antibiotics complicates the development of improved tuberculosis chemotherapies. We identified the Mtb proteins CinA and FecB as major determinants of multidrug tolerance and resistance, respectively, and their inhibition as potential strategies for the shortening of TB chemotherapy. Genetic and drug metabolism experiments indicate that CinA mediates drug tolerance via cleavage of NAD-drug adducts. The mechanism by which FecB mediates resistance to antibiotics remains unclear but likely involves safeguarding Mtb's cell envelope integrity. I will discuss recent approaches and discoveries that provide insight into the physiological functions of CinA and FecB.

It's about time: Antibiotic resilience as a driver of treatment failure in tuberculosis

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Antibiotics are a cornerstone of medicine, placing bacterial pathogens under intense pressure to evolve new survival mechanisms. We performed a population genomic analysis of more than 50,000 Mtb genomes. We identified a previously uncharacterized essential regulator, here named resR, as a frequent target of positive (adaptive) selection associated with the acquisition of drug resistances. ResR mutants do not demonstrate canonical drug resistance or drug tolerance but instead have significantly faster recovery after drug treatment across all antibiotics and combinations tested, a phenotype which we term antibiotic resilience.

Exploring horizontal gene transfer in predominantly clonal tuberculosis-causing mycobacteria

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Current models of horizontal gene transfer (HGT) in mycobacteria are based on "distributive conjugal transfer" (DCT), an HGT type described in the fast-growing, saprophytic model organism *Mycobacterium smegmatis*, which creates genome mosaicism in resulting strains and depends on ESX-1 and ESX-4 type VII secretion systems. In contrast, only few data on inter-strain DNA transfer are available for tuberculosis-causing mycobacteria, for which chromosomal DNA transfer between two *Mycobacterium canettii* strains was reported, a process which, however, was not observed for *M. tuberculosis* strains.

Here, we have studied a wide range of human and animal-adapted members of the *Mycobacterium tuberculosis* complex (MTBC), using an optimized filter-based mating assay, together with three selected strains of *M. canettii* that acted as DNA recipients. Unlike in previous approaches, we obtained a high yield of thousands of recombinants containing transferred chromosomal DNA fragments from various MTBC donor strains, as confirmed by whole-genome sequence analysis of several dozens of randomly selected clones. While the genome organizations of the obtained recombinants showed mosaicisms of donor DNA fragments randomly integrated into a recipient genome backbone, reminiscent of those described as being the result of ESX-1-mediated DCT in *M. smegmatis*, we observed similar transfer efficiencies when ESX-1-deficient donor and/or recipient mutants were used, arguing that in tubercle bacilli, HGT is an ESX-1-independent process. On the other hand, our preliminary data point to possible involvement of the ESX-4 secretion system in the process.

These findings provide new insights into the genetic events driving the pathoevolution of *M. tuberculosis* and radically change our perception of HGT in mycobacteria. Indeed, these results argue that HGT in tubercle bacilli is organized in a different way than that of the most widely studied *Mycobacterium smegmatis* model, a finding that is also relevant beyond tubercle bacilli, given that many mycobacteria, like for example *Mycobacterium avium* or *Mycobacterium abscessus* are naturally devoid of an ESX-1 secretion system, but show recombinogenic, mosaic-like genomic population structures.

The Mycobacterium tuberculosis protein O-phosphorylation landscape

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Well-coordinated cellular signaling is the basis for bacterial adaptations. In bacteria, phosphosignaling was long synonymous with two component systems, but increasingly, protein Ser, Thr, and Tyr phosphorylation (*O*-phosphorylation) is also being recognized as an important signaling mechanism. *Mycobacterium tuberculosis* (*Mtb*) produces a similar number of Ser/Thr kinases (STPKs) and two component systems, suggesting a balance between these two arms of phosphosignaling. In a deep phosphoproteomic study of the *Mtb O*-phosphorylation system, we now expand the number of known *O*-phosphorylation sites by >10,000 unique sites, showing that *Mtb O*-phosphorylation gives rise to an expansive, distributed, and cooperative signaling network of a size and complexity not previously seen in bacteria. Using a comprehensive panel of STPK loss- and gain-of-function mutant strains and quantitative mass spectrometry (MS)-based phosphoproteomics, we find that >80% of *Mtb* proteins are phosphorylated on Ser, Thr, and Tyr, suggesting that a much larger share of *Mtb* physiology than previously thought is controlled by *O*-phosphorylation. Remarkably, >30% of gene expression is affected by STPK perturbation, identifying a large interface between *O*-phosphorylation and transcription. Our high-content and kinase-specific map of the *O*-phosphorylation network identified >3,700 direct kinase-substrate interactions, among them many between STPKs and transcription factors (TFs). We predict dozens of functional phosphorylation events on TFs by integrating phosphoproteomic and transcriptional data and delineate signaling pathways from STPK to gene in molecular detail. These data provide the deepest bacterial *O*-phosphoproteome to date, provide >3,700 individual STPK-substrate relationships, identify a large interface between *O*-phosphorylation and transcription, and together establish *O*-phosphorylation in *Mtb* as a dominant signaling mechanism with a complexity that has so far only been seen in eukaryotes.

Pheno-tuning: A strategy to undermine mycobacterial cells

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Tuberculosis remains a major global health challenge, and better therapeutic interventions are constantly needed to combat this infection. Phenotypic variation of clonal cells promotes adaptive mechanisms that are associated with stress tolerance and persistence and can suggest original strategies to tackle the tubercular pathogen. Based on the association between phenotypic variation in DNA damage response and differential susceptibility to treatment, we hypothesized that fine-tuning phenotypic variation could help sensitize clonal mycobacterial cells to standard antitubercular drugs, ultimately aiming to shorten tuberculosis therapy. We developed a multi-condition microfluidic platform and used it in conjunction with quantitative time-lapse microscopy, to screen for pheno-tuning compounds against our RecA fluorescent reporter of DNA damage response. We identified four main hits that induce RecA expression and decrease cell-to-cell RecA variation, aiming to make the population more susceptible to treatment and to homogenize single-cell responses. Finally, we characterized the mechanism of action of three hits and showed that one of them can potentiate some standard anti-tubercular drugs in vitro. Our results confirm that targeting phenotypic variation using single-cell approaches holds promise for drug discovery.

OPTIKA, a new high content *in vitro* kill-kinetic assay to evaluate the efficacy of novel anti-TB drug combinations

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Tuberculosis (TB) is one of the top 10 causes of death worldwide. The FDA approved combination of bedaquiline, pretomanid and linezolid (BPaL) was recently recommended by the WHO for 6 months XDR-TB treatment. Understanding how to efficiently develop such regimens remains however a challenge.

The main limitation of traditional *in vitro* methods used to develop drug combinations, such as the checkerboard (CBA) or the DiaMOND assays, is the use of growth inhibition as a metric of drug activity. This requires secondary validation assays typically performed by the gold-standard *in vitro* proxy, i.e., time-kill assays (TKA). Unlike CBA and DiaMOND, TKA rely on a bactericidal parameter (Log_{10}CFU/mL), being this one of the most valuable *in vitro* assays, and the basis of pharmacometrics modelling of antimicrobial drug action. However, compared to the fixed endpoint for CBA or DiaMOND, TKA rely on CFU enumeration at different time points, which requires large culture volumes, long readout times and has a limited throughput capacity. This creates a barrier to validate interactions of more than 3 drugs per experiment.

In this context, we developed a new methodology named **OPTIKA (Optimized Time Kill Assays)** that dramatically increases traditional TKA capacity, and allows for facile and dynamic interrogation of drug interactions with a CFU-free methodology. OPTIKA is based on the CARA assay and replaces the use of CFU with a resazurin-based fluorescence readout in a 96-well plate format. This technique has been optimized to robustly analyse up to 770 unique conditions (in quadruplicate: 3080 samples) at the same time and allows data to be delivered after 10 days, almost 2 weeks earlier than traditional CFU-based methods.

OPTIKA coupled to pharmacometrics modelling can play a critical role in the preclinical and clinical development of novel anti-TB combinations.

Targeted protein self-degradation as a novel therapeutic strategy for tuberculosis

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With the ever-increasing incidence of multi-drug resistant TB, there is an urgent need for novel *Mycobacterium tuberculosis* (*Mtb*) therapeutics. Conserved bacterial proteases present an attractive, yet underdeveloped, set of potential drug targets. Recently, targeted protein degradation has been exploited as a cancer therapeutic through proteolysis-targeting chimeras (PROTACs); these molecules feature bi-specific domains joined by a linker and induce proximity of a target substrate to an E3 ligase, resulting in polyubiquitination and subsequent proteasomal degradation of that target. To determine if an analogous system could be used in mycobacteria, we created dimerizable domain fusion proteins which would permit small molecule-induced proximity of target substrates to the mycobacterial ClpC1P1P2 proteolytic complex. Using flow cytometry, immunoblotting, and time lapse microscopy, we show that induced proximity to this Clp proteolytic complex is sufficient to cause the degradation of several endogenous proteins in a non-pathogenic model of *Mtb*, *M. smegmatis*. We also find that targeted degradation of various essential, natively tagged proteins inhibits *Msm* growth, both alone and in synergy with antibiotics targeting the same complexes. Here, we establish early proof-of-concept experiments and highlight targeted degradation as a potential novel drug development strategy against the deadly pathogen *Mtb*.

Sanfetrinem Cilexetil - From (re-) discovery to clinical trial

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New TB treatments are urgently needed to shorten therapy and cure all forms of the disease; however, developing new antimicrobials is costly and lengthy. Drug repurposing represents a potentially rapid approach toward this goal.

β-lactams have an exceptional record of clinical safety. Used for decades to treat bacterial infections, they were regarded as ineffective against Mycobacterium tuberculosis; however, the clinical efficacy of meropenem was recently shown [PMID: 27433841]. Unfortunately, meropenem can only be administered intravenously (not practical in under-resourced settings where oral drugs are needed) and requires co-dosing with a β-lactamase inhibitor like clavulanic acid.

Together with multiple collaborators, GSK performed screens looking to identify β-lactams better suited to TB treatment. These screens identified sanfetrinem, a tricylcic carbapenem developed by GlaxoWellcome in the 1990s and its prodrug sanfetrinem cilexetil as promising otions. Importantly, sanfetrinem displayed activity against TB even in the absence of clavulanic acid. And the cilexetil prodrug shows oral bioavailability that allowed the drug to achieve positive results in three Phase 2 trials against respiratory tract infections. Time-kill assays and confocal time-lapse microscopy confirmed sanfetrinem's intracellular and rapid bactericidal activity. And mouse studies confirmed the equipotency of sanfetrinem cilexetil versus a combination of meropenem and amoxicillin/clavulanate.

Given its clinical pedigree, GSK has been able to move directly toward a Phase 2 EBA study of sanfetrinem cilexetil for TB. This study is now underway in South Africa.

Design Principles of Combination Therapies for TB

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Treatment of TB requires multidrug regimens to ensure the killing of a heterogeneous bacterial population present in different lesion types. Design of more effective, treatment-shortening therapies should realize the vast drug-combination space's potential early in development. We have developed a pipeline based on systematic, efficient *in vitro* measurement of the drug combination space to predict treatment outcomes in pre-clinical animal models. Measurement in a suite of *in vitro* conditions that model different aspects of the host environment were critical to improving model accuracy. We found that *in vitro* potency and synergy metrics of pairwise drug combinations can be used as building blocks to articulate principles of designing optimized combinations of three and four drugs. Using these tools and an expanded dataset of drug combination responses in *M. tuberculosis*, we have predicted the treatment outcomes of >7000 drug combinations in the BALB/c relapsing mouse model. We propose that this empirical approach for early drug combination design can be used for iterative model improvement and as a resource to understand the principles of drug combination design.

Mycobacterial growth control in natural vs vaccine induced protection

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BCG vaccination gives variable protection, protection in infants and young children against severe forms of TB and against heterologous pathogens is considerable. However, protection in adolescents and adults is highly variable. Recently, BCG revaccination in adolescents was shown to protect against sustained infection. BCG is well known for its heterologous protection against other pathogens, likely as a result of sustained innate immune activation in addition to specific adaptive responses. To delineate the specific players involved in effector responses responsible for mycobacterial growth control we investigated an adult BCG vaccination cohort in the Netherlands. In the current study we assessed the functional capacity to control BCG outgrowth, based on these results samples were classified and scRNAseq data used to identify pathways responsible for bacterial control.

A Mycobacterium tuberculosis fingerprint in human breath allows tuberculosis diagnosis

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An estimated one third of tuberculosis (TB) cases go undiagnosed or unreported. Sputum samples, widely used for TB diagnosis, are inefficient at detecting infection in children and paucibacillary (smear negative) patients. Indeed, developing point-of-care biomarker-based diagnostics that are not sputum-based is a major priority for the WHO. Here, we tested exhaled breath condensate (EBC) for *Mycobacterium tuberculosis* (Mtb) molecules and assessed whether this approach allows pulmonary TB diagnosis. Mtb-specific lipids, lipoarabinomannan lipoglycan, and proteins present in EBCs unambiguously differentiate TB patients from controls. We used EBCs to track the longitudinal effects of antibiotic treatment in Mtb-infected children. In addition, Mtb lipoarabinomannan and lipid structure in EBC revealed specific metabolic and biochemical states of Mtb in the human lung. Our data collectively indicate that EBC analysis can unequivocally diagnose TB across all patient populations and monitor treatment efficacy. This affordable, rapid and non-invasive approach seems superior to sputum assays and can potentially easily be implemented at point-of-care.

Mycobacterial virulence and macrophage mitochondrial metabolism face off

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Necrosis of granuloma macrophages represents a major pathogenic event in tuberculosis. I will discuss our findings that increased mitochondrial energy metabolism in response to infection constitutes an early defense against the cryptic mitochondrial damaging activity of the mycobacterial secreted protein ESAT-6. The finding that the host can effectively counter this early critical mycobacterial virulence mechanism simply by regulating energy metabolism may explain why *Mycobacterium tuberculosis*, albeit humanity's most lethal pathogen, is successful in only a minority of infected individuals.

Human Antibodies Targeting a Transporter Mediate Protection Against Tuberculosis

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Immune response to Mycobacterium *tuberculosis* (Mtb) is traditionally thought to be T cell dependent. However, recent evidence suggest that in a subset of individuals with history of exposure to Mtb, anti-Mtb antibodies are mounted and may be of protective nature. Still, it is not known whether patients with active tuberculosis disease develop anti-Mtb protective antibodies. Also, what bacterial antigens are targeted by these potentially protective antibodies is currently unknown. In the present study, we screened a cohort of 26 actively infected patients from Israel for their anti-Mtb serum responses. We focused on PstS1, a subunit of the Mtb phosphate transporter, and a dominant antigen during infection. Using single B cell sorting we generated a panel of monoclonal antibodies (mAbs) isolated from memory B cells of one patient, P004, who showed elevated anti-PstS1 serum responses. We isolated and analyzed 85 anti-PstS1 mAbs naturally elicited during active Mtb infection in donor P004, 16 of which were members of four distinct B cell clones. Two antibodies p4-163 and p4-36, members of Clones 1 and 3, demonstrated robust anti-Mtb inhibitory activity and showed protective efficacy against Mtb in macrophage infection, *ex vivo* human whole blood assay, and in aerosol-infected mice, reducing bacterial lung burden in the latter model by 50% after a single injection prior to infection. Crystal structures of the two protective antibodies complexed to PstS1 were determined at a resolution of 2.1Å and 2.4Å revealed the precise epitopes recognized by the antibodies, indicating that the two antibodies are directed against two nonoverlapping epitopes on PstS1. Germline versions of p4-163 and p4-36 had no detectable binding to Mtb lysate compared with the mature antibodies, implying that somatic hypermutation occurred during infection was necessary for their activity. Our study shows that inhibitory B cell responses arise during tuberculosis infection, and identifies PstS1 as a potential target for elicitation of protective anti-Mtb antibodies.

Roles of antibody characteristics in the protection against TB - from epitope to Fc and isotype significance

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Antibodies have protective *in vitro* and *in vivo* efficacy against *Mycobacterium tuberculosis* (*Mtb*) suggesting that inducing both cell-mediated and humoral immunity could be complementary. However, a better understanding of protective antigens, their epitopes, and mechanisms of antibody-mediated protection remain a gap of knowledge in the TB field. The focus of this talk are antibodies targeting the mycobacterial surface glycan arabinomannan (AM)/lipoarabinomannan (LAM). The data presented will enhance the knowledge on the importance of glycan epitope specificity in combination with FcγR-mediated effects for antibody-mediated protection against TB.

BCG::ESAT6-PE25SS – a new recombinant BCG strain – insights into delivery and protective mechanisms

A. Kupz
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In my presentation, I will discuss our latest results and ongoing projects regarding the further development and pre-clinical evaluation of the recombinant BCG strain, BCG::ESAT6-PE25SS. I will cover the foundational studies that have led to the rational design of the vaccine candidate and will provide data demonstrating how this strain uncouples virulence from immunogenicity. I will show data regarding safety and efficacy in several pre-clinical animal models, including mouse models of diabetes and reactivation of latent TB. My presentation will also touch on results showing reduced mycobacterial meningoencephalitis after infection of severely immunocompromised mice and increased T cell activation in the lung following mucosal vaccine administration relative to conventional BCG and other recombinant BCG strains. Finally, I will provide insights into ongoing studies designed to complete pre-clinical evaluation and process optimisation leading towards future human trials.

MTBVAC from the lab to clinical efficacy trials, the moment of truth 100 years after BCG

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MTBVAC is the only live attenuated TB vaccine candidate in clinical development. MTBVAC's 25 years of vaccine discovery, construction and characterization have strictly follows Pasteur's principles of vaccination: isolation of the human pathogen, attenuation by inactivation of selected genes, protection in animals, and evaluation in humans. Inside various European-TB vaccine projects and the European Tuberculosis Vaccine Initiative_TBVI, in collaboration with the Pasteur Institute of Paris, the University of Zaragoza, developed MTBVAC from a clinical isolate of *M. tuberculosis* by the stable deletions in two major virulence genes, *phoP* and *fadD26*. The clinical *M. tuberculosis* strain used to construct MTBVAC belonging to modern Lineage-4, representing the most geographically widespread lineage in Europe, Africa and America. Preclinical studies have shown the safety and protection of MTBVAC in TB-relevant animal models conducted by independent collaborative-laboratories from mouse, guinea pigs to non-human primates.

The industrial partner Biofabri is responsible for Industrial and Clinical Development of MTBVAC for newborns and for adolescent/adults. From 2008-2012 Biofabri performed GMP development of freeze-dried MTBVAC and from 2012 until present: industrial development and scale-up production of MTBVAC. First-in-human Phase 1a of MTBVAC was performed in healthy adults in Lausanne, Switzerland (Spertini *et al* The Lancet Respir Med 2015). Successful Phase 1a completion leads to first-in-human Phase 1b evaluation of MTBVAC in newborns in Worcester, South Africa (SATVI) (Tameris *et al* The Lancet Respir Med 2019). Two Phase 2 dose-defining studies in healthy newborns (NCT03536117) and in adults (NCT02933281), which commenced in 2019 and were carried out by SATVI, are at their final completion stages. Safety and immunogenicity results of the two Phase 2 dose-defining trials, will allow the dose-definition of MTBVAC for entry into planned multi-center Phase 3 efficacy evaluation in newborns in South Africa, Madagascar and Senegal (NCT04975178) estimated to initiate at 2022.

Future perspectives in research of MTBVAC: At the present the research of our team is focused in the study of the therapeutic effect of MTBVAC in the treatment of bladder cancer, and asthma. In addition, we are interested on alternative delivery routes of MTBVAC, mainly in clinically feasible aerosol route, could facilitate universal administration of MTBVAC.

Mind the Gap: Functional Characterisation of the Mycobacterial Virulence Protein Erp

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Throughout *M. tuberculosis*' complex life cycle in the host – infection, growth, transmission – mycobacteria must continually resist host defences. A major mycobacterial survival mechanism lies in the unusual and complex cell envelope. The mycobacterial cell envelope provides a robust permeability barrier that helps the bacteria tolerate the variety of host insults it encounters. Many mycobacterial virulence factors are localised in the cell envelope. Erp (exported repetitive protein) was the first mycobacterial virulence factor to be identified by targeted deletion, yet how it performs this function remains relatively poorly understood. Erp is a cell envelope associated, critical virulence factor, that is required for survival and growth specifically in host macrophages. Loss of Erp increases the permeability of the cell envelope, sensitising mycobacteria to both hydrophobic antibiotics and a range of antimicrobial molecules enriched in macrophages. Erp appears to anchor the cell wall to the inner membrane, thereby preserving cell envelope ultrastructure and outer membrane organisation. Localisation of Erp specifically to the cell wall is required to maintain the cell envelope as an impermeable barrier. Erp is mycobacterium-specific, and widely conserved in both saprophytic and pathogenic mycobacteria, suggesting that it co-evolved with the mycobacterial cell envelope to maintain a permeability barrier that was essential to counter ubiquitous environmental antimicrobial factors. Erp's function then allowed mycobacteria to evolve into intracellular pathogens.

ESX-1-proficient BCG vaccines: challenges and opportunities

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Bacille Calmette-Guérin (BCG) continues to play a key role in the vaccination programs of many countries with high incidence of tuberculosis (TB) thanks to its recognized efficacy against miliary and meningeal TB in babies and young children. In contrast, BCG vaccination confers variable, mostly only limited protection against pulmonary TB in adolescents and adults, and massive BCG vaccination for decades has not been able to stop the spread of the TB pandemic.

For this reason, new generations of improved anti-TB vaccines are eagerly awaited. As one promising approach to increase protective innate and adaptive immune signaling, recombinant BCG strains expressing variants of the ESX-1 type VII secretion system, which is absent from BCG due to the deletion of region of difference RD1, have shown improved protection in preclinical models. ESX-1-proficient strains permeabilize the phagosome of the host cell and establish contact with the cytosol, a process which is thought to underlie the improved protection observed in different preclinical models and routes of vaccine administration.

Mycobacterial resistance to metal stress during infection

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Although essential in trace amounts, transition metals are toxic when in excess, and their intracellular concentration must be tightly regulated. We and others previously discovered that immune cells exploit transition metals, namely zinc and copper, to intoxicate bacterial pathogens, and that metal efflux systems are involved in bacterial virulence. Specifically, we showed that, in human macrophages, zinc accumulates in phagocytosis vacuoles containing *Mycobacterium tuberculosis*, and that the metal efflux pump CtpC, a member of the P-ATPase superfamily, is required for *M. tuberculosis* to resist zinc intoxication and multiply inside these cells. In addition, we had found that CtpC is encoded together with a 93-residue protein, Rv3269, renamed PacL1 for “P-ATPase-associated chaperone-like protein 1”, in a zinc-inducible operon. Two other *M. tuberculosis* P-ATPases, namely CtpG and CtpV, are encoded together with PacL proteins, namely PacL2 and PacL3. We recently found that PacL1 is a zinc-binding chaperone-like protein that plays an unexpected dual role in resistance to zinc poisoning: its metal binding domain is required for survival only at high concentrations of zinc, consistent with a metallochaperone; however, PacL1 is required for survival even at low zinc concentrations, independent of its metal binding domain. We found that PacL1 additionally acts as a scaffold to stabilize CtpC at the plasma membrane in previously undescribed P-ATPase-containing functional membrane foci that resemble functional membrane microdomains, which we propose to call “metal efflux platforms”. Over 400 other P-ATPases are encoded in operons with PacL proteins across bacteria, suggesting the broad conservation and function of metal efflux platforms. Here, I will discuss the concept of “metal efflux platform” in mycobacterial resistance to metal intoxication, and more broadly the potential importance of membrane compartmentalization in mycobacterial physiology and virulence.

Pre-existing heterogeneity of inducible nitric oxide synthase expression drives differential growth of Mycobacterium tuberculosis in macrophages

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Mycobacterium Tuberculosis (Mtb) is a highly effective pathogen infecting nearly a third of the world's population. Part of its success comes from its ability to survive and replicate inside macrophages, cells that are usually charged with repressing bacterial infections. The interactions between *Mtb* and macrophages are heterogeneous and can lead to diverse outcomes, ranging from the death of the pathogen to its replication and subsequent killing of the host cell. The origins of this heterogeneity are so far not well characterized, and we are striving to understand how diversity in the bacteria and the host populations influence the outcome of an infection.

Thanks to automated time-lapse microscopy, we can follow single live macrophages infected with fluorescent *Mtb* over the course of several days. By using *Mtb* strains expressing different fluorescent proteins, we observe 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 inter-macrophage differences disappear when inhibiting inducible nitric oxide synthase (iNOS), a known defense mechanism against *Mtb*. By using a macrophage reporter cell line for iNOS expression, we then demonstrate that iNOS levels fluctuate in time at the single cell level, independently to other known IFNg-mediated defense mechanisms, and that this pre-existing heterogeneity in iNOS expression is linked to the control of intracellular *Mtb* in both activated (+IFNg) and unactivated (-IFNg) samples.

As alveolar macrophages are considered to be the initial cells in contact with *Mtb*, 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 *Mtb,* an infection may initiate from a single bacterium coming in contact with a single host macrophage. Thus, the level of iNOS this macrophage expresses when it is infected could influence how well it controls the initial infection and, eventually, how the disease progresses.

Is *Mycobacterium tuberculosis* the greatest cell biologist in the world?

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Making mistakes in the goldilocks zone: mistranslation as a novel form of mycobacterial virulence

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Increasing evidence suggests that errors in protein synthesis – mistranslation – can allow microbes to adapt to hostile environments via proteome innovation. We had previously identified that specific errors in protein synthesis, mistranslation, were necessary for tolerance of *M. tuberculosis* (Mtb) to rifampicin, and that the small molecule kasugamycin could both reduce mistranslation rates and potentiate rifampicin *in vitro* and *in vivo*. We now show that kasugamycin, as a single agent, can substantially attenuate Mtb in animal infection, despite never reaching its minimum inhibitory concentration *in vivo*. Kasugamycin is not acting on the host, since infection of mice with a kasugamycin-resistant mutant is not attenuated with kasugamycin treatment. Mistranslation increases survival of Mtb in macrophages, and alters the lung immune milieu to benefit the pathogen. Our data support the notion that mycobacterial mistranslation is necessary for full virulence of Mtb in animals. Nonetheless, too high a mistranslation rate leads to error catastrophe. We identified mutants in a suppressor screen that could suppress high mistranslation rates conferred by a mutation in *gatA*, which causes high specific rates of error due to the indirect tRNA aminoacylation pathway. Many of these mutations mapped to the 16S rRNA methyltransferase *gidB*. Deletion of *gidB* increased discrimination of misacylated tRNA in the high mistranslation mutant, but surprisingly, had no phenotype on a wild-type background grown in normal axenic culture. However, when wild-type mycobacteria were grown under conditions that enriched for high mistranslation, Δ*gidB* increased translational fidelity. Solving the structure of the mycobacterial ribosome from wild-type and high mistranslating strains with and without *gidB* showed subtle changes in ribosomal structure, specifically when *gidB* was deleted in a high mistranslating background, potentially explaining a mechanism by which (lack of) methylation of a specific residue in 16S rRNA allows for discrimination of misacylated tRNAs. Our work provides a potential mechanism by which microbes allow for modest, beneficial rates of translational error whilst insuring against runaway error catastrophe. It also suggests that the mycobacterial ribosome is capable of discriminating against misacylated tRNA, challenging a prevailing dogma in the proof-reading mechanisms of the ribosome.

Drivers of diversity in tuberculosis *in vivo*

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One Health analysis of *Mycobacterium bovis*

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This presentation will focus on comparative analyses of *Mycobacterium bovis* and *Mycobacterium tuberculosis*, the exemplar animal- and human-adapted members of the *Mycobacterium tuberculosis* complex (MTBC). Comparative studies across human and animal TB pathogens offer a unique 'One Health' perspective to explore host tropism and evolutionary trajectories of the MTBC.

Our work is founded on comparative experimental infections in cattle using *M. bovis* and *M. tuberculosis* strains, work that revealed distinct infection outcomes in cattle with these bacilli. To explore the mechanistic basis for these differences, we have focussed on understanding the role of the MPB70 protein which is constitutively secreted at high levels by *M. bovis* as compared to *M. tuberculosis*. These analyses explored the role of MPB70 in *M. bovis*-macrophage interactions and extended to infection of cattle with an *M. bovis* MPB70 mutant. We have also explored the role of the TbD1 locus, present in *M. bovis* but deleted from 'modern' *M. tuberculosis* lineages, using *in vitro* comparisons of *M. bovis* mutants.

By exploring such genetic differences using a range of 'omics, *in vitro* and infection systems, we aim to define the molecular basis of distinct phenotypes across these archetypal TB bacilli.

Drosophila melanogaster as a host model to study mycobacterial host-pathogen interactions

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is the world's deadliest infectious disease (current SARS-CoV-2 pandemic excluded) with one death every 20 seconds. To improve TB treatment and prevention, we must better understand the underlying mechanisms of the disease. Central to this are host-pathogen interactions (HPIs): the interplay between the host response and the pathogen's virulence factors. Novel host infection models to dissect HPIs within reasonable cost and time frames may be key to the future control of TB.

Drosophila melanogaster (Drosophila) is one of the most extensively studied animal models we have, with a short generation time and a broad, advanced and organized research community with tools and host mutants readily available at low cost. Yet, Drosophila has barely been exploited to understand the underlying mechanisms of mycobacterial infections, including those caused by Mtb. In fact, only 10 research papers in total have been published using Drosophila or Drosophila cell lines to study mycobacterial infections. Drosophila is as such an untapped resource to study HPIs relevant to Mtb.

We hypothesized that Drosophila is a powerful future host model to study mycobacterial virulence factors, using *Mycobacterium marinum* (Mmar) to model mycobacterial pathogens. First, we validated that an established mycobacterial virulence factor, EccB1 of the ESX-1 Type VII secretion system, was required for Mmar growth within the flies. Second, we identified Mmar virulence factors in Drosophila in a high-throughput genome-wide manner using transposon insertion sequencing (TnSeq). Of the 181 identified virulence genes, the vast majority (91%) had orthologs in Mtb, suggesting that the encoded virulence mechanisms may be conserved across Mmar and Mtb. Finally, we validated one of the novel Mmar virulence genes we identified, a putative ATP-binding protein ABC transporter encoded by *mmar_1660*, as required for full virulence during both Drosophila and human macrophage infection. Hence, Drosophila is apt to study and identify mycobacterial virulence genes in a manner that is relevant to infection of human cells. Future applications of the Mmar-Drosophila infection model comprise identifying genetic HPIs specific to host response pathways to provide deeper insights into mycobacterial pathogenesis that may be targeted therapeutically.

Implementation of a cynomolgus macaque model of tuberculosis

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Tuberculosis (TB) is still causing millions of deaths worldwide and new therapeutics are urgently needed as multi-drug resistant strains are threatening. Non-human primate models are very valuable for drug development, and especially cynomolgus macaque model, which reproduces the full spectrum of human TB and allows deep granuloma analysis for efficacy studies. As part of the ERA4TB consortium, we developed a cynomolgus macaque model of TB in IDMIT (Infectious Diseases Models for Innovative Therapies, Paris, France) for future drug efficacy studies. After exposure with 25 CFU of Erdman strain by intra-bronchial route, disease progression was followed using whole body PET-CT imaging, bronchoalveolar lavage culture, whole blood transcriptomic, along with clinical and immune monitoring. After 24 weeks, half of the animal developed an active TB while the other declared subclinical to latent TB during the follow-up. The data confirmed that clinical forms observed in cynomolgus macaques were very close to the spectrum of human disease and ongoing analysis will reveal early innate immune response to TB in an NHP model.

Efficacy of new TB regimens in an NHP model: focusing on pathology

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New chemotherapeutics or new combinations of therapeutics are needed to shorten Tuberculosis (TB) treatment. Bedaquiline (BDQ), Delamanid, and Pretomenid (PA) are newer agents used specifically for MDR-TB salvage treatment and are being explored for a pan-TB regimen. The BPaL regimen combines BDQ and PA with oxazolidinone linezolid (L). Recently, we deconstructed the standard 4-drug HRZE regimen in "NexGen EBA" clinical trial to identify PET/CT signatures of regimen components along with an MRZE arm where Moxifloxacin was substituted for H. This study was repeated in *Mycobacterium tuberculosis*-infected common marmosets, a small, non-human primate (NHP), to add 2-month treatment assessments to the 2-week EBA. These regimens along with their component parts were evaluated using similar in-vivo imaging plus terminal histologic and bacterial assays. Serial PET/CT scans during treatment were computationally evaluated to assess changes in TB-associated abnormalities. After 2 mo., bacterial burden and lesion histology were assessed and compared with standard treatment (HRZE) and with the human responses. Similar disease responses to H, Z, and HZ were seen in both species including that FDG-avid lesions responded more to Z and that synergy of HZ was observed in dense-disease reduction. Although 2-weeks of HRZE was not distinguishable from MRZE in either species, 2 months of HRZE promoted a significantly more rapid lesion resolution when compared to MRZE in the marmoset. In the BPaL regimen deconstruction, the addition of L to BDQ or PA did not increase bacterial killing over either agent alone, but animals treated with BL experienced more consistent reductions in disease volume and FDG uptake. Ex-vivo drug penetration into caseum also predicted the activity of the BPaL agents in lesions. The chosen therapeutic agent also appeared to affect the distribution of lesion types observed at necropsy. Both the BPaL regimen and HRZE reduced the MTB burden by 4.5 Log10 over 2 mo. Quantitative reductions in lesion volume and FDG uptake were more tightly correlated with lower bacterial loads in the BPaL regimen than in the HRZE regimen, suggesting that the measures might be useful in determining treatment duration and supporting further use of PET/CT in clinical studies of shorter TB regimens.

Mycobacterium abscessus fitness modulation *in vivo*: a bacterial trait or ... a bacterial trait

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Mycobacterium abscessus is a fast-growing mycobacterium, an opportunistic pathogen for humans, responsible for cutaneous-mucosal infections and respiratory infections, sometimes severe, especially in subjects with cystic fibrosis. *M. abscessus* has two type VII secretion systems (T7SS), Esx-3 and Esx-4, whose involvement in the intracellular lifestyle of the mycobacterium has recently been revealed (Laencina et al., 2018; Lagune et al., 2022). In comparison, strict human, and animal pathogenic mycobacteria, such as *Mycobacterium marinum* and *Mycobacterium tuberculosis*, have 4 and 5 (Esx-1 to Esx-5) respectively. These T7SS, mainly Esx-1 and Esx-5, have been extensively studied in *M. marinum* and *M. tuberculosis*, with their structure recently revealed. However, little information on the functionality of Esx-4 has been published, except on mycobacterial conjugation and even on phagosomal lysis. We have shown for the first time in a mycobacterium, M. abscessus, that Esx-4 is functional, involved in intracellular survival, and allowing the secretion of small proteins Esx-U and Esx-T, homologs of EsxA and EsxB belonging to the esx-1 locus. We will see that depending on the genetic modifications made within the esx-4 locus of M. abscessus, the physiopathological behavior of the bacterium will not be the same. In particular, the absence of EsxU and EsxT makes M. abscessus even more virulent in animals (Lagune et al., 2022).

The small RNA B11 is a master regulator of ESX-4 secretion and M. abscessus pathogenesis

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Introduction: Small non-coding RNA play an important role in virulence regulation of many pathogens. However, their role in mycobacterial pathogenesis remains unknown. *Mycobacterium abscessus* is an emerging pathogen, but only little is known on its mechanisms of virulence in general, and those of sRNA in particular. We used a novel transposon system to identify genes involved in *M. abscessus* pathogenesis and physiology.

Results: We identified a transposon mutant with a colony morphology reminiscent of mutants defective in the pathogenesis-essential ESX-4 secretion system. This transposon mutant had markedly reduced expression of the sRNA B11, also present in Mtb, whos role is unknown. Secretomic analysis showed dramatically (~50 fold) increased secretion of the ESX-4 system. We therefore created a full targeted deletion mutant of B11, and confirmed it by PCR and northern blotting. This mutant showed: **A)** an obvious "Rough" morphology; **B)** clumping and biofilm-like growth when grown without tween; **C)** a 30-fold increase in ESX-4 secretion, and; **D)** a markedly hyper-virulent phenotype in THP-1 cells, as compared to the parent ATCC19977 strain.

An *in-silico* analysis suggested at least part of B11's effect is mediated by binding to the RBS of *Mabs_0628*, the first gene in the regulatory *espI* operon.

Conclusions and significance: The role of sRNA in mycobacterial physiology and virulence is unknown, although extrapolating from other bacteria, this role must be of significance. Here we show that a sRNA, also present in Mtb, is a pivotal negative regulator of *M. abscessus* virulence, specifically (but not limited to) by negative regulation of ESX-4 secretion (which is a "substitute" for ESX-1 in Mtb), and possibly alteration of outer cell-wall composition. Deletion of B11, first by transposon and then directed mutagenesis, caused transformation to "Rough" morphology, biofilm-like growth, ~50 fold increase in ESX-4 secretion, and a dramatic **hyper-**virulent phenotype in THP-1 cells. The equivalent sRNA in Mtb is understudied, and may play a similar role. This is the first direct implication of sRNA in pathogenesis-related physiology in any mycobacteria. As mycobacteria have dozens of sRNA molecules, this finding opens a new field of mycobacterial genetic research, including possible therapeutic interventions by sRNA delivery.

A drug candidate against Mycobacterium abscessus and other cystic fibrosis pathogens

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Non-tuberculous mycobacteria (NTM) infections are becoming a threat to the life of people with cystic fibrosis (CF) as well as a major public health menace. *Mycobacterium abscessus* (*Mab*) is the pathogen of greatest concern amongst NTMs worldwide. *Mab* is intrinsically resistant to most available drugs, which generally lack bactericidal activity. Therefore, there is an urgent need of novel efficacious drugs against *Mab*.

In this study, we screened more than 700 compounds synthesized by Dr. V. Makarov and identified only one, 11226084, with bactericidal activity against *Mab* (MIC=0.25 mg/ml). 11226084 is also active against other NTMs including multi-drug resistant clinical isolates and other CF pathogens, such as *Staphylococcus aureus* and *Acinetobacter baumannii*. The molecule is also active against *Mab* biofilm and suitable for combinatorial therapy since no antagonism was identified among a panel of drugs clinically used for NTM treatment. Furthermore, 11226084is active in *Mab*-infected mice by intranasal administration, similar to amikacin, the drug comparator and is not toxic to the mice with good bioavailability.

Transcriptomic analysis showed that treatment with a 11226084 derivative inhibits essential metabolic pathways, such as cell division, protein biosynthesis, ATP production and gene transcription. Further studies elucidating the mode of action suggest that 11226084 could affect *Mab* cell division through FtsZ inhibition. In support of this finding, 11226084*Mab* treated cells displayed an elongated phenotype as evident from scanning electron microscopy studies.

The following experiments are in progress:

- Further pharmacokinetic, metabolism and toxicity studies;
- In depth characterization of its mechanism of action by heterologous production of *Mab* FtsZ;
- Determination of its activity against both *Mab* non-replicant cells and in macrophages model;
- Study of its activity in combination with gene correctors against both planktonic cells and biofilm.
- Evaluation of its activity in *Mab*-infected mice using different concentrations by intranasal administration.

  Our results indicate that 11226084 is a promising anti-*Mab* drug candidate.
- This work has received support from the "Fondazione Italiana Ricerca Fibrosi Cistica (FFC#18/2021, FFC#14/2020, FFC#19/2018)".

Unpacking the molecular determinants of Mycobacterium abscessus infections

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Mycobacterium abscessus (*Mab*) resistance mechanisms have been partially characterized with shared features observed in *Mycobacterium tuberculosis*, yet a knowledge gap remains in understanding persistent infections via phenotypic and genetic changes. The morphological transition from smooth (S) to rough (R), controlled predominantly by the glycopeptidolipid (GPL) gene locus, is linked to reduced GPLs and increased virulence. In the R morphotype, bacteria form cords and escape the host's response, leading to infection persistence. By pairing culture-based and molecular methods the link between phenotypic behaviour and genetic determinants will be further elucidated. Clinical *Mab* strains were isolated from 20 long-term cystic fibrosis patients in Vancouver, Canada. Multiple analytic methods were used including WGS, antibiotic testing, lipid analysis, and infection modelling. Strains were separated by microscopy into S and R, with half of the original strains presenting both. Morphotypes were classified as S and R, however observed colony features deviated from the discrete morphologies described in the literature. GPL profiles from lipid analysis matched the assigned morphotypes, except in four strains, which presented as R yet expressed GPLs. A subset (n=35) belonging to four patients were evaluated for bacterial burden and cording using THP-1 macrophage infections. A single experiment has been completed with subsequent replicates to be performed. MICs of nine clinically-relevant antibiotics fell within published ranges, with some outliers (amikacin >128µg/mL; meropenem >128µg/mL) including a 1-4x-fold change in six of the nine antibiotics from a mixed S and R strain to a R-only strain isolated from the same patient. Strains will further undergo WGS to find putative explanatory mutations and indels linking phenotype to genotype. Microscopy is a frequently-used diagnostic method, however, the morphotypes are ill-defined and subject to observer interpretation. GPLs have been a metric for classifying morphotypes, however, these results indicate clinical strains present greater variability with what appear to be 'transition' morphologies. Preliminary results link the transition from a mixed population to an exclusively R population with changes in drug tolerance, showing higher resistance. This could indicate that virulence, persistence, and resistance are a consequence of mechanisms beyond the S-to-R transition, to be further evaluated by WGS.

M. orygis: zTB's missing link

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In the era of DNA microarrays, our lab determined that mutations in sigma K (*sigK*) explain the variable production of MPT70/MPT83 by BCG strains and that independent mutations in its regulator (*rskA*) explain the high production of these proteins by *Mycobacterium bovis* and the Oryx bacillus (later renamed *M. orygis*). It was subsequently shown that the *M. orygis*-specific mutation in *rskA* was not merely a broken anti-sigma factor but rather, resulted in constitutive MPT70 expression when introduced into *M. tuberculosis*. We had a newly named bacterium with a curious *in vitro* phenotype in search of relevance. Given that *M. orygis* cultures had been described in both animals and patients from South Asia, an epidemiologic study was done at a referral centre in Vellore, India looking for either *M. bovis,* a well-defined contributor to zoonotic TB (zTB), or *M. orygis*. *M. orygis* was detected in 7 patients and was enriched in extrapulmonary samples, consistent with a potential zoonotic reservoir. Surprisingly, *M. bovis* was not detected at all. An analysis of an additional 715 publicly available *M. tuberculosis* complex sequences from South Asia also found no *M. bovis.* Together, this suggests that *M. orygis* may be the South-Asian counterpart of *M. bovis.* To better understand the capacity of *M. orygis* to infect and cause disease, we have established gavage and aerosol murine infection models. Following 10(8) bacilli by gavage, infection is well tolerated (up to 6 months) and we detect bacteria in the mesenteric lymph nodes, spleens and lungs. However, following infection with ~200 bacilli by aerosol, infection leads to mortality beginning at 4 weeks post infection. Mortality is postponed but not reversed by prior BCG vaccination, whether the vaccine produces MPT70 (BCG Russia) or not (BCG Danish). The ensemble of studies suggest that *M. orygis* is a hypervirulent member of the *M. tuberculosis* complex with an undetermined reservoir and to-be-determined virulence mechanism.

Exploiting MAP-kinase signaling as a host directed target in tuberculosis

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Host-modulating therapies for improved management of tuberculosis (TB) are coming increasingly into focus of anti-TB drug development strategies. One promising approach is to manipulate signaling pathways that contribute to immunopathology by causing hyperinflammation, necrosis and tissue damage. Mitogen-activated protein kinases (MAPK) represent central regulatory hubs involved in multiple pro-inflammatory and cell death pathways of immune cells. Among the different MAPK, it is primarily the p38-MAPK that seems to be involved in pathology of *Mycobacterium tuberculosis (Mtb)*. We have shown that this kinase is essential for cytokine release and host cell death following infection of human macrophages with *Mtb*. Treatment of *Mtb*-infected mice with a p38 MAPK inhibitor led to reduced inflammation, granuloma formation and lung pathology. P38 MAPK inhibition also improved performance of conventional antibiotics *in vivo*. Since p38 MAPK is a validated target in autoimmune and inflammatory diseases with several completed or ongoing clinical trials, repurposing of these drugs as an adjunct therapy for TB could be a promising approach. However, biomarker guided patient selection for TB clinical trials may be an essential aspect for successful application of these drugs. To address this bottleneck, we have been matching whole blood p38 MAPK gene expression levels of a TB patient cohort with different severities of disease. The data can help to implement p38 MAPK as a host directed target in future TB treatment regimen.

WNT6/ACC2-induced storage of triacylglycerols in macrophages is exploited by Mycobacterium tuberculosis

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In view of emerging drug-resistant tuberculosis (TB), host-directed adjunct therapies may be of use to to improve treatment outcomes with currently available anti-TB therapies. One approach is to interfere with the formation of lipid-laden "foamy" macrophages in the host, as they provide a nutrient-rich host cell environment for Mycobacterium tuberculosis (Mtb). Here, we provide evidence that Wnt family member 6 (WNT6), a ligand of the evolutionarily conserved Wingless/Integrase 1 (WNT) signaling pathway, promotes foam cell formation by regulating key lipid metabolic genes including acetyl-CoA carboxylase 2 (ACC2) during pulmonary TB. Using genetic and pharmacological approaches, we demonstrated that lack of functional WNT6 or ACC2 significantly reduced intracellular triacylglycerol (TAG) levels and Mtb survival in macrophages. Moreover, treatment of Mtb-infected mice with a combination of a pharmacological ACC2 inhibitor and the anti-TB drug isoniazid (INH) reduced lung TAG and cytokine levels, as well as lung weights, compared with treatment with INH alone. This combination also reduced Mtb bacterial numbers and the size of mononuclear cell infiltrates in livers of infected mice. In summary, our findings demonstrate that Mtb exploits WNT6/ACC2-induced storage of TAGs in macrophages to facilitate its intracellular survival, a finding that may open new perspectives for host-directed adjunctive treatment of pulmonary TB.

Targeting human macrophages to improve the efficacy of anti-TB drugs

L. Tailleux
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Despite considerable efforts, tuberculosis (TB) remains a major public health problem. An effective vaccine against TB is still not available and multidrug resistant strains of *Mycobacterium tuberculosis* (MTB) are continually emerging. New strategies are thus urgently needed. In addition to the development of classical TB drugs targeting key factors in MTB physiology, host-directed therapy (HDT) has emerged as a promising approach to be used in adjunct with existing or future antibiotics. MTB manipulates host-signaling pathways to subvert immunity. It might thus be possible to reprogram the host immune system to better control or even kill intracellular MTB.

Macrophages are central to TB pathogenesis and represent an attractive target for HDT. Recently we showed that bedaquiline increases macrophage defenses against MTB and potentiates the activity of other anti-TB drugs, independently of its bactericidal activity on MTB. By screening libraries of chemical compounds in MTB-infected cells, using high throughput automated confocal microscopy, we identified other compounds with similar properties.

Host regulators of phagosomal membrane integrity in TB infection

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In a typical pathogen invasion, macrophages respond by engulfing the alien organism and containing it in a specialized orgenelle called phagosome. The nascent phagosome undergoes maturation through series of fusion and fission events creating a hostile intraphagosomal environment with acidification and accumulation of antimicrobial peptides, ultimately fusing with a lysosome. *Mycobacterium tuberculosis* (*Mtb)* has evolved mechanisms to rupture the phagosome membrane meant to contain it, leading to the escape into the cytosol. This leads to necrotic death of the host cell and spread of *Mtb* to the neighboring cells. The host has evolved mechanisms to sense and repair the membrane ruptures, thereby overcoming the damage caused by the phagosomal membrane breach. Endosomal Sorting Complex Required for Transport (ESCRT) machinery has been shown to repair the phagosomal membrane damage. In this work we developed a genome-wide pooled CRISPR-KO screen in THP-1 macrophages to identify the host factors responsible for keeping the phagosomal membrane intact. Prior to the screen, we performed RNAseq analysis to identify the transcriptional correlates of phagosomal membrane damage. We then developed an mRNA flow-FISH based assay to moniter the phagosomal membrane damage at the single cell level. Using this assay as a readout, we performed the screen and identified multiple hits including members from ESCRT machinery. While the identification of ESCRT proteins validated the rationale as well as the screening approach, we followed up with cherry picked hits to understand the role of other proteins and their involvement in phagosomal repair. We also demonstrated the importance of these factors in restricting the intracellular bacterial growth and potential therapeutic application of this findings.

Neutrophil-driven immunosuppressive feedback loops underlying susceptibility to *M. tuberculosis*

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For reasons that remain poorly understood, control of *Mycobacterium tuberculosis* (*Mtb*) breaks down in only a fraction of infected humans. In these individuals, active tuberculosis disease correlates with a neutrophil-driven type 1 interferon (IFN) signature that is absent in wild-type C57BL/6 (B6) mice. Chronic type I IFNs have well-documented immunosuppressive effects, and indeed, we found that B6.*Sst1*^{s} ("Kramnik") mice are *Mtb*-susceptible due to their strong IFN response. We also identified *Sp140* as the gene responsible for the *Sst1*^{s} phenotype. Using *Sp140*$^{-/-}$ mice as a model that better recapitulates active human disease, we first sought to ascertain which cells are producing IFN *in vivo*. A genetic reporter of IFN expression and single cell RNA-sequencing defined interstitial macrophages, monocytes, and plasmacytoid dendritic cells (pDCs) as the main producers of IFN in infected lungs. Antibody and genetic depletion of pDCs partially rescued the susceptibility of *Sp140*$^{-/-}$ mice suggesting that pDCs are one of the major IFN producers during *Mtb* infection. pDCs from *Sp140*$^{-/-}$ mice appear more sensitive to stimulation by DNA ligands *in vitro* than B6 pDCs, leading us to speculate that pDCs respond to extracellular DNA from abundant neutrophil NETs *in vivo*. We then identified cells responding to type I IFN by scRNA-seq. Interstitial macrophages and neutrophils had a strong IFN response signature, specifically in *Sp140*$^{-/-}$ mice. Myeloid cell specific deletion of the type I IFN receptor rescued the susceptibility of *Sp140*$^{-/-}$ mice, while neutrophil specific deletion did not, suggesting that IFNs act primarily on macrophages, and not simply on neutrophils, to drive *Mtb*-susceptibility in *Sp140*$^{-/-}$ hosts. Importantly, IFN receptor deletion did not rescue the *Mtb*-susceptibility of *Acod1*$^{-/-}$ and *Nos2*$^{-/-}$ mice, suggesting that *Sp140*$^{-/-}$ mice uniquely model the IFN-driven disease observed in humans. Interestingly, neutrophil depletion rescues all three susceptible mouse models. Neutrophils are major producers of the immunosuppressive IL-1 receptor antagonist, deletion of which also reverses *Mtb*-susceptibility in all three mouse models. Our data suggest a model in which excessive neutrophilic inflammation suppresses effective immunity to *Mtb* via stimulation of type I IFNs by pDCs and by production of IL-1 receptor antagonist.

Is intracellular activity a potent driver for novel TB drug development?

P. Brodin

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Over the past 17 years, with the development of high-throughput, high-content phenotypic screens, we have systematically studied the effect of small molecules and host inhibitors for their ability to inhibit Mycobacterium tuberculosis intracellular trafficking and replication in macrophages. This approach has given rise to the concept of host-directed therapy (HDT), which could be used to optimise combination treatment strategies for TB. Examples of successes and pitfalls will be presented here.

Exploiting a Bacterial Enzyme for Selective Prodrug Activation: Mycobacterium tuberculosis N-acetylates 5-aminomethyl oxazolidinones

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Linezolid is a drug with proven human antitubercular activity whose use is limited to highly drug-resistant patients because of its toxicity. This toxicity is related to its mechanism of action – linezolid inhibits protein synthesis in both bacteria and eukaryotic mitochondria. To develop a more selective oxazolidinone for use specifically in treating tuberculosis, we identified a series of highly active molecules bearing a 5-aminomethyl moiety in place of the typical 5-acetamidomethyl moiety of linezolid. Linezolid resistant mutants were cross-resistant to these molecules, but not vice versa. Resistance to the 5-aminomethyl molecules mapped to an N-acetyl transferase (Rv0133) and these mutants remained fully linezolid susceptible. Purified Rv0133 was shown to catalyze the transformation of the 5-aminomethyl oxazolidinones to their corresponding N-acetylated metabolites and this transformation was also observed in live cells of *Mycobacterium tuberculosis*. Mitochondria, which lack an appropriate N-acetyltransferase to activate these prodrugs, were not susceptible to inhibition with the 5-aminomethyl analogs. Several of these compounds that were more potent than linezolid with acceptable pharmacokinetics were taken into C3HeB/FeJ mice and were shown to be highly efficacious and one of these (MRL662) was taken into marmosets where it was also found to be highly active at a very low dose. Penetration of these 5-aminomethyl oxazolidinone prodrugs into caseum was excellent and this likely contributed significantly to their exceptional *in vivo* performance.

Expression of a novel mycobacterial phosphodiesterase successfully lowers cAMP levels resulting in reduced tolerance to cell-wall targeting antimicrobials

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Antimicrobial tolerance, the ability to survive exposure to antimicrobials via transient nonspecific means, promotes the development of antimicrobial resistance (AMR). The study of the molecular mechanisms that result in antimicrobial tolerance is therefore essential for the understanding of AMR. In gram-negative bacteria, the second messenger 3',5'-cyclic adenosine monophosphate (cAMP) is known to be involved in AMR. In mycobacteria, the role of cAMP in antimicrobial tolerance has been difficult to probe because mycobacterial cAMP signaling is particularly complex. In order to address this difficulty, we identified a novel cyclic nucleotide-degrading phosphodiesterase enzyme (Rv1339) and developed a system to significantly decrease intracellular cAMP levels by plasmid expression of Rv1339. In *Mycobacterium smegmatis* mc^{2}155, recombinant expression of Rv1339 reduced cAMP levels 3- fold and resulted in altered gene expression, impaired bioenergetics and a disruption in peptidoglycan biosynthesis that resulted in decreased tolerance to antimicrobials with cell wall synthesis targets. This work increases our understanding of the role of cAMP in mycobacterial antimicrobial tolerance. Our observations suggest targeting nucleotide signaling as a new avenue for development of antimicrobial therapies.

Targeting mycolic condensation enzymes: from screens to insights

H. Marrakchi
IPBS, Toulouse, France

Mycobacterium tuberculosis (*Mtb*) possesses a unique and complex cell envelope whose biogenesis represents a niche of potential anti-tubercular drug targets. Accordingly, targeting enzymes involved in cell envelope synthesis has been of major interest for anti-TB drug discovery. The talk will focus on efforts to target FAAL32, a bi-functional enzyme involved in the last (condensation) step of the biosynthetic pathway of the cell wall mycolic acids. The chemical probe development to study this enzyme as well as key insights that emerged from the structural analysis will be presented. The highlight will be on how the chemical probes and the structural insights were used to build a miniaturized high-through screening platform for a drug repurposing campaign that led to the discovery of salicylanilide closantel. We show that this compound and some of its derivatives are potent inhibitors of *Mtb*, which suggests that salicylanilide represents a potentially promising pharmacophore for novel anti-tubercular candidates targeting FAAL32. Taken together, this work illustrates the relevance of FAAL32 as a druggable target, and the value of drug repurposing campaigns to discover new leads in challenging drug discovery fields.

Solution structure of the type I polyketide synthase Pks13 from Mycobacterium tuberculosis

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Type I polyketide synthases (PKSs) are multifunctional enzymes responsible for the biosynthesis of a group of diverse natural compounds with biotechnological and pharmaceutical interest called polyketides. The diversity of polyketides is impressive despite the limited set of catalytic domains used by PKSs for biosynthesis, leading to considerable interest in deciphering their structure-function relationships, which is challenging due to high intrinsic flexibility. Among nineteen polyketide synthases encoded by the genome of *Mycobacterium tuberculosis*, Pks13 is the condensase required for the final condensation step of two long acyl chains in the biosynthetic pathway of mycolic acids, essential components of the cell envelope of *Corynebacterineae* species. It has been validated as a promising druggable target and knowledge of its structure is essential to speed up drug discovery to fight against tuberculosis.

We report here a quasi-atomic model of Pks13 obtained using small angle X-ray scattering of the entire protein and various molecular subspecies combined with known high-resolution structures of Pks13 domains or structural homologues. As a comparison, the low-resolution structures of two other mycobacterial polyketide synthases, Mas and PpsA from *Mycobacterium bovis* BCG, are also presented. This study highlights a monomeric and elongated state of the enzyme with the apo and holo forms being identical at the resolution probed. Interestingly, dimerization of the enzyme occurs following the loading of a C16-CoA substrate analogue onto the acyltransferase domain. Catalytic domains are segregated into two parts, which correspond to the condensation reaction per se and to the release of the product, a pivot for the enzyme flexibility being at the interface. The two acyl carrier protein domains are found at opposite sides of the ketosynthase domain and display distinct characteristics in terms of flexibility.

The Pks13 model reported here provides the first structural information on the molecular mechanism of this complex enzyme and opens up new perspectives to develop inhibitors that target the interactions with its enzymatic partners or between catalytic domains within Pks13 itself.

C. Bon *et al. Solution structure of the type I polyketide synthase Pks13 from Mycobacterium tuberculosis.* Accepted for publication in BMC Biology.

Novel insights into mycobacterial cell division mechanisms using *C. glutamicum* as a model organism

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The detailed molecular mechanisms by which bacteria divide remain poorly understood even in well studied model organisms, such as *E. coli*. In *Corynebacteriales*, the suborder of *Actinobacteria* that includes *Mycobacterium tuberculosis*, many of the well characterized divisome components are missing from the genomes, likely because they have evolved specific mechanisms linked to their complex cell wall and polar growth mode. We use MS-based interactomics to identify corynebacterial-specific divisome components in the model organism *Corynebacterium glutamicum*, and through extensive bioinformatic and evolutionary analysis chose the most promising candidates that could be part of a conserved ancestral core divisome of *Corynebacteriales*. Here I will present two examples of such complexes. I will describe the activation mechanism of the cell wall endopeptidase RipA through actinobacterial-specific protein-protein interactions and the role of the newly identified GLP-GLPR complex at the heart of the divisome-elongasome transition.

Compartment-specific proximity labeling uncovers the exposure of Type VII ESX secretion system substrates to the mycobacterial periplasm

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The cell envelope of mycobacteria must contain the machinery for critical processes, from nutrient acquisition to lipid transport to protein secretion. This compartment has been extensively interrogated, yet remains replete with poorly defined pathways of murky mechanism. Advances in our understanding are limited in part by persistent experimental needs, including the reliable localization and identification of periplasmic, mycomembrane, and cell surface proteins. We adapted proximity labeling by the engineered peroxidase APEX2 to tag proteins in mycobacteria and validated the accuracy and specificity of this method for subcellular compartments. In identifying the cell wall proteome labeled by APEX2 in *M. tuberculosis*, we detected substrates of at least two Type VII ESX secretion systems, providing the first experimental evidence that these proteins are exposed to the periplasmic environment. This result has key implications for the mechanism of Type VII secretion through the mycobacterial cell wall and our approach provides immediate opportunities for probing how export to the periplasm depends on the ESX core complex.

Structure and dynamics of a mycobacterial type VII secretion system

T. Marlovits

Institute of Structural and Systems Biology, Hamburg, Germany

T7 secretion systems (T7SS) are specialised protein transport systems central to the virulence of M. tuberculosis, and are also crucial for nutrient and metabolic transport across the mycobacterial cell envelope. Here, I will discuss the molecular details of the intact T7SS inner-membrane complex from M. tuberculosis providing evidence for assembly, complex stabilization and substrate transport.

Human Genetics of tuberculosis : the TYK2 story

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The pathogenesis of tuberculosis (TB) remains poorly understood, as no more than 5–10% of individuals infected with *Mycobacterium tuberculosis* go on developing clinical disease. The contribution of human genetics to TB pathogenesis has been amply documented by means of classic genetics since the turn of the twentieth century. Over the last 20 years, following-up on the study of Mendelian susceptibility to mycobacterial disease (MSMD), monogenic disorders have been found to underlie TB in some patients. Rare inborn errors of immunity, such as autosomal recessive, complete IL-12Rβ1 and TYK2 deficiencies, impairing the IL-12- and IL-23-dependent induction of IFN-γ, were initially identified in a few patients. More recently, homozygosity for a common variant of *TYK2* (P1104A) that selectively disrupts cellular responses to IL-23 was found in two cohorts of TB patients. It shows high penetrance in areas endemic for TB and appears to be responsible for about 1% of TB cases in populations of European descent. The marked decrease in frequency of the TYK2 P1104 variant after the Bronze age in Europe is also strongly suggestive of negative selection. Recent reports of additional TYK2 variants further indicate that impaired IL-23 dependent induction of IFN-γ underlies mycobacterial disease in patients with inherited TYK2 deficiency. Both rare and common genetic etiologies of TB affect IFN-γ immunity, providing a rationale for novel preventive and therapeutic approaches for TB control, including the use of recombinant IFN-γ.

Systems immunomonitoring to support development of new treatments and vaccines for TB

D. Duffy
Institut Pasteur, Paris, France

Tuberculosis remains a major global public health challenge with an urgent need for improved vaccines and treatments. Clinical studies to develop such new approaches can benefit from immunomonitoring that identifies robust correlates of protection and successful treatment responses. We propose the use of standardized whole blood stimulation systems with multi-functional readouts that show more robust results over conventional approaches. As a proof of concept we demonstrated their improved ability to classify active TB disease from latent infection. Exploratory analysis combining digital ELISA and transcriptomic data sets showed that LTBI donors with high IFNγ clustered with patients with active TB, suggesting the possibility to identify subclinical disease. In parallel the integration of multiple phenotypic readouts allowed us to identify a novel immune-metabolic association between pregnane steroids, the PPARγ pathway and elevated plasma IL-1ra in TB disease. We are now testing the ability of this approach to predict early response to antibiotic treatment. Finally, we aim to integrate results from population-based studies that are identifying major determinants of immune variability to support future precision immunology studies.

Transcriptional signatures reveal the immune response underlying progression and pathogenesis in tuberculosis

A. O'Garra
The Francis Crick Institute, London, United Kingdom

Tuberculosis remains a major cause of mortality in infectious diseases, with most people infected with *M. tuberculosis* remaining asymptomatic, termed latent, and only a fraction progressing to active tuberculosis disease. We identified a whole blood transcript signature for active tuberculosis correlating with the radiological extent of disease, which was dominated by a neutrophil-driven interferon (IFN)-inducible gene profile, consisting of both IFN-g and Type I IFNαβ signaling and suggested a hitherto under-appreciated role of Type I IFNαβ signalling in human tuberculosis pathogenesis. Our recent findings show that the modular blood transcriptional tuberculosis signature in susceptible mice infected with a clinical isolate of *M. tuberculosis* resembles that of active human tuberculosis disease, with dominance of a type I IFN response, which we show contributes to neutrophil activation, lung pathology and infection burden. This now provides a tractable model to further study targets and mechanisms underlying TB pathogenesis. In our past studies we defined a blood transcriptional signature of active TB, absent in the majority of latently exposed asymptomatic individuals and healthy controls. Building upon our findings of the blood transcriptional signature of human active TB findings our recent TB research has provided mRNA expression tools to support early diagnostics and treatment monitoring of TB, which will now be developed for clinical use by our collaborators. With clinical collaborators in Leicester, UK, we have now demonstrated heterogeneity in the blood transcriptome of a cohort of recent TB contacts where a small proportion expressed a persistent TB signature and subsequently progressed to active TB disease. These findings form the basis our future studies to determine the early responses in the airways of contacts with recently acquired *M.tuberculosis* infection who control the infection or progress to active TB disease in TB resistance or progression in humans and in experimental TB models. We will define early airway events in TB resistant and susceptible mice to identify additional targets that parallel the host factors associated with outcome in human TB, so as to test and investigate mechanisms of outcome to infection.

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Using Biomarkers to Predict TB Treatment Duration

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Stellenbosch University, Stellenbosch, South Africa

Drugs hug, bugs chug - genetic-chemical synergy in mycobacteria

E. Rubin
Harvard University, Boston, United States

POSTER SESSION 1

Tuesday, September 13, 2022

1.45 pm to 3.30 pm

Genomic epidemiology of *Mycobacterium bovis* in symaptric cattle and badgers from a bovine tuberculosis hotspot

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Background: Animal tuberculosis (bTB) is a costly, epidemiologically complex, multi-host, endemic disease with zoonotic potential. Pathogen whole genome sequencing can improve the resolution of epidemiological tracing. We genome sequenced an exceptional data set of 619 *Mycobacterium bovis* isolates from badgers and cattle in a 100km^2 bTB 'hotspot' over a 30 year period, albeit with some temporal biases. Historical molecular subtyping data permitted the targeting of an endemic pathogen lineage, whose long-term persistence provided an opportunity to study genome epidemiology in detail. To assess whether badger population genetic structure was associated with the spatial distribution of pathogen genetic diversity, we microsatellite genotyped hair samples from 769 badgers trapped in this area.

Results: Eight lineages of *M. bovis* were circulating in the study area, seven of which were likely non-endemic, and imported by cattle movement. The endemic lineage exhibited low genetic diversity with an average inter-isolate genetic distance of 7.6 SNPs (s.d. ± 4.0), consistent with contemporary transmission. Bayesian phylogenetic methods determined an evolutionary rate of 0.30 substitutions per genome per year for this lineage, estimating its emergence 40-50 years before present, while Bayesian Skyline analysis identified significant population expansion of the endemic lineage in the 1990s and again in 2011-2012. The phylogeny revealed distinct sub-lineages, all of which contained isolates from both cattle and badger hosts, indicative of the sharing of closely related strains and inter-species transmission. Phylodynamic methods indicated cattle-to-cattle transmission was the most common dissemination pathway while interspecies transmission was rare and badger-to-badger transmission apparently rarer still. Correspondingly, the presence of significant badger population genetic structure was not associated with the spatial distribution of *M. bovis* genetic diversity.

Conclusions: Our data provided unparalleled detail on the evolutionary history of an endemic *M. bovis* lineage. Findings are consistent with ongoing interspecies transmission in the study area but suggest that badgers may not be major drivers of persistence. The approaches and methods used here may be applicable in other epi-systems in which zoonotic TB is an issue.

Mycobacterium tuberculosis complex NGS made easy: data analysis step-by-step - an educative resource

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NGS has a great potential to improve tuberculosis (TB) diagnostics and surveillance, in particular in the case of drug-resistant TB. The need for expertise guiding NGS implementation in laboratories and the lack of bioinformatic expertise, are main obstacles hindering the implementation into TB programs.

We have conceived a series of publicly available on-line training materials aiming at improving the basic and applied knowledge on NGS technology, targeting TB health practitioners and researchers with limited experience on NGS. Pre-recorded webinars and hands-on bioinformatics tutorials present state-of-art TB-specific solutions for generating and analyzing NGS data. All materials are hosted in Galaxy, an open-source, web-based platform for accessible, reproducible, and transparent computational biological research where bioinformatic pipelines can be used both for training and data analysis purposes even without any programming knowledge.

A first interactive edition was carried out as an asynchronous 5-Day course, where forty participants, mostly laboratory and technical staff, have autonomously followed the training on-line. The learners interacted with a panel of experts via real-time chat and live discussion sessions. This format enabled delivering the same didactic content to trainees with heterogeneous backgrounds and geographic origins. The live sessions have shown that despite the digital nature of the activities, trainees felt motivated and engaged. Learning objectives were attained in at least 50% of the participants as measured by formative assessments.

This resource is freely available for the community, providing training opportunities that lead to the acquisition of skills necessary to bring NGS and bioinformatics closer to high TB burden communities.

Whole genome sequencing for resistance prediction and transmission analysis of *Mycobacterium tuberculosis* complex strains from Namibia

M. Claassens*[1], E. Nepolo[1], V. Dreyer[2], Q. Mokomele[3], G. Van Rooyen[3], N. Ruswa[4], G. Guenther[5], S. Niemann[2]

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Background: Namibia is among the thirty high burden tuberculosis (TB) countries with an estimated incidence of 460 per 100,000 population and around 800 new multidrug resistant (MDR) TB cases per year. Still, data on transmission and evolution of drug-resistant *Mycobacterium tuberculosis* complex (Mtbc) strains are not available.

Methods: Whole genome sequencing data of a convenient sample of 136 rifampicin resistant (RIFr) Mtbc strains obtained in 2016, 2017, and 2018 were used for a phylogenetic classification, resistance prediction and cluster analysis (12 single nucleotide polymorphism threshold) and linked with classical phenotypic drug susceptibility (pDST) data.

Results: High first line drug resistance rates were detected with roughly 50 % of the strains investigated being resistant against all first line drugs. Furthermore, 13 % of the MDR Mtbc strains were already pre-extensively drug resistant (XDR). The cluster rate was high with 74.6 % among MDR, and 85 % among pre-XDR strains and clusters ranging in size from two to 25 isolates (cl22). A significant proportion of strains had borderline low-level resistance mutations e.g., *inhA* promotor mutations or *rpoB* L430P. Accordingly, 25 % of the RIFr strains tested susceptible by pDST. Finally, we determined a potentially new bedaquiline resistance mutation (Rv0678 D88G) occurring in two independent clusters.

Conclusions: High first line resistance rates in line with emerging pre-XDR and likely bedaquiline resistance linked with ongoing recent transmission of MDR Mtbc clones underline the urgent need for the implementation of interventions that allow rapid diagnostics to break MDR-TB transmission chains in the country. A borderline RIFr mutation in the dominant outbreak strain causing discrepancies between phenotypic and genotypic resistance testing may require breakpoint adjustments, but also allow individualized regimens with high dose treatment.

Transmissibility of drug-resistant tuberculosis in a high-endemic setting as explained by bacterial genetic epistasis

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The role that bacterial factors play in the transmission of drug-resistant tuberculosis (DR-TB) remains unclear. It is well established that drug resistance mutations often decrease replicative fitness in bacteria, and that this fitness cost can be alleviated by additional compensatory mutations. However, whether compensatory evolution also increases replicative fitness *in vivo* and, ultimately enhances transmission, is still controversial. In this study, we sequenced all bacterial genomes available from patients routinely diagnosed with rifampicin-resistant tuberculosis in Khayelitsha, Cape Town, South Africa, between 2008, and 2018. We performed Bayesian phylodynamic analysis to delineate transmission of DR-TB and assess host and bacterial factors associated with being a transmitter. Multivariable regression analysis showed multidrug-resistance (versus mono-resistance), bacterial genetic background (lineage 2), the rifampicin resistant mutation rpoB S450L, compensatory mutations and later diagnosis date to be associated with being a transmitter. Interestingly, the transmission benefit provided by the mutation rpoB S450L was dependent on the genetic background in which it evolved. Additionally, we show that compensatory mutations are associated with higher sputum bacterial load, and with the acquisition of drug resistance mutations. Our findings suggest that bacterial factors largely determine the epidemiological success of certain genotypes, and that previous measures of replicative fitness *in vitro* are mirrored *in vivo*, and are linked to bacterial transmissibility.

A cloud-based analytics platform for antimicrobial susceptibility testing of *Mycobacterium tuberculosis* from WGS data

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Globally, the incidence of tuberculosis (TB) has been decreasing since 2005. However, drug-resistant strains of *Mycobacterium tuberculosis (Mtb)* are emerging worldwide, and are often associated with poor treatment outcomes, posing a significant threat to global TB control. Phenotypic drug susceptibility-testing (pDST) is too slow to inform treatment decisions and targeted molecular approaches fail to test for all resistances. Previous studies have shown that such methods can lead to underdiagnosis of drug resistance, resulting in inadequate treatment and higher mortality. These studies also demonstrate the superiority of whole-genome sequencing (WGS) in providing the complete susceptibility profile. As such, clinical laboratories are increasingly adopting WGS for diagnostics and surveillance of *Mtb*, and infectious diseases in general. Despite the promises WGS holds in clinical microbiology diagnostics, bioinformatics data analysis remains a bottleneck, restricting the effective implementation of NGS technologies in clinical settings.

We are developing an intuitive cloud-based analytics platform for predicting resistance to anti-TB drugs from WGS data. By identifying drug resistance-conferring variants and predicting treatment options following WHO guidelines, our platform will guide clinicians towards individualised TB therapies. This ensures patients are treated with sufficiently active drugs, have improved treatment outcomes, and prevents the selection of additional resistances. The platform additionally provides a phylogenetic classification of the strain and identifies transmission clusters and outbreaks. The clinician can inform the platform about treatment outcomes for patients with similar susceptibility profiles, to ultimately better inform patient diagnosis and treatment.

Mtb proves to be an ideal candidate for developing our platform due to its slow growth rate, the clonality of its genome and the high correlation between pDST and genomic variants. In the future, we plan to extend our analytics platform to other pathogens, further contributing to the fight against antimicrobial resistance.

Estimating tuberculosis transmission bottleneck from donor-receptor genomic diversity

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Background: Based on studies of infection via intratracheal injection in animal models, it has been considered that even a single bacillus is capable of infecting and causing tuberculosis. However, whether this is true in clinical settings is unknown. Determining the infection dose and the associated bottleneck in genetic diversity of *M. tuberculosis* is important to interpret genomic epidemiology studies and also to understand the likelihood of transmission of variants associated with drug resistance. Our objective is to estimate the founder population/bottleneck between donor-recipient pairs to understand the transmission dynamics of *M. tuberculosis.*

Methods: We sequenced the whole pathogen genome from 1277 tuberculosis culture-positive cases occurring in the Valencia Region during 2014-2018. We performed clustering analysis using pairwise genetic distances between isolates (≤10 SNPs), identifying 181 transmission clusters including 627 isolates. From the data, we selected 21 donor-recipient pairs based on: epidemiological data, genetic distance (≤2 SNPs), size of the transmission cluster and contact tracing information. We applied the beta-binomial method to estimate the bottleneck, in both versions: approximate -which assumes infinite sequencing depth-; and exact -which considers sequencing depth-.

Results: The estimated range of bottlenecks for the 21 pairs evaluated was 21-166, with a mean of 64 bacilli. We corroborated these ranges in a second, unrelated cohort. We plot the serial interval (as the time elapsed between donor and recipient symptoms) vs bottleneck size; we defined two groups with low (≤1 year) and high (>1 year) serial interval. We observed an inverse and significant relationship between the serial interval and bottleneck (*Wilcoxon rank sum test with continuity correction*, p-value = 0.02).

Conclusions: Our results suggest that natural TB infections are unlikely to be caused by a single *M. tuberculosis* bacillus. Instead, our minimum estimate was 21 (18-25) bacilli to initiate an active tuberculous infection. The serial interval negatively correlates with the bottleneck size suggesting that the lower the number of bacilli transmitted, the longer the time needed to establish the infection.

MEX-TB Genomic epidemiology and functional analysis of *Mycobacterium tuberculosis* strains in Mexico

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Genomic epidemiology of Tuberculosis (TB) has achieved remarkable success in understanding the genetic basis of infection and survival mechanisms of the pathogen *Mycobacterium tuberculosis* (*Mtb*). The increasing number of studies based on whole genome sequencing (WGS) of *Mtb* strains has allowed the creation of well-curated catalogs of variants associated with lineage and drug resistance. However, it is unclear how applicable this knowledge is to *Mtb* isolates from geographical regions with low genomic representativeness, such as Latin America. Here, we provide WGS-based genotyping of 133 *Mtb* clinical isolates from Mexico, including 53 newly sequenced strains, as stated by the global lineage and drug resistance catalogs. According to strain classification, we found a predominant prevalence (96.2%) of Lineage 4 (Euro-American), featuring a uniform distribution of the sub-lineages X-type (33.08%), LAM (22.56%), and Haarlem (21.05%). Regarding drug-resistance prediction, we found that the most frequent variants associated with rifampin resistance were *rpoB*D435V, H445N and L452P, whereas for isoniazid resistance were *ahpC* D73H and *katG* S315T. Interestingly, we obtained low Cohen's kappa coefficient values for all the antibiotics tested, indicating poor concordance between phenotype and genotype. Subsequently, to better understand the genetic basis of these discrepancies, we conducted a functional analysis of the variants at genome-scale by developing a new bioinformatic tool, named FuN-TB. This tool performs a comparative analysis of genomic variants between two or more groups of samples with specific attributes, delivering a functional network that represents the absence/presence of genes containing mutations between the selected groups. In a comparison of groups of samples with a monoresistant phenotype to the five first-line antibiotics, we found several genes with non-synonymous convergent mutations that could be related to global mechanisms of drug resistance. For example, point mutations in the *hpt, etgB* and *ligC* genes, which are involved in prodrug metabolism, virulence, and DNA repair pathways, converged in at least three groups of monoresistant samples. These results suggest that the widespread application of functional genomics approaches to thousands of Latin American *Mtb* genomes will provide new drug resistance screening targets for the design of effective diagnostic tools and accurate TB treatment regimens.

Constitutive heterogeneity in *Mycobacterium tuberculosis* clinical strains' genome structure recapitulates evolutionarily fixed events

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Bacterial populations are known to be heterogeneous yet are typically represented as a single consensus genome. While small variant heterogeneity is frequently investigated, heterogeneous structural variants (SVs) have received less scrutiny. Here, we develop a framework to discern heterogeneous structural variation from long-read sequencing data. Applying this framework to 137 *Mycobacterium tuberculosis* complex (MTBC) clinical isolates identified high-confidence heterogeneous SVs present in multiple strains. Several of these SVs interrupt cis-regulatory and coding regions of clinically noteworthy genes. These include a proposed vaccine component and diagnostic marker for active TB, a cell envelope constituent, and important virulence mediators. Most heterogeneous SVs recapitulated known MTBC macroevolutionary events on a microevolutionary timescale, suggesting the evolution of *M. tuberculosis* genome structure exhibits recurrent properties. These findings spotlight homologous recombination and IS-transposition as influential determinants of MTBC evolution that constitutively generate structural heterogeneity to confer a more plastic genome structure than previously appreciated.

Whole-genome sequencing and phylogenetic analysis in clinical isolates of *Mycobacterium tuberculosis* resistant to first- and second-line drugs

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Background: Tuberculosis is a potentially serious infectious due to increased cases of multi (MDR) and extreme drug resistance (XDR) worldwide, which remains the challenge for tuberculosis control. In Ecuador, genomic studies related to lineage and resistance characterization of *Mycobacterium tuberculosis* by sequencing are limited. This study assesses the characterization of lineage and molecular resistance profile in clinical isolates of *Mycobacterium tuberculosis* through whole-genome sequencing (WGS).

Methods: DNA was extracted using the CTAB method from 24 isolates resistant, microbial characterized previously. Genomes were sequenced on the MiniSeq platform with High Output Reagent Kit (2 x 150 bp) following tagmentation-based library according to the manufacturer's instructions. The sequencing reads were mapped to the *M. tuberculosis* H37Rv reference genome, and associated resistance and lineage mutations were identified by TB-Profiler.

Results: From 24 microbiological MDR isolates, 2 pre-MDR, 16 MDR, 4 pre-XDR, and 2 XDR were identified by genomic analysis of mutation present in associated resistance gene; the principal family was LAM (53,88%), and clades X, T and S were identified. In pre-XDR and XDR isolates the LAM family (66,6%) and X-clade (33,33%) were distributed. 105 associated mutations were identified being the more frequent the missense mutation (82.86%) in gene rpoB (23.81%), katG (18.10%), embB (14.29%), and pncA (12.38%) associated with resistance to rifampicin, isoniazid, ethambutol, and pyrazinamide, respectively. 12.38% of mutations were in a gene related to second-line drugs resistance, 46.15% in non-coding, 30.77% in the frameshift, and 23.08% in missense regions.

Conclusion: This study highlights the necessity to apply WGS to characterize mutations, that can cause serious problems generating MDR and XDR strains, and lineage as a part of routine surveillance programs in Ecuador, due to the presence of different lineages circulating, and mutations associated with first and second-line antibiotic resistance genes between tuberculosis patients.

Modern history of epidemic MDR strains of *Mycobacterium tuberculosis* complex lineage 2 and lineage 4 in Central Asia

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The emergence and spread of multidrug resistant (MDR) strains of *Mycobacterium tuberculosis* (Mtbc) is a major health and economic threat, especially in Central Asian countries where MDR Mtbc account for more than 20% of new infections. This region has recently experienced several MDR Mtbc outbreaks linked to the emergence and rapid expansion of modern lineage 2 "Beijing" strains. These strains are characterized by high resistance rates and rapid spread compared to strains of other major Mtbc lineages, such as lineage 4.

The meta-analysis presented here combines 20 years of sampling of Mtbc strains obtained through collaborations from the five Central Asian republics: Uzbekistan, Turkmenistan, Tajikistan, Kazakhstan and Kyrgyzstan. The dataset includes 2247 high quality whole genome sequences from 2001 to 2019. Of these, 1001 strains were MDR, 229 strains were pre-XDR and 15 were XDR. Genomic transmission analysis has allowed us to establish an overview of the recent spread and evolution of MDR Mtbc strains. Phylogenetic classification of the strains showed that L2 strains are dominant (n=1611), followed by L4 strains (n=592). Bayesian reconstructions of the effective population through time correlate recent crises, such as the 1992-1997 civil war in Tajikistan and the 2008 energy crisis in Central Asia, with the rapid expansion of modern L2 epidemic clones, e.g. "W148" and "Central Asian Outbreak", in three overlapping waves. Inferences from ancestral geographic states reveal that L2 has experienced more inter-country movement than L4, consistent with more recent local implementation and efficient spread of the former.

Although sampling bias must be taken into account, the results point to a recent history of replacement of lineage 4 TB strains by the more epidemically successful modern lineage 2 clones in Central Asia.

Unraveling the role smallRNAs in drug resistance in *Mycobacterium tuberculosis*

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Shreds of evidence on the role of small non-coding RNAs (sRNAs) in drug resistance (DR) development are nowadays available. In this study, we applied different computational and biological strategies to unravel putatively new mechanisms of DR and their regulation in *M. tuberculosis* (MTB).

WGS data and Minimum Inhibitory Concentration (MIC) to 13 anti-TB drugs from more than 8000 clinical isolates were made available by the CRyPTIC Consortium. We performed a genome-wide association study (GWAS) to identify mutations relevant for MIC increase mapping in 575 previously identified MTB sRNAs. A phylogenetic analysis was also performed to evaluate the distribution of the mutations among MTB lineages and was used to complete the list of sRNAs putatively relevant for DR. sRNA *in silico* target prediction was performed by IntaRNA3 and sRNARFTarget and refined using a neural network model previously trained to remove false predictions. Finally, Gene Ontology (GO) was performed on the top-40 predicted targets. Overexpression of *ncRv0842c* in MTB isolates (L1, L5, L4, and L6) and reference strain H37Rv was achieved using a pMV261 plasmid backbone. qPCR was carried out to assess the expression of sRNA and its target in both basal and rifampicin (RIF)-induced stress. RIF MIC was determined by microplate AlamarBlue assay (MABA).

GWAS reported the association between 9 mutated sRNAs and increased MIC for 7 different drugs. For each drug, GO produced significant terms associated with biological processes related to DR mechanisms. The phylogenetic analysis highlighted that the sRNA *ncRv0842c* (cis-encoded to the efflux pump *Rv0842*, involved in RIF tolerance) is expressed only in modern lineages. Experimentally, we demonstrated that, under RIF-stress, *ncRv0842c* is down-regulated in L4 isolates ($p<0.001$; $\beta=2.5$, 95% Cis 1.7;3.3). Overexpression of the sRNA caused a MIC reduction to RIF. As expected, a MIC reduction was not observed in ancient strains, lacking *ncRv0842c.* The validation of the other candidates is still ongoing as well as a target prediction that considers the sRNA mutations list.

This is the first study revealing the role of sRNAs in mechanisms related to drug tolerance in MTB, and putatively involved in DR development.

Holistic evidence for accelerated mutation rate and increased adaptive landscape in strains belonging to the lineage 2 of *M. tuberculosis*

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The *M. tuberculosis* lineage 2 is one of the most widely dispersed lineages globally and is particularly associated with the spread of multi-resistant strains in Eurasia. Yet, the reasons behind the success of Lineage 2 are only partially understood. In the present study, we wanted to develop an integrative approach that combines predictive simulations, sequencing data and laboratory fluctuation assays to answer this question.

One of our first objectives was to determine whether Bayesian phylogenomic approaches (BEAST algorithm) were able to distinguish contrasting mutation rates between lineages, or even to detect a potential acceleration of mutation rates along branches during an outbreak, based on the mutational parameters presented by Ford et al. (2015). According to our predictive simulations on SLIM, mimicking population sizes and demographic parameters sticking to recent outbreaks observed on the L2 and L4 lines, Bayesian inferences are able to detect relatively fine differences in mutation rates at the population level (around 10%). Furthermore, forward simulations and random sampling of sharply expanding *M. tuberculosis* populations under a constant mutation rate of 1×10^{-7} substitutions/nucleotide/year did not show any signal of accelerated mutation rates under the Beast analyses (tip-dated trees), excluding therefore a potential confounding effect of the demographic component. Once reassured on this point, we compared two recent clones responsible for outbreaks belonging respectively to the L2 and L4 lineage (W148 and Haarlem/Hamburg) and presenting the characteristics of measurably evolving populations. Interestingly, the inferred mutation rate estimates confirmed that L2 outbreak strains evolve faster ($P < 0.01$) than L4 outbreak strains. Even more surprising, was the fact that the L2 strains displayed a significant pattern of mutation rate acceleration along the branches, and this at a scale of only 30 years. Last but not least, our fluctuation assays showed that Lineage 2 modern Central Asia strains acquire rifampicin resistance approximately twice as fast as L2 ancestral strains or Lineage 4 strains.

Here, we demonstrate that derived L2 strains present substantially higher mutation rates than those of other lineages, that are likely to increase their adaptive landscape, as well as their propensity to acquire resistance/compensatory mutations.

Back-to-Africa introductions of *Mycobacterium tuberculosis* as the only cause of tuberculosis in Dar es Salaam, Tanzania

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Dar es Salaam is a high-endemic tuberculosis (TB) setting in East Africa and has the highest TB notification rate in Tanzania. We investigated the TB epidemic in the Temeke District of Dar es Salaam by analyzing 1,082 genomes of *Mycobacterium tuberculosis (Mtb)* isolated from patients and their clinical data collected between 2013 and 2019. Our results revealed that four *Mtb* lineages circulate in Dar es Salaam, whereby L3 (47%) was the most abundant followed by L4 (31%), L1 (14%), and L2 (8%). Placing the Dar es Salaam genomes into a global collection of genomes (n = 10,733) revealed that the TB epidemic in Dar es Salaam is exclusively driven by *Mtb* strains that have been introduced from other parts of the world during the last 300 years. Some introductions spread more successfully than others and we found L3.1.1 to be the most prevalent sublineage resulting from a single introduction. Molecular dating analyses revealed that L3.1.1 was introduced earlier than other sublineages, however in general we did not find a correlation between the date of introduction and the number of cases generated. Investigating the transmission rates suggested L2.2.1 and L3.1.1 having increased transmission rates compared to other abundant sublineages.

In summary, we found a high diversity within *Mtb* strains circulating in Dar es Salaam resulting from early and late introductions and the predominance of L3.1.1 being a composite of an early introduction and enhanced transmission rate.

MazF-mt9 toxin mediates upregulation of multiple pathways important for *Mycobacterium tuberculosis* virulence

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B.L. Abbadi and J. Puffal contributed equally to this work

Emerging studies on the molecular underpinnings of bacterial stress survival generally point to a major role for toxin-antitoxin (TA) systems. The *M. tuberculosis* genome contains ~90 TA systems, yet the roles of individual TA system toxins in stress survival are poorly understood. We had previously demonstrated that the *M. tuberculosis* MazF-mt9 endoribonuclease toxin exclusively inactivates $tRNA^{Lys-UUU}$ by cleavage at a single site within its anticodon. Depletion of $tRNA^{Lys-UUU}$ then results in genome-wide ribosome stalling and mRNA cleavage at cognate Lys AAA codons, representing a novel mode for remodeling mycobacterial physiology. Here we performed RNA-seq and quantitative mass spectrometry to determine how MazF-mt9 targeting of this single tRNA followed by codon-specific ribosome stalling reprogrammed *M. tuberculosis* to survive stresses encountered during infection. The most striking and consistent alteration mediated by MazF-mt9 was upregulation of the mRNAs and enzymes they encode involved in biosynthesis of phthiocerol dimycocerosate lipid (PDIM). PDIMs are involved in recruitment of permissive macrophages while evading recruitment of microbicidal macrophages. PDIMs achieve this by masking the pathogen-associated molecular patterns (PAMPs) to suppress the toll-like receptor-dependent recruitment of microbicidal macrophages. These genome-scale findings were further validated by direct quantification of radiolabeled PDIMs in *M. tuberculosis* cells with and without MazF expression. We also detected upregulation of nearly all enzymes involved in mycolic acid synthesis upon MazF-mt9 expression. Mycolic acids make *M. tuberculosis* cells more impermeable, protect them from dehydration, limit the efficacy of antibiotics used for TB treatment, and enable growth inside macrophages. Finally, MazF-mt9 mediated upregulation of transcripts encoding the mycobacterial master regulator of intrinsic antibiotic resistance WhiB7 and its regulon. Therefore, this toxin-mediated, codon-driven shift in the transcriptome and proteome appears to preferentially activate a subset of pathways crucial for *M. tuberculosis* survival upon infection and treatment with antibiotics.

Nitric oxide-inducible Fe-S cluster biogenesis enables *Mycobacterium tuberculosis* to persist in mice

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The persistence of*Mycobacterium tuberculosis*(*Mtb*) is a major problem in managing tuberculosis (TB). Host-generated nitric oxide (NO) is perceived as one of the signals by *Mtb*to reprogram metabolism and respiration for persistence. However, the mechanisms involved in NO sensing and reorganizing *Mtb'*sphysiology are not fully understood. Since NO damages, Fe-S clusters of essential enzymes, the mechanism(s) involved in regulating iron-sulfur (Fe-S) cluster biogenesis could help Mtb to persist in host tissues. Here, we show that a transcription factor SufR (*Rv1460*) senses NO via its 4Fe-4S cluster and promotes persistence of *Mtb*by mobilizing the Fe-S cluster biogenesis system; *suf*operon (*Rv1460-Rv1466*). Analysis of anaerobically purified SufR by UV-visible spectroscopy, circular dichroism, and iron-sulfide estimation confirms the presence of a 4Fe-4S cluster. Atmospheric $O_2$and H_2O_2gradually degrade the 4Fe-4S cluster of SufR. Furthermore, electron paramagnetic resonance (EPR) analysis demonstrates that NO directly targets SufR 4Fe-4S cluster by forming a protein-bound dinitrosyl-iron-dithiol complex. DNase I footprinting, gel-shift, and *in vitro*transcription assays confirm that SufR directly regulates the expression of the *suf* operon in response to NO. Consistent with this, RNA-sequencing of *MtbΔsufR*demonstrates deregulation of the suf operon under NO stress. Strikingly, NO inflicted irreversible damage upon Fe-S clusters to exhaust respiratory and redox buffering capacity of *MtbΔsufR*. Lastly, *MtbΔsufR*failed to recover from a NO-induced non-growing state and displayed persistence defect inside immune-activated macrophages and murine lungs in a NO-dependent manner. Data suggest that SufR is a sensor of NO that supports persistence by reprogramming Fe-S cluster metabolism and bioenergetics.

Uncovering hidden messages within the *Mycobacterium tuberculosis* genome through toxin-mediated ribosome stalling

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The *M. tuberculosis* endoribonuclease toxin, VapC4, specifically cleaves and inactivates $tRNA^{Cys}$. This leads to depletion of the pool of $tRNA^{Cys}$ followed by highly selective ribosome stalling at Cys codons within actively translating mRNAs. This observation enabled application of codon-specific ribosome stalling as a novel tool for reliable detection of new *M. tuberculosis* ORFs. Upon genome mapping of the hundreds of transcripts harboring Cys-stalled ribosomes we unmasked ~100 unannotated ORFs, of which 75% are <100 amino acids and 50% are small ORFs (sORFs) encoding Cys-rich proteins ≤50 amino acids. Even though these small proteins are notoriously difficult to detect by mass spectrometry because they contain few, if any, protease cleavage sites and are often not abundant enough for detection, we identified 18% of these unannotated ORFs in *M. tuberculosis* mass spectrometry datasets. Although we have recently demonstrated that a few of these sORFs appear to function as Cys-responsive attenuators to control translation of downstream genes, other unannotated ORFs encode stable Cys-containing proteins that map to a variety of locations: immediately before a gene, upstream of a gene in the opposite orientation, overlapping a gene in the opposite orientation or overlapping a gene in the same orientation. The sequences of several of these ORFs also reveal functional clues, e.g. some contain zinc-binding motifs and four unannotated sORFs encode novel EsxB-like proteins. Overall, our ability to detect ribosome stalling within actively translated transcripts suggests that the *M. tuberculosis* genome has adapted to expand its protein coding potential by engaging an abundance of unconventional ORFs.

How epistatic interactions of drug-resistance mutations shape the *M. tuberculosis* proteome

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Antimicrobial resistance (AMR) in *Mycobacterium tuberculosis* (*Mtb*) accounts for 12% of the 1.5 million annual deaths due to tuberculosis (TB). Yet, little is known on the factors shaping the within-host evolution of AMR in *Mtb*. Rifampicin (RIF) and fluoroquinolones (FQ) are key drugs in the treatment of TB, the latter as part of the multidrug-resistant TB treatment regimen. Resistance to RIF occurs through mutations in *rpoB,* encoding the β-subunit of RNA polymerase and resistance to FQ is mediated by mutations in *gyrA* and *gyrB* that encode subunits of DNA gyrase. In absence of drug, AMR often causes a reduction in bacterial fitness. Moreover, drug tolerance seems to facilitate the development of AMR, possibly through upregulation of efflux pumps.

Previously, we reported a positive correlation between proteome dysregulation and the fitness cost caused by one particular RIF resistance mutation in *Mtb*. In this project, we expand this work and study the effects of additional AMR mutations on their own, as well as in combination, to explore mutation-specific and potential epistatic effects on the proteome composition of drug-resistant *Mtb*. Specifically, we have evolved high and low fitness *rpoB* and *gyrA* single- and double-mutants in different clinical *Mtb* strains and are currently characterizing them using proteomics and various fitness assays. The results of this work lead to a better understanding of the impact of AMR on the physiology of *Mtb*, strengthening future treatment regimens.

Unveiling the effect of rifampicin selective pressure on the evolution of the *Mycobacterium smegmatis* genome

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Rifampicin is the most important first-line antibiotic against tuberculosis (TB), a disease that today remains one of the top causes of death worldwide. Rifampicin-resistant and multidrug-resistant TB constitute a major global public health problem that makes the control of the disease more difficult. Thus, studies to understand antibiotic resistance mechanisms in *Mycobacterium tuberculosis* and to develop new treatments are crucial. With the aim of exploring the effect of rifampicin on mutation rate and studying the evolutionary adaptation to this antibiotic in mycobacteria, in this work we have performed a mutation accumulation (MA) assay in presence of increasing concentrations of rifampicin in a wild type and a hypermutator Δ*nucS* strain of the nonpathogenic model species *Mycobacterium smegmatis*. Genomic DNA of the MA lines was extracted and sequenced by whole genome sequencing (WGS). When compared with a previous MA experiment in absence of antibiotic, we determined that the treatment with rifampicin generated an increase in mutation rate, more marked in the wild type strain, where the mutation rate was doubled. Moreover, by sequencing the *rpoB* gene and analyzing the rifampicin resistant level of the MA lines during the different weeks of evolution, we observed that the absence of *nucS* accelerated the acquisition of *rpoB* mutations and led to higher levels of resistance. Lastly, the use of the *nucS*-deficient strain has proved to be helpful to search for new *rpoB*-independent mechanisms involved in rifampicin resistance, being remarkable the presence of mutations in several genes encoding transmembrane transport proteins and metabolic enzymes. This work is the first one exploring the total mutations of the genome by MA/WGS under rifampicin selective pressure in a wild type mycobacterium and its hypermutator derivative, putting the focus on mutation rate and the evolutionary trajectory to drug resistance in mycobacteria.

Gene evolutionary trajectories in *Mycobacterium tuberculosis* reveal temporal signs of selection

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Genetic differences between different *Mycobacterium tuberculosis* complex (MTBC) strains are likely associated with different disease and epidemiological phenotypes. Said differences usually emerge through de novo mutations and are maintained or discarded by a balance of evolutionary forces which includes different types of selection and random population processes. Purifying selection is thought to be weak in MTBC but dominant. Only around 10% of the genes have evidence of genetic drift or positive selection, the latter mainly associated with drug resistance. Using a dataset of ~5,000 strains representing global MTBC diversity and a methodology that reconstructs the evolutionary trajectory of each gene since the emergence of the MTBC, we have determined the action of past and present selective forces through time, for every single gene.

- Almost **half of the genes** seem to have been under **positive selection and/or genetic drift** at some point in time, in contrast with previous estimates of 10% or less
- Temporal signals identify genes under **positive selection in the past but highly conserved in the present**. This includes **epitopes** that tend to accumulate older mutations, suggesting very early adaptation to host populations.
- Temporal signals identify genes that were **conserved in the past but under positive selection in the present**. Beyond drug-resistant genes, we detected several sensor proteins of **two-component systems** and **toxin-antitoxin systems**.
- When applied to an enriched drug resistance dataset from high-burden countries our approach correctly identifies **changing selection patterns linked to first-line drug use** and reveals **candidate genes** associated with resistance to **second-line drugs**. We functionally validated one of these genes, **Rv1830**

In conclusion, our novel approach allows to incorporate the joint analysis of past and present evolutionary dynamics. Our results reveal hidden signals of the action of evolutionary forces and can be adapted to identify genes involved in different selective pressures.

Mycobacteria naturally form viable wall-deficient cells that are undetectable by conventional diagnostics

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The cell wall is considered essential for most bacteria and the enzymes involved in cell wall synthesis are therefore among the prime targets of effective antibiotics. Considering the importance of the cell wall, it is surprising that under specific stressful conditions some bacteria transiently shed their cell wall to form cell wall-deficient cells. These cells are insensitive to cell wall-targeting antibiotics, are more competent for DNA uptake and can revert to their walled state when the stressful conditions have ended. Recent observations suggest a link to chronic infections, during which such wall-deficient *Escherichia coli* cells could be isolated from clinical recurrent urinary tract infection samples. This raises the question whether similar cells are also formed by other pathogens, such as mycobacteria, responsible for devastating diseases such as Tuberculosis and leprosy. Here we show that a wide range of mycobacterial species, including *Mycolicibacterium smegmatis, Mycobacterium avium* and mycobacterial clinical isolates, are able to naturally form cell wall-deficient cells and that this formation is stimulated by the presence of cell wall-targeting agents. Confocal microscopy and cryo-transmission electron microscopy confirm that these cells contain DNA but lack their cell wall. We furthermore show that these cells are viable and can revert to a walled state. Importantly, conventional diagnostic media and stains used for detection of mycobacteria do not sustain these cells, perhaps indicating that such cells have been largely overlooked in clinical settings.

Mycobacterium tuberculosis cysteine desulfurase (IscS) coordinates redox balance, central metabolism, and bioenergetics for persistence *in vivo*

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Mycobacterium tuberculosis adapts and persists in response to host-generated reactive oxygen species (ROS) and nitrogen intermediates (RNIs) during infection. Since Fe-S cluster-containing proteins are the most sensitive targets of ROS and RNIs, Fe-S cluster biogenesis and repair mechanisms are likely to be important in this human pathogen. Fe-S clusters are generally assembled by multiple proteins belonging to SUF and/or ISC systems in bacteria. While *Mtb* expresses a complete SUF system comprising seven proteins (Rv1460-Rv1466), the ISC pathway is downsized to only one protein- cysteine desulfurase (Rv3025c; IscS). Surprisingly, IscS can assemble Fe-S clusters on aconitase and succinate dehydrogenase without any auxiliary proteins *in vitro* and protects *Mtb* from hydrogen peroxide (H_2O_2). Because Fe-S cluster proteins are essential for central metabolism, respiration, antibiotic resistance, and persistence of *Mtb*, we explored the contribution of IscS in these processes. Of particular interest to us is to examine the crosstalk between SUF and ISC systems in *Mtb*. An IscS-deficient strain (*MtbΔiscS*) showed defective growth under aerobic conditions. Using multiple biochemical and genetic techniques, we confirmed that the aerobic growth defect was associated with the accumulation of ROS and labile Fe in *MtbΔiscS*. Contrary to our expectation, treatment of *MtbΔiscS* with a ROS scavenger (thiourea), an antioxidant (N-acetylcysteine), and Fe-chelator (bi-pyridyl) did not rescue aerobic growth defect, but rather survival of the mutant further declined. Metabolomics and Seahorse XF bioenergetics assay revealed a significant downregulation of glycolysis, PPP, TCA cycle, and respiration in *MtbΔiscS.* RNA sequencing further confirmed the suppression of central carbon metabolism, respiration, and DosR regulon pathways in *MtbΔiscS*. *MtbΔiscS* displayed exceptional sensitivity to oxidants but not with the nitric oxide donor. Interestingly, while the survival of the mutant was severely attenuated in macrophages, the mutant showed a hypervirulent phenotype in mice. The increased growth of *MtbΔiscS* in mouse lungs was associated with a compensatory increase in the expression of the SUF system. Reducing the expression of SufS in *MtbΔiscS* to wild-type *Mtb* levels abolished the hypervirulent phenotype of the mutant. Our study provides an elegant example of how coordinated regulation of SUF and IscS systems is necessary to achieve persistence in *Mtb*.

Proteomic studies to deciphering the physiological role of Rv2577 phosphatase from Mycobacterium tuberculosis

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Mycobacterium tuberculosis (Mtb) is the causative agent of human Tuberculosis (TB), an airborne disease, endemic in Argentine and Latinamerica and the second leading cause of death worldwide, after COVID-19. In order to control the pathogen, several virulence factors have been proposed as new targets of drug action such as the phosphatases PtpA, PtpB, SapM. Its biological function allows inhibiting the microbicide phage-lysosome fusion, thus favoring the persistence and survival of the bacteria in the macrophage. Rv2577 is a new *Mtb* virulence factor, with dual phosphatase and phosphodiesterase activity, belongs to the metallophosphoesterase (MPE) superfamily of proteins sharing their catalytic site and the typical tyrosine for the phosphatase activity. The objective of this work is to explore the physiological role of Rv2577 phosphatase through shotgun proteomic studies, protein-protein interactions (PPI) and lipids analysis. We analyzed the *M. smegmatis´s* proteome when overexpreewsed the Rv2577wt or the Rv2577-Y220A, an inactive variant of the enzyme, using the empty bacterium (*Msmeg*) as control. Nano-HPLC- MS/MS analysis following a spectrum studies with PatternLab V revealed the 53% of the proteome by Maximun parsimony. The up and down-repressented proteins between Rv2577wt vs Rv2577-Y220A conditions which were equal observed in the Rv2577wt vs Control condition were identified and a PPI performed as STRING network by Cytoscape. In present of Rv2577wt phosphatase, the network revealed the AccD3 protein (Rv0904c) as central node in connection with in lipid metabolism related proteins (i.e. Icl, FadE26, PimB). Secondly, in the presence of the Rv2577-Y220 inactive enzyme, a network centred in NadE (Rv2438c) and redox and stress related proteins was obtained. Then, the lipids pattern in *Msmeg*, the bacterium overexpressing the Rv2577wt or the Rv2577-Y220A inactive enzyme was analysed. As expected, an increased in TAGs and FAMEs and a decreased of DAGs lipids were observed in present of Rv2577wt. A opposite lipids pattern was observed in the *Mtb* Rv2577 mutant strain shared with *Mtb*wt and complemented strains. In summary, all results together suggest that Rv2577 phosphatase could be related with lipid metabolism. Nevertheless, additional analyses are needed to deepen the putative role of Rv2577 in lipid metabolism and/or in regulatory pathways in mycobateria.

Comparative functional analysis of the genomic architecture of isoniazid resistance in *Mycobacterium tuberculosis* reveals lineage-specific differences

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Genetic background can significantly change the effect of mutations on the bacterial phenotype, including antibiotic resistance mutations. Here, we have studied the influence of genetic background on the genomic architecture of isoniazid resistance in *M. tuberculosis* using genomewide insertion mutagenesis on representatives of lineages 1 (L1), 2 (L2), and 4 (L4). We exposed the saturated insertion-mutant pools to isoniazid and determined resistance-associated regions using transposon sequencing.

We found significant similarities, with a total of 65 totally concordant regions and ~120 additional genomic regions showing similar resistance phenotypes across all lineages. Resistance regions shared by all three lineages were enriched in energy and redox metabolism genes, highlighting the central role of redox balance in the mode of action of isoniazid. In contrast, no enrichment was found for cell envelope or cell wall biosynthesis genes, which are both normally keystones of the intrinsic resistome.

One key finding was that the genomic architecture of L2 was markedly different from those of L1 and L4, contrary to what we would expect based on phylogenetic similarity. This points to the existence of a different adaptative landscape with respect to isoniazid resistance for L2 compared to L1 and L4. Additionally, regions associated with isoniazid resistance in L2 were enriched in genes involved in replication, transcription, and DNA repair, suggesting an enhanced ability to acquire resistance mutations through an increased mutation rate.

In sum, the genomic architecture of isoniazid resistance has a clear common component across different lineages that is centered on redox metabolism. However, there are significant differences that could explain observed disparities in the acquisition of antibiotic resistance in natural populations.

Nitric oxide treatment induces Resuscitation promoting factor (RPF)-dependency in Mycobacterium tuberculosis

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Tuberculosis (TB) killed ~1.4 million people in 2019 and despite the recent advances in development of effective regimens, TB treatment failure and relapse remain a global challenge. Resuscitation-promoting factor (RPF)-dependent bacteria of *Mycobacterium tuberculosis* (Mtb) are detected in TB patients in high abundance and linked to TB relapse in mice. RPF-dependent Mtb are formed during infection, presumably in response to host-imposed stresses. We hypothesise that nitric oxide (NO), produced by the host cells, can induce RPF-dependency in Mtb. In this study, the NO donor 3-cyano-5-nitropyridin-2-yl diethyldithiocarbamate was used to test this hypothesis. The NO-treated Mtb were enumerated using colony forming unit (CFU) and most probable number assays in 7H9 media or Mtb culture supernatant containing media (CSN). The data revealed that after 24 hour of NO exposure, the CFU count was significantly reduced ($p<0.05$). This dramatic change in CFU counts was accompanied by the downregulation in the expression of *rpf* A, *rpfB* and *rpfE* genes. Incubation of NO-treated Mtb in 7H9 medium or in CSN resuscitated $1.2x10^5$ cell ml^{-1} and $1.7x10^6$ cell ml^{-1}, respectively. The addition of a specific Rpf inhibitor (3-nitro-4-thiocyanato-phenyl)-phenyl-methanone) abolished the resuscitation of NO donor treated Mtb in both 7H9 and CSN. Lsr2, the global transcriptional regulator, controls expression of RPFs in Mtb and Δ*lsr2* Mtb mutant showed a growth defect on solid media and decreased expression of *rpf* genes. Upon exposure to the NO donor, Δ*lsr2* Mtb strain only resuscitated in CSN, containing RPFs, but failed to recover in 7H9. In conclusion, these results showed that the NO donor induced RPF-dependency in Mtb.

CspA of *Mycobacterium tuberculosis*: autoregulation and expression at low temperatures

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Cold shock proteins (CSPs) are common in almost all bacteria and are essential components that allow bacteria to overcome the negative effects of low temperatures. CSPs are chaperone proteins that are able to non-specifically bind single-stranded nucleic acids. The binding of CSPs to RNA secondary structures destabilizes the latter, which leads to the maintenance of transcription and translation processes, which is one of the most important factors in bacterial adaptation to cold stress. However, the role of CSPs is not limited to response to low temperatures. The CSPs of many bacteria are involved in various processes such as pili and biofilm formation or adaptation to osmotic and acid stresses. CSPs are also virulence factors in some pathogens (*B.subtilis*, *L.monocytogenes*, *S.aureus*).

Mycobacterium tuberculosis has two cold shock-like proteins, CspA (Rv3648c) and CspB (Rv0871). Both proteins are currently poorly understood and their functions are unknown. Here, we show CspA autoregulation loop in *M.tuberculosis* and suggest that its function is not related to the response to cold stress. To assess the CspA expression profile at low temperatures in dynamic we created the *M.smegmatis* strain with the β-Galactosidase gene integrated into the chromosome under the *M.tuberculosis cspA* promoter. The activity of β-Gal remains unchanged when *M. smegmatis* is incubated at 15 oC for 24 hours and only slightly increases by 48 hours. Evaluation of *cspA* transcription by real-time PCR also showed only a slight increase in gene expression. This is a strong argument for the hypothesis that the function of *M.tuberculosis* CspA is not associated with adaptation to low temperatures, since the classical behavior of CSPs under such conditions is a transient increase in expression during the first hours of the acclimation phase. We also showed a decrease in β-Gal activity during episomal expression of MTB *cspA* in a previously created strain. Real-time PCR showed that the amount of the *cspA-lacZ* transcript does not change, which indicates that *cspA* autoregulation occurs precisely at the translational level.

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Carbon source-specific vulnerabilities in *Mycobacterium tuberculosis* identified by genome-wide CRISPR interference screen

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Our ability to comprehensively study the central metabolism in *Mycobacterium tuberculosis* (*M.tb*) has thus far been limited by a lack of techniques that can investigate essential genes on a genome-wide scale. The recent development of CRISPR interference (CRISPRi) in *M.tb* has opened up the possibility for in-depth study of these pathways to identify novel drug targets.

Here we performed a CRISPRi screen to simultaneously interrogate essential and non-essential genes required by *M.tb* when grown on a single carbon source including cholesterol, oleic acid, butyrate, dextrose, and glycerol. Utilizing a genome-scale CRISPRi library containing 96,700 sgRNAs (Bosch et al., 2021) we were able to systematically titrate the expression of nearly all *M.tb* genes. This has permitted us to identify genes that exhibit high vulnerability during growth on in vivo relevant carbon sources, i.e. genes that only require minimal inhibition to cause large fitness cost for the bacteria.

The screen confirmed the role of various previously known genes involved in carbon metabolism including the need for dedicated cholesterol and fatty acid uptake and degradation systems. Beyond this, we were able to identify several novel key players in carbon metabolism, as well as novel links between pathways, including amino acid synthesis and protein degradation, and the metabolism of specific carbon sources.

Deciphering *Mycobacterium tuberculosis* sulfur metabolism

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In view of the dramatically increasing number of drug-resistant strains of *Mycobacterium tuberculosis* (*Mtb*), new approaches to fight tuberculosis are urgently needed. Among them, identifying promising targets to tackle *Mtb* survival in the host requires a better understanding of how *Mtb* adapts its metabolism in the infectious context. On the contrary to carbon or nitrogen metabolisms, *Mtb* sulfur metabolism has been understudied in the context of infection due to the multiplicity of potential sulfur sources. However, sulfur assimilation and the downstream cysteine synthesis are key elements of mycobacteria's metabolism as they are required for the production of mycothiol, the main redox buffer in mycobacteria. Thus, there is a tight link between sulfur acquisition and redox balance control that could be used to increase *Mtb* susceptibility to specific antibiotics whose effects rely on the redox state of the bacteria. To investigate *Mtb* sulfur metabolism during infection, we first seek to identify the sulfur sources that can be used by *Mtb* in the host. To answer this question, NanoSIMS (Secondary Ion Mass Spectrometry) technology is used to follow sulfur sources incorporation into bacteria in infected macrophages and the survival of strains inactivated in sulfur source transporters is studied in the murine model. Downstream of sulfur import, several metabolic pathways leading to cysteine synthesis have been identified in *Mtb*, including redundant pathways that complicate the understanding of sulfur metabolism in this pathogen. By studying single- and multiple-mutated strains, we try to identify the significance of each of these pathways in response to stressful conditions that are encountered by *Mtb* in the host, such as oxidative stress and hypoxia but also to antibiotics treatments. Collectively, our project will lead towards a better understanding of *Mtb* sulfur metabolism in the context of infection and may help identify new promising targets to develop novel antibiotics, or to sensitize *Mtb* to existing antibiotics through unbalancing the bacterial redox state.

Network-centric Functional Dissection of Ser/Thr Kinase Regulation in *Mycobacterium tuberculosis*

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The transmembrane Ser/Thr protein kinases (STPKs) of *Mycobacterium tuberculosis* (Mtb) play pivotal roles in mediating stress response and host-pathogen interactions that impact infection fate and treatment outcome. Deciphering the functional and fitness consequences of STPK regulation should uncover novel biology and reveal novel druggable therapeutic intervention targets. We are developing network-based computational and experimental approaches to systematically characterize the impact of STPK perturbations on Mtb gene expression, metabolism, and survival. These characterizations are based on a previously described library of genetically barcoded STPK mutant strains, each of which can either disrupt or induce the upregulation of a different Mtb STPK. Sequencing for strain-specific genetic barcodes facilitates the quantification of relative abundance of each strain within the population of a pooled culture or infection over time, and thus enables a highly parallelized forward-genetic screen to quantify fitness across STPK perturbations from a single experiment. Fitness phenotypes identified by this screen can be integrated with transcriptional profiling and network analysis of the STPK mutant strains to generate hypotheses of mechanisms underlying altered Mtb growth and survival conveyed by STPK perturbation. Using this approach, we have identified STPKs that convey Mtb growth defects when induced, and modeling suggests that these defects can at least in part be linked to perturbations in Mtb metabolism.

The impact of treatment history on drug tolerance in *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (*Mtb*) which causes tuberculosis (TB) remains globally intractable. An under-researched phenomenon in *Mtb* is drug tolerance, where drug susceptible bacteria can survive extended durations in killing concentrations of bactericidal antibiotics.

Tolerance can contribute to drug resistance evolution under combination drug treatment, which is the standard of care for TB patients. We and others have shown that within-host evolution of *Mtb* under antibiotic therapy can result in compensatory evolution for deleterious drug resistance mutations. Notably, mutations in RNA polymerase subunit C (*rpoC*) compensate for the fitness costs of mutations in RNA polymerase subunit B (*rpoB*), which confer resistance to the crucial frontline drug rifampicin (RIF). The effects of such mutations on future therapies is unknown. We hypothesize that previous drug exposure could manifest mutations that mediate tolerance to drugs used to treat multidrug resistant TB (MDR-TB).

To explore this, we have generated a panel of spontaneous *rpoB* mutants from pan-susceptible clinical isolates, along with laboratory-evolved *rpoB/rpoC* strains. With this panel, we are investigating the impact of RIF resistance and compensation on tolerance to novel anti-TB drugs through comparative time-to-kill assays.

Our data will provide insights into the impact of drug treatment history on the prognosis for new drugs such as bedaquiline and increase our understanding of the role of drug tolerance in *Mtb* evolution.

Investigating novel genetic regulators of *Mycobacterium tuberculosis* cell division

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Mycobacterium tuberculosis is an exceptionally successful human pathogen largely because the bacteria can resist killing by the host immune response and antibiotic treatment. Recently, an analysis of clinical isolates identified mutations in several transcriptional regulators indicating that these genes are under positive selection in the global *M. tuberculosis* population. Clinically relevant mutations in two transcriptional regulators, WhiA and WhiB2, were further found to be associated with acquisition of drug resistance and treatment failure. WhiA and WhiB2 are uncharacterized in *M. tuberculosis* but have putative roles in cell division according to data published on homologs in other organisms. We aim to understand the roles of WhiA and WhiB2 in *M. tuberculosis* cell division and how these functions contribute to the bacteria's success during infection. We constructed inducible CRISPR interference (CRISPRi) strains and have characterized the consequences of targeted knockdown of either *whiA* or *whiB2.* Understanding the role of WhiA and WhiB2 in *M. tuberculosis* pathogenesis and how their function relates to altered drug susceptibility and poor treatment outcomes can aid with improving current tuberculosis therapy.

Elucidating the importance of aspartic surface proteases of pathogenic mycobacteria

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Surface proteins of pathogenic mycobacteria play an important role in host-pathogen interactions. Recently, we identified a novel aspartic protease, PecA (PE_PGRS35) on the mycobacterial cell surface, which is secreted by the specialized type VII secretion system ESX-5. PecA of *Mycobacterium marinum* was shown to be responsible for the processing of secreted PE_PGRS proteins, including itself. Specifically, PecA appears to remove the PE domain of these proteins and cleaves in or near the type VII secretion motif. We have expanded our observations by proteomic analysis on secreted surface-associated protein fractions of wild type and *pecA* mutant (Δ*pecA*) strains. Many new putative PecA substrates were identified, predominantly members of the PE and PPE protein families. Furthermore, analysis of semi-tryptic peptides resulted in the identification of a potential consensus cleavage site.

Both *M. marinum* and *M. tuberculosis* contain two additional predicted aspartic proteases with similar secretion domains (PE_PGRS16 and PE26). We have generated frameshift mutants in genes encoding for PE_PGRS16 (PecB) and PE26 (PecC) and studied the effect of these mutations on cell surface proteins using mass spectrometry. This analysis revealed that PecB or PecC are also involved in processing specific substrates, including PE and PPE proteins, although to a lesser extent than PecA.

Surface localization of these proteases suggests they could also be involved, directly or indirectly, in host-pathogen interactions. In line with this hypothesis, the *M. marinum* Δ*pecA* strain showed reduced bacterial outgrowth in zebrafish larvae as compared to infection with wildtype *M. marinum*. This effect could be complemented with an intact copy of PecA but not with an active site mutant, demonstrating that proteolytic activity of PecA is contributing to virulence *in vivo.* Currently, the function of PecB and PecC are evaluated during macrophage and zebrafish infection to further elucidate the importance of these aspartic proteases.

The PDIM paradox: new solutions for a persistent problem

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Phthiocerol dimycocerosate (PDIM) is a long-chain apolar lipid found in the outermost layer of the *Mycobacterium tuberculosis* (*Mtb*) cell envelope. PDIMs form a permeability barrier, protecting the bacteria from antimicrobial compounds, and are important virulence factors involved in several aspects of *Mtb* pathogenesis. Paradoxically, despite these important functions, PDIM is dispensable *in vitro*, and its loss confers a growth advantage leading to the selection of spontaneously occurring PDIM negative clones in laboratory cultures, adversely affecting experimental reliability and reproducibility. PDIM loss leads to reduced cell wall permeability and virulence attenuation, producing misleading results in infection experiments, drug susceptibility testing, and genetic manipulations. This necessitates routine screening of strains for PDIM; however, current approaches (whole genome sequencing and lipid-based analyses, including mass spectrometry and thin layer chromatography) suffer various limitations related to throughput, cost, limited functional information, laborious processing, and requirement for specialized skills or equipment.

Here, we present a newly developed, straightforward, and cost-effective functional assay to screen *Mtb* for PDIM production. The method is amenable for high throughput and integrates well into existing laboratory procedures. Using our new assay, we have studied the dynamics and conditions under which PDIM loss is selected for/against and examine the use of modified culture media to prevent PDIM loss. The ability to maintain, select and screen for PDIM positivity is especially important during strain construction and passage, and our approach offers an easy and elegant solution to this dilemma. Further, we investigate how modifications to standard growth media modulate central metabolic pathways to drive downstream processes (i.e. PDIM production) and how this affects bacterial phenotypes such as growth and drug resistance.

Together, these advances will be of significant value to the TB research community, increasing the reliability and reproducibility of host-pathogen interaction studies, drug screening and genetic manipulations, and further expand our understanding of the important role of *Mtb* metabolism in virulence and drug resistance.

Iron deprivation enhances transcriptional responses to growth arrest of *Mycobacterium tuberculosis*

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The establishment of *Mycobacterium tuberculosis* (Mtb) long-term infection *in vivo* depends on several factors, one of which is bacterial availability of key nutrients such as iron. The relation between Fe deprivation inside the granuloma and the capacity of Mtb to accumulate lipids and persist in the absence of growth is not well understood. In this context, current knowledge of how Mtb modifies its lipid composition in response to iron starvation is scarce. To shed light on these matters, in this work we have compared genome-wide transcriptomic and lipidomic profiles of Mtb at exponential and stationary growth phases using axenic cultures with glycerol as a carbon source, in the presence or absence of iron.

We found that the main driver that differentiated the transcriptomic response was the growth phase, and the influence of iron availability was strongly associated with the stationary phase. In up to 711 genes, we found significant iron effects on the transcriptomic responses to growth arrest, in a majority of which, cultures grown in iron deprived media showed larger responses to growth arrest than iron-rich samples. Significantly, larger upregulated genes were involved in responses devoted to maintaining the ion metals homeostasis, and several stressful cues considered cardinal features of the intracellular environment and pathogen-host interaction. Furthermore, genes being more tightly repressed upon growth arrest in iron deprived media disclosed energy production processes such as the TCA cycle, NADH dehydrogenation (operon nuo) and cellular respiration.

A main component of the cell envelope, lipid PDIM, was not detected in the stationary phase regardless of iron availability. This suggests that changes in the lipid repertoires during Mtb adaptation to non-dividing phenotypes appear to be iron-independent.

Taken together, our results indicate that environmental iron levels act as a key modulator of the intensity of the transcriptional adaptations that take place in the bacterium upon its transition between dividing and dormant-like phenotypes *in vitro*.

Mycobacterial extracellular vesicles as a novel drug tolerance mechanism

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Our studies and others have clearly established a role of mycobacterial extracellular vesicles (MEVs) in immunomodulation and shown that MEVs deliver factors that impair macrophage effector functions, inhibit T cell activation, and modify the response of host cells to infection. Although the importance of MEVs has been recognized, hardly anything is known regarding the molecular mechanisms underlying vesicle formation in mycobacteria and how they contribute to its survival strategy within the host. Understanding the molecular mechanisms of MEV formation can lead to novel therapeutic or prophylactic interventions urgently needed to strengthen TB control efforts. We have identified two distinct conditions that stimulate vesiculogenesis in Mtb: iron limitation and deletion of the gene encoding the surface protein VirR. Common transcriptional responses indicate upregulation the *iniBAC* operon, that have been previously involved in the response to drugs targeting the mycobacterial cell envelope. In fact, we can measure concomitant enhancement of MEV production and overexpression of *iniBAC*, suggesting a link between these two phenomena. The connection between antimicrobial resistance and production of EVs has been shown in different ways and mainly in Gram-negative bacteria yet, it has never been investigated in Mtb. Through the combination of genetics and drug screenings approaches we show that MEVs determine the ability of Mtb to tolerate antibiotic exposure; and identify molecules that can either repress or stimulate vesicle production in Mtb, suggesting that vesiculogenesis may represent a novel druggable target.

Morphological phenoprinting for drug mechanism of action elucidation in mycobacteria

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Background: Mycobacterium tuberculosis (*Mtb*) presents a profound challenge for new tuberculosis (TB) drug discovery and development. Among manifold obstacles, the inability to determine mechanism of action of *Mtb* growth inhibitory compounds in early-stage preclinical drug discovery pipelines can complicate triage and design of medicinal chemistry programme efforts toward optimization of physicochemical and pharmacological properties. Here, we describe the adaptation of our previously described mycobacterial phenoprinting approach for higher throughput drug mechanism of action elucidation.

Objectives: This study aimed to couple essential gene knockdown morphologies to known drug target-induced phenoprints to yield a database allowing high-confidence mechanism of action predictions for existing and experimental anti-TB drugs with unknown mechanism of action.

Methods: Utilizing the non-pathogenic *M. smegmatis::parB-mCherry* bioreporter as background strain, multi-variable cytological profiles were generated in antibiotic-exposed cells and in an expanded library of CRISPRi hypomorphs targeting essential *M. smegmatis* genes with homologs in *Mtb*.

Results: A database comprising >300 gene-specific morphotypes was generated which was augmented with corresponding phenoprinting data of cells exposed to a panel (>20 compounds) of known antimycobacterial agents with different antimycobacterial targets. Notably, the antibiotics clustered tightly according to known mechanism of action. Moreover, in proof-of-principle experiments combining the comprehensive resource with a semi-automated analytical pipeline, putative drug targets were identified for several novel compounds with anti-Mtb activity, while previously predicted mechanism of action assignments were excluded for others.

Conclusion: Our results suggest the CRISPRi-based morphotyping pipeline can be usefully incorporated in early-stage pre-clinical drug development as one of a suite of complementary biological tools enabling rapid selection of the most promising leads for further development.

Towards optogenetic control of gene expression in mycobacteria

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Chemically induced genetic switches such as the tetracycline- and arabinose-responsive promoter systems have transformed the study of mycobacterial gene function and pathogenesis. Key investigations enabled by such switches include the analysis of gene function (especially essential genes) individually and at scale, protein overexpression for structural and other analyses, and control of gene expression in cellular and animal infection models, thereby providing invaluable tools for drug target identification and validation. In contrast, light-based control systems have been much less extensively applied in mycobacteria, yet offer several potential benefits, including that they are fast, non-invasive, and often reversible. Since these features have been leveraged in the wider application of optogenetics in eukaryotic systems, the potential to develop optogenetic systems for spatiotemporal control of gene expression and other key cellular processes in model bacterial organisms, including mycobacteria, appears worth exploring. However, the optimization of optogenetic systems for new models or organisms is not trivial.

Here, we propose a “toolbox” of considerations to inform adaptation of available optogenetic systems to (myco)bacteria, including (1) species compatibility, (2) nature of the light-responsive protein and its cognate wavelength, (3) system size and complexity, and (4) system mechanism – for example, whether it provides transcriptional or translational control. Using *Mycobacterium smegmatis* as an experimental model, we demonstrate the incorporation of the essential components of an optogenetic module into a fluorescent protein-reporter system, and evaluate baseline characteristics including the potential for phototoxicity, and impact on cell viability. We also describe the combined use of flow cytometry and microscopy to determine system performance in moving towards optimization of experimental conditions to allow fine tuning of reporter expression. Our results support the potential development of optogenetics systems in mycobacteria, and might provide a guide to the future adaptation of similar systems in mycobacteria and other microbiological models.

Structure and lipid transport mechanism of triacylglyceride exporter Rv1410c

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Mycobacterium tuberculosis is the most devastating bacterial pathogen worldwide, a serious threat to public health due to the spread of multidrug-resistant tuberculosis. It relies heavily on its complex cell envelope to protect it from the hostile host environment and antibiotic therapy. We are interested in a conserved mycobacterial operon encoding an inner membrane-embedded major facilitator superfamily membrane transporter Rv1410c and a lipoprotein LprG (Rv1411c) tethered to the inner membrane. Both Rv1410c and LprG are involved in transporting cell envelope components, triacylglycerids, from the cell to the envelope and thus contribute to the intrinsic mycobacterial antibiotic resistance by securing the impermeability of the envelope to certain drugs. However, the exact mechanisms of transport and interplay between transporter and lipoprotein are not clear. So far, no physical interactions have been detected between the pair neither *in vitro* nor *in vivo*. To shed light on these problems, we have solved the structure of an Rv1410c homologue by combining the approaches of crystallography and cryo-EM with nanobody technology. The structure adopts an outward-open conformation, displays a small, yet very hydrophobic cavity suitable for lipid transport, and exhibits transmembrane helix 11 and 12 extensions that protrude ~20 Å into the periplasm. An extensive mutational analysis of Rv1410c, guided by the structure and molecular dynamics simulations, has revealed several interesting features of the transporter. A prominent ion-lock located at the center of the transporter is uniquely present in Rv1410c and its homologues and is crucial for proper functioning of the transporter. Potential entrances and exits for the substrate have been characterized as well. What is more, linker helices A and B connecting the N- and C-domains seem to be essential for the proper functioning of the transporter. We propose a triacylglyceride transport model in which the substrate enters the transporter cavity from the membrane via lateral clefts when the transporter is in inward-facing conformation and leaves the transporter in outward-facing state via the central cavity opening, with TM11 and TM12 extensions being necessary for the exit. The extensions may be required for the extraction and transfer of triacylglycerids to the hydrophobic lipid binding pocket of LprG.

Characterization of RscA, a new anti-sigma factor of *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis genome encodes thirteen sigma factors, small subunits of the RNA polymerase that allow rapid adaptation of the bacterium transcriptional landscape, playing a fundamental role in stress-response and pathogenesis. Among them, alternative sigma factor C (σ^C) is still poorly characterized: its function is seemingly linked to copper uptake and biofilm formation, and no anti-sigma has been associated to it yet, although the transmembrane protein Rv0093c represents a suitable candidate.

In order to investigate σ^C role in the physiology of *M. tuberculosis*, mutants lacking its structural gene, as well as *rv0093c*, were generated and analysed in a number of contexts such as different culture conditions, σ^C-related gene expression and biofilm development.

Although no significant differences were found between *M. tuberculosis* wildtype strain and its mutants when cultured in low-copper conditions, a marked sensitivity to high copper concentrations was detected for the *rv0093c* null mutant compared to the wildtype strain. Moreover, genes that had been reported as part of σ^C regulon were indeed upregulated in the *rv0093c* mutant, supporting the hypothesis of Rv0093c effectively being σ^C specific anti-sigma factor, and redefying σ^C implication in copper metabolism.

Evidence of physical interaction between the two proteins was also obtained experimentally, by setting up a mycobacterial protein-fragment complementation assay, executed in the non-pathogenic model species *Mycobacterium smegmatis*.

Confirmation of this interaction and a finer analysis of the σ^C regulon through RNA sequencing experiments will allow to better characterize this poorly studied sigma factor, and to investigate its role in *M. tuberculosis* very complex physiology and virulence.

Metabolic adaptation of mycobacteria pathogens to metals availability

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At the onset of infection, the host triggers a response called “nutritional immunity” to halt pathogen replication. The nutritional immunity response consists in the variation of metal (micronutrients) availability, aimed at either starve or intoxicate pathogens. To survive this condition and be able to persist in host tissues, successful pathogens have developed high affinity metal-binding molecules and efficient efflux pumps. Despite its generality and importance in host-pathogen interactions, the effect of alterations in metal availability has been poorly characterised, in comparison with macronutrients, such as carbon and nitrogen sources. Here, we investigate the effects of Fe^{3+} starvation and wide range Mn^{2+} concentrations on *Mycobacterium tuberculosis* and *Mycobacterium abscessus* physiology. We observed that i) Fe^{3+} starvation causes a re-modelling of the central metabolism to contrast the reduced ATP production and *pmf* alteration due to the down regulation of iron-containing enzymes of Krebs cycle and electron transport chain; ii) *M. abscessus* shows distinct growth phenotypes depending on the carbon source present; iii) both *M. tuberculosis* and *M. abscessus* have adapted to grow in a wide range of Mn^{2+} concentrations (from 0 to 800 mM). This last result appears particularly interesting, considering that mycobacteria are routinely grown in media that contain less than 1 µM of Mn^{2+} or even no source of this metal, suggesting the possibility that several mycobacterial processes related to Mn^{2+} are not active in traditional growth media. On the basis of these observations, we compared the proteomes of *M. abscessus*grown in the presence of low and high concentration of Mn^{2+}, in two distinct carbon sources, acetate and glucose, to identify metal- and/or carbon source-dependent differential expression of proteins and unmask potential new mechanisms related to Mn^{2+} homeostasis.

The quest for novel targets in TB-therapy: CRISPRi-mediated repression of PG modifications in *Mycobacterium smegmatis* promotes beta-lactam susceptibility and intracellular killing

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Background: Tuberculosis (TB) is a global health concern owing to the lack of effective therapeutics against emerging multidrug and extensively-drug resistant strains of *Mycobacterium tuberculosis* (*Mtb*). With 1.5 million TB-related deaths annually, identifying therapeutic targets that will lead to the development of novel antibiotics is imperative. For that, probing the peculiar complexity of the mycobacterial cell wall (CW) is crucial. The peptidoglycan (PG) layer of the CW features unique modifications, of which the *N*-glycolylation of muramic acids and the amidation of D-*iso*-glutamate are of special interest.

Methods & Aims: To understand the role of these PG modifications in the susceptibility of *Mycobacterium smegmatis* to beta-lactams and in the modulation of host immune responses, the genes encoding the enzymes responsible for these PG modifications were silenced using CRISPR interference (CRISPRi). Afterwards, the knockdown efficiency was evaluated by quantitative PCR (qPCR) and the mutants were phenotypically characterized through spotting dilutions. Minimum inhibitory concentration (MIC) assays were performed to test for antibiotic susceptibility; ≥4-fold MIC differences were considered significant. Furthermore, the intracellular survival was evaluated by counting colony forming units (CFUs), following infection of J774A.1 murine macrophages.

Results: The qPCR results confirmed the efficient repression of the targeted genes, along with polar effects and differential repression depending on PAM strength and target site. The phenotypical characterization validated qPCR results and facilitated essentiality classification. Both PG modifications were found to promote resistance to β-lactams. Whereas the depletion of PG *N*-glycolylation resulted in a significant hypersusceptibility to tested β-lactams, a reduction in the D-*iso*-glutamate amidation of PG led to increased β-lactam susceptibility, specifically to cefotaxime. Moreover, the repression of the *N*-glycolylation of PG was found to promote a significantly faster bacilli clearance by J774 macrophages. Preliminary results suggest that the D-*iso*-glutamate amidation of PG also influences the intracellular survival in host macrophages.

Conclusion: Our results suggest that the characteristic modifications of mycobacterial PG could be considered potential therapeutic targets since they simultaneously contribute to β-lactams resistance and promote intracellular survival inside host macrophages. Henceforth, we hope to provide insight on how these PG modifications modulate antibiotic susceptibility and host-pathogen interactions in *Mtb*.

High-throughput phenotyping of 158 Mtb clinical strains during host-relavant carbon metabolism and antibiotic stress

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Mtb is a successful pathogen because of the acquired and intrinsic ability to evolve and adapt to selective pressures, namely antibiotics and macrophage-mediated host challenges. One of the most well-characterized selective forces of the macrophage is immune activity, however carbon source availability is also prominent host features of which Mtb must adjust to for growth and survival. With selective pressure comes variation, however due to more facile technology such as whole-genome sequencing, genetic variation is much more appreciated compared to phenotypic heterogeneity. However, understanding the diversity of the Mtb phenotypic landscape is important because it can potentially inform the design of improved antibiotics and treatment regimens, host-directed therapies, and strain-specific personalized patient care. Towards investigating Mtb phenotypic heterogeneity, convention might suggest evaluating fitness responses to different pressures in isolation without respect to how selective forces might converge. But importantly, with the goal of targeted and synergistic interventions in mind, there is a benefit to identifying how drugs, carbon sources, and host immune pathways with different mechanisms of action, metabolic processes, and bacteriostatic/bactericidal activity lead to similar Mtb fitness phenotypes. To address this, we performed a large-scale systematic and comparative assessment of Mtb fitness heterogeneity during growth in disparate selective pressures to identify commonalities in bacterial responses. This required development of a method for high-throughput phenotyping of Mtb strains. We tagged 158 Mtb clinical isolates from three different lineages with unique genetic barcodes that enable pooled competition experiment to assess relative competitive fitness of each strain in a growth-based assay. We completed competition experiments in liquid media using host-relevant defined carbon source media and also with first- and second-line antibiotic treatment. We then compared the fitness data in these stress conditions to Mtb fitness in macrophages with different immune backgrounds. Our robust technique for Mtb clinical strain phenotyping not only revealed a large range of growth and survival phenotypes in these conditions, but we also observed associations between distinct pressures, strain genetic background, transmission, and patient treatment outcomes. Not only does our comparative analysis have implications for patient care, it also suggests fundamental pathways that are crucial to Mtb control.

Pangenome Analysis of Drug Resistant *Mycobacterium tuberculosis* Clinical Isolates: Highlighting Mechanisms of Diversity

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Background: *Mycobacterium tuberculosis* is believed to have low genetic diversity, despite its ability to persist and evade host and antimicrobial pressures. To evaluate diversity, we conducted a pangenome analysis of 109 *de novo* assembled genomes from globally sourced clinical isolates of patients with significant drug resistance (1) and H37Rv reference strain.

Methods: Prokka-annotated GFF files were inputted into Panaroo (2), a recently published pangenome clustering tool for prokaryotic genomes to estimate the core (genes shared in ≥99% of genomes) and accessory pangenomes (<99% of genomes). Analyses were conducted in R, Panther, and Cytoscape.

Results: Out of 4325 total genes in the resulting pangenome, the majority of genes were conserved (core pangenome N=3767, 87%), with the accessory pangenome being significantly enriched for phospholipase activity and disruption of host cellular components. Hierarchical cluster analysis confirmed that, among the five lineages present in our sample, Euro-American lineage indeed had more diversity in its genomes (sharing 87% of its genes vs. 94-97% other lineages). About 7% of the pangenome had genes that were identified by Panaroo but not annotated by Prokka, with 45% of these genes being conserved in the core pangenome. Several of these unannotated genes were highly correlated in our principal component analysis. Additionally, tens of genes were discovered to have duplicates within at least one isolate, with the majority (66%) having a gene copy found in all genomes, but only a few of the isolates had the second copy. From the network analyses, on average each gene node had 2.34 neighbors. Few gene clusters drive the network heterogeneity, with PE/PGRS, PPE and unannotated genes having high degrees of node connectedness. There are also several known insertion sequence elements in some areas with high variation in the gene networks.

Conclusion: The pangenome of these drug resistant isolates is largely conserved, though the global characteristics seen from the network analyses highlight areas of heterogeneity. Genes yet unannotated in *M. tuberculosis* may be influential in driving the difference between lineages and structural networks.

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Reprogramming *Mycobacterium tuberculosis* for stress survival by the VapC36 tRNase toxin

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The *M. tuberculosis* genome harbors an abundance of toxin-antitoxin (TA) systems, including 50 members of the VapBC family. Since one proposed role of TA systems is to protect bacteria from the stresses encountered in their environmental niches, *M. tuberculosis* TA systems are implicated in enabling this pathogen to evade killing by the host immune system and persist inside macrophages as a latent tuberculosis infection. All VapC toxins are ribonucleases that contain a conserved catalytic PilT N-terminus (PIN) domain. We used a multi-omics approach to identify the primary and downstream targets of one *M. tuberculosis* VapC toxin, VapC36, that enabled us to track the molecular events that underlie VapC36-medited growth arrest and reprogram its physiology. We first enlisted a specialized RNA-seq method, 5' RNA-seq, and determined that VapC36 cleaves and inactivates a single tRNA isoacceptor, $tRNA^{Ser\text{-}CGA}$, within its anticodon sequence. Consequently, VapC36 cleavage of $tRNA^{Ser\text{-}CGA}$ resulted in its depletion, leading to ribosome stalling at cognate Ser UCG codons. Finally, depletion of $tRNA^{Ser\text{-}GCA}$ leads to surgical reprogramming of the *M. tuberculosis* transcriptome and translatome based on codon-usage, i.e. global reduction in the abundance of transcripts and synthesis of new proteins with one or more UCG codons. This genome-wide shift manifested in upregulation of the master regulator of mycobacterial intrinsic resistance WhiB7 as well as discrete pathways for defense against oxidative stress. Therefore, VapC36 does not simply inhibit global translation to cause growth arrest, instead VapC36 helps *M. tuberculosis* adapt its physiology to evade killing by antibiotics and the host immune response.

ESX-2 secretion system contributes to *Mycobacterium tuberculosis* survival in nutrient starvation

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Among the ESX secretion systems expressed by *Mycobacterium tuberculosis (Mtb)*, ESX-2 is the less extensively characterized. It has been reported that the *esx-2* locus regulation might involve the WhiB5 transcriptional factor, the expression of which is induced in nutrient starvation and is required for *Mtb* reactivation in a murine model of chronic tuberculosis. In this work, the expression of *esx-2* genes was tested in various *in vitro* conditions: i) different growth phases in standard laboratory medium, at optimal O_2 concentration; ii) nutrient starvation (incubation in PBS); iii) reactivation after hypoxia-induced dormancy. No differences were observed in the expression of genes encoding for ESX-2 structural components (e.g. $eccC_2$ and $eccD_2$), in all conditions tested. In contrast, an increased expression was observed for *esxD/esxC* genes both in nutrient depletion and reactivation, as well as for $espG_2$ and $mycP_2$ during reactivation. Consistently with the expression data, a panel of *Mtb* ESX-2 mutants (deleted for the entire *esx-2* locus, the $eccC_2$ or the *esxC* genes) displayed a progressive reduction of survival during incubation over an 8-day-period in PBS, while the *Mtb* wild-type strain was able to survive over the same period. Comparative analyses on growth kinetics and ability to resume growth revealed no differences between the ESX-2 mutants and the *Mtb* wild-type, when starting cultures were grown to exponential phase. However, all ESX-2 mutants showed a reduced ability to resume growth (delayed kinetics as compared to the wild-type strain) when cultured to the early stationary phase. Most importantly, the ESX-2 deletion mutant was able to survive under low-oxygen conditions (Wayne model of progressive oxygen depletion) over a 40-day period, but was impaired in growth reactivation from a dormant state as compared to the *Mtb* wild-type. These findings suggest a potential role of ESX-2 and its substrates in *Mtb* survival in nutrient-starvation and in growth resumption.

Exploring Galsomes, an mRNA based nanovaccine, for protection against intracellular bacterial infections

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Within our research group, we developed an advanced mRNA vaccination platform, Galsomes. This vaccination platform consists of a lipid-based nanovaccine co-encapsulating antigen-encoding, nucleoside-modified mRNA and the immuno-activator α-Galactosylceramide (αGalCer). Galsomes are preferentially targeted to APCs and provide antigen signaling to both conventional T cells, B cells as well as invariant natural killer T cells (NKT cells), resulting in a broad and multi-faceted immune response involving both innate and adaptive immune responses.

In contrast to other mRNA vaccines which only contain antigen-encoding modified mRNA, we showed that αGalCer, presented in the MHC-like molecule CD1d, evokes the activation of NKT cells and mRNA-transfected APCs, which coincides with the release of Th1/Th2 cytokines. We were able to prove that this vaccine can achieve superior cellular-mediated immunity (CD4 and CD8 T-cells) and obtain higher (IgG) antibody titers compared to the currently approved nucleoside-modified mRNA vaccine format[1].

Besides that, NKT cell activation can also offer a major advantage in Mtb vaccination as they were identified to play a key role in its host defense[2]. It has been demonstrated that Mtb derived lipid antigens as phosphatidylinositol mannosides bind CD1d and activate human NKT cells. Active TB patients had decreased percentages of NKT cells in peripheral blood or bronchoalveolar lavage samples, with respect to subjects with latent infection[3]. Activated NKT cells can limit bacterial replication and have a direct bactericidal effect thereby reducing bacterial burden in Mtb infected mice[4]. Mice immunized with αGalCer and BCG or subunit vaccines have a significantly lower bacterial burden after Mtb challenge[3]. We have also shown that Galsome vaccination can deplete immunosuppressive cells, which is known to improve clinical outcome and vaccine efficacy for TB[5]. αGalCer also induces the proliferation of educated NK cells, defined as important players in trained immune responses to mycobacteria. As a first proof of concept, we used a *Listeria monocytogenes* infection model, where we were able to show enhanced protection by inclusion of αGalCer in an mRNA vaccine encoding a newly identified, immunodominant Listeria antigen.

All together this indicates that our mRNA Galsome platform holds enormous potential to develop more effective vaccines against intracellular bacteria as TB.

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In vitro killing dynamics of the diarylquinolone TBAJ-587 and its main metabolites against *Mycobacterium tuberculosis*

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The first-in-class diarylquinoline bedaquiline was recently added to WHO essential medicines list for drug-resistant tuberculosis (TB). Improved efficacy of bedaquiline may be possible, beyond current doses, but reported adverse effects limit evaluation of higher exposures. TBAJ-587 is a next-generation diarylquinoline with anti-Mtb activity and better properties than bedaquiline in terms of safety and efficacy in animal models. Its dose optimization for new combinatorial regimens is currently under evaluation within the ERA4TB consortium.

The *in vitro* antibacterial activity of TBAJ-587 and its metabolites M2, M3, and M12 (structurally similar to its parent compound) were evaluated against *Mycobacterium tuberculosis* H37Rv under conditions with differing carbon sources found in the granuloma, including standard *in vitro* conditions (dextrose), cholesterol, long-chain fatty acids, butyrate, acetate, and pyruvate. First, minimum inhibitory concentrations (MIC) were determined by the resazurin microtiter assay. Then, 28-days dose-response time-kill assays (TKA) were performed to obtain both pharmacodynamic (PD) (Log_{10} CFU/mL) and pharmacokinetic (PK) parameters (concentration of the drug at every time-point of the kill kinetic) in standard and two lipid conditions. TKA drug concentrations were measured by LC-MSMS.

The MIC of TBAJ-587 and its main metabolites was conserved between the different carbon sources with average MIC90 values of MIC_{587}= 0.031-0.062 µg/mL; $MIC_{587\ (M2)}$= 0.4-1.6 µg/mL; $MIC_{587\ (M3)}$= 0.062-0.125 µg/mL; and $MIC_{587\ (M12)}$= 2.2-9 µg/mL. In TKA, all four compounds showed bactericidal activities at ≥5xMIC, and bacteriostatic activities at 1xMIC and 2xMIC with regrowth by day 28. Patterns of activity were independent of the carbon source used in the assay. Drug concentrations in all media suffer a drastic drop of up to 90% by day 1, probably due to unspecific binding to the plastic ware, similar to observed for bedaquiline. The remaining drug was stable during the full length of the experiment.

This *in vitro* study applies a multimodal approach and links for the first time the activity of a compound (PD) to its effective concentration (PK) at every time point. Our data suggest that the actual *in vitro* activity of TBAJ-587 might have been underestimated.

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Nitronaphthofuran derivatives: promising anti-TB compounds killing replicating and non-replicating *M.tuberculosis*

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Multidrug Resistant Tuberculosis (MDR-TB) is threatening the efforts to control and eradicate TB. The WHO estimates that 3.3% of new and 18% of previously treated TB cases occurring worldwide in 2019 had MDR/rifampicin-resistant (RR)-TB, which translates into close to half a million new cases of MDR/RR-TB. In this study, a high-throughput whole-cell screening of a chemical library composed of 35,860 compounds using *M. aurum* ATCC23366 as a surrogate of Mtb led to the discovery of nitronaphtofuran (nNF) derivatives as novel molecules with anti-TB activity. The most active compounds showed bactericidal activity against both replicating (MIC of 0,03 – 8 mg/ml) and non-replicating *Mtb*. After testing their cytotoxicity profile in Vero and HepG2 cell lines, we selected 7 derivatives for which the Selective Index was ≥ 50, or the acute toxic concentration (IC_{50}) was higher than 100 mg/ml. A selection among the non-cell-toxic analogues was tested in *Mtb*- and *M. marinum*- infected Bone Marrow derived Macrophages (BMDM), in which a significant reduction of viable intracellular bacteria was shown in a concentration-dependent manner (up to 4 logs for some compounds at the maximum concentration tested). Compounds showing high or intermediate *in vitro* activity against Mtb were also tested for their toxicity and efficacy using the zebrafish TB model. One of the compounds (C4) showing a high SI, was found to be toxic in the zebrafish embryo model. However, another derivative (C11) which showed intermediate *in vitro* activity, significantly reduced the burden of the *M.marinum* infection in zebrafish embryos with no signs of toxicity at the maximum dose tested (50 mg/kg). In order to determine the mechanism of action of the active compounds, spontaneous Mtb resistant mutants were isolated and subjected to whole-genome sequencing using Illumina MiSeq 2x300bp. Most of the resistant mutants showed non-synonymous SNPs or INDELs in rv2466c, a proposed mycothiol-dependent nitroreductase (Mrx2). Finally, NO release was shown in BCG cultures when exposed to the compounds in a concentration-dependent manner. Further investigation on highly promising nNF anti-TB candidates is guaranteed.

New ionic liquids to treat mycobacterial infections

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The incidence of nontuberculous mycobacterial (NTM) infections has been increasing in the last decades, mainly those caused by species belonging to the *Mycobacterium avium* complex (MAC). Management of NTM infections is challenging and prone to failure. First-line treatments are based on a multidrug regimen taken from months to years. This is associated with a myriad of problems, from high toxicities to low patient compliance, low clinical success, and high chances of relapse. Thus, it is urgent to find new strategies to treat these infections. Ionic liquids (ILs) are organic salts made by combining two molecules with opposite polarities. Besides improving the bioactivity and cytotoxicity properties of the parental drug, using ILs can avoid polymorphism and solubility issues presented by solid conventional drugs, improving their absorption and desired dissolution rate. By testing ILs based on conventional antimycobacterial drugs against *M. avium* in axenic culture and inside bone marrow-derived macrophages (BMM), we aim to find new drugs with better bioavailability and antimicrobial activity, as well as lower toxicity to the host cells. Our results show that ILs derived from one of two fluoroquinolones, ofloxacin or norfloxacin, and the antimycobacterial drug clofazimine have a similar extracellular and intracellular activity to clofazimine and are more active than the fluoroquinolones. Moreover, these ILs are not more toxic than clofazimine to the host cells. Chemical analysis shows that the ILs are more soluble and more stable thermically than the parental compounds, which corroborates the potential of these new formulations as alternatives to conventional antimycobacterial drugs. Our next objective is to test the ILs in more complex *in vitro* models of infection, such as biofilms, in vitro granulomas, and lung organoids in order to better predict their clinical outcome.

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In-silico investigation of the mannose-transfer mechanism of Phosphatidyl-myo-inositol mannosyltransferases A and B'

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The emergence of antibiotic resistance and the long duration of treatment regime compel us to search for novel therapeutic drugs against Mycobacterium tuberculosis (Mtb). The complex cellular envelope of Mtb is one of the major reasons behind their virulence and survival in hostile conditions. Phosphatidyl-myo-inositol hexamannoside (PIM6), lipomannan (LM), and lipoarabinomannan (LAM), capable of modulating the host immune functions, are important structural elements of the cell envelope. A common membrane constituent, phosphatidylinositol (PI) is mannosylated by phosphatidyl-myo-inositol mannosyltransferase A (PimA) at the 2-position of the inositol group using GDP-mannose (GDPM) as the mannose-donor to produce phosphatidyl-myo-inositol monomannoside (PIM1) and GDP. This PIM1 is further mannosylated at the 6-position of the inositol group by phosphatidyl-myo-inositol mannosyltransferase B' (PimB') utilizing GDPM to synthesize phosphatidyl-myo-inositol dimannoside (PIM2) and GDP. PIM2 is mannosylated, acylated, and further modified to synthesize Ac1/2PIM6 or LM/LAM.

The lack of counterparts in humans make PimA/PimB' attractive targets to design novel drugs. The mechanism behind different regioselectivity on the PI moiety of these enzymes is not yet known. How the lipid substrates (PI/PIM1) or the mannosylated products (PIM1/PIM2) interact with PimA/PimB' is not understood. How both the substrates or both the products interact at the catalytic center of these enzymes is not studied. In this study, interactions of PI and PIM1 with PimA (PIM1 and PIM2 with PimB') are analyzed by molecular docking. Molecular dynamics (MD) simulations showed that both PimA and PimB' are least dynamic when bound to both their substrates. Our studies demonstrated the ‘open to closed’ motions of PimA. -helices formed by residues 73-86 of PimA and 72-86 ofαAmphipathic PimB' interact with PI and PIM1, respectively. Partly ordered residues 120-130 of PimA/PimB' interact with their products. The residues 59-70 of PimA and 281-288 of PimB' contain unique insertions and interact with their respective lipid substrates and products, indicating their possible roles in the regioselectivity of the mannosyltransferase activities. MD analyses further identified crucial roles of the residues R196, R201, and K202 of PimA (R206, R210, and K211 in PimB') in the mannosyltransferase activities. These findings may help design novel drugs against mycobacterial PimA and PimB'.

Large-scale profiling of mycobacterial drug tolerance reveals clinical outcomes independent of drug resistance

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Antimicrobial susceptibility testing is critical for drug development and guiding treatment of mycobacterial infections. However, *in vitro* assessments often do not translate into clinical outcomes, leading to unexplained treatment and trial failures. Conventional susceptibility measures evaluate growth across different antibiotic concentrations but not bacterial killing, which may be required to clear long-lasting infections. By overcoming the poor scalability and reproducibility of killing assessments, we establish the relevance of sterilising anti-mycobacterial activity in *Mycobacterium abscessus*, a particularly difficult-to-treat pathogen causing increasing rates of chronic pulmonary infections globally.

We developed Antimicrobial Single-Cell Testing (ASCT), an experimental live-cell imaging and analytical platform for quantifying bacterial killing at scale. Herein, we immobilise, successively image and automatically track millions of bacteria for four days using AI-driven image analysis, to evaluate single-cell dynamics and population time-kill kinetics. With ASCT, we assessed over 7000 kill curves in *M. abscessus* isolates from more than 300 patients and found various time-kill profiles across and within the drugs tested, indicating that bacterial killing is a drug property and a fundamental bacterial phenotype. The distinct killing rate of each isolate (drug tolerance) was not related to drug resistance (MIC) and was genetically determined, as revealed through whole-genome sequencing. Cefoxitin and Imipenem killing, which were most rapid and presumably drive mycobacterial clearance in *M. abscessus* lung disease, were associated with clinical outcomes in patients (tolerant isolates with persisting infection, non-tolerant isolates with *M. abscessus* clearance). Importantly, antimicrobial tolerance was independent and additive to resistance measurements and predicted, in contrast to drug resistance, lung function decline during mycobacterial infection. Finally, by applying a genome-wide association study (GWAS) approach, we discovered several potential molecular mechanisms underlying mycobacterial multi-drug tolerance.

We present Antimicrobial Single-Cell Testing for assessing sterilising antibiotic activity, demonstrate highly diverse killing kinetics in *M. abscessus* and validate its importance via predicting clinical outcomes, thereby reducing the gap between *in vitro* assessments and *in vivo* antibiotic efficacy. Our findings highlight the relevance of antimicrobial killing mechanisms, potentially improve treatment decisions in patients and may advance anti-mycobacterial drug development.

Evolution of drug resistance under sub-inhibitory drug concentrations in *Mycobacterium tuberculosis*

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Drug-resistant *M. tuberculosis* variants may evolve in single patients even under optimal treatment conditions. It is believed that sub inhibitory drug concentrations (sub-MIC) contribute to treatment failure, by giving resistant variants, omnipresent in populations of sufficient size, a selective benefit over drug-susceptible variants. *M. tuberculosis* infections are treated with at least 4 active antituberculous drugs- However, the influence of drug combinations at sub-MIC on the evolution of drug-resistance in *M. tuberculosis* is unknown. Here we present a study investigating the influence of sub-MIC of two drugs, rifampicin (RIF) and isoniazid (INH), alone and in combination on the evolution of drug resistance in; 3 drug-susceptible clinical *M. tuberculosis* isolates from 3 different genetic background. The strains were passaged for ~180 bacterial generations in the presence increasing concentrations of RIF and INH alone and in combination, starting at a fraction of the wt MIC and ending at 4 x MIC. Whole genome sequencing was performed of three endpoint cultures per treatment and strain. We report the detection of drug-resistance conferring mutations in most cultures that survived until the end of the experiment. The results, will help us to develop interventions to slow or prevent the evolution of highly resistant *M.tuberculosis* strains.

GSK-839 inhibits the *Mycobacterium tuberculosis* tryptophan synthase TrpAB

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The tryptophan biosynthesis is an essential pathway for bacterial growth, but it is absent in humans, thus representing a potential target for novel antituberculars. Recently, several compounds inhibiting this pathway have been reported, including inhibitors of the tryptophan synthase. *Mycobacterium tuberculosis* tryptophan synthase TrpAB is an heterotetrameric enzyme, that catalyses the conversion of indole-3-glycerol phosphate (IGP) and L-serine into L-tryptophan. In particular, the α-subunit hydrolyses IGP into glyceraldehyde 3-phosphate and indole, while the β-subunits catalyses the reaction of indole with L-Ser to produce L-Trp. In this context we identified a compound, namely GSK-839, a potent antitubercular regardless the location of the bacteria extra or intracellularly, equally potent against clinical isolates with no cross resistance with other anti-TB drugs and with low Frequency or Resistance. This profile makes it well suited for further exploration in the clinic to be part of new PanTB regimen. Isolation and sequencing of spontaneous resistant mutants strongly suggested an involvement of TrpAB in its mechanism of action (MOA). To demonstrate that TrpAB is directly inhibited by GSK-839, the enzyme was expressed in *Escherichia coli* and purified. The catalytic activity of the α-subunit was determined using a spectrophotometric coupled assay with glyceraldehyde 3-phosphate dehydrogenase, while the β-reaction was monitored following the increase in absorbance at 290 nm of L-Trp. Using these assays, the recombinant enzyme showed catalytic constants in the same range of the values previously reported, thus suitable to investigate the inhibition by GSK-839. In particular, the compound was demonstrated to specifically inhibit the β-subunit showing an IC50 of 0.89 ± 0.04 µM. By contrast, no effects were found against the α-reaction. An in deep characterization of the mechanism of inhibition revealed that GSK839 behaves as a mixed uncompetitive inhibitor, with a Ki value of 0.25 ± 0.01 µM. Finally, the compound was found to have a residence time of 1.5 min. With these results we have shed light into the MOA of a novel promising antitubercular, and further confirmed the L-Trp biosynthesis is a good target for drug development.

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The thin-layer agar method allows a fast determination of the minimal inhibitory concentration for bedaquiline.

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Since 2018, WHO guidelines recommend bedaquiline (BDQ) for the treatment of MDR-TB. The genotypic background of BDQ resistance and its association with the resistance phenotype and treatment outcome remain however unclear. Therefore, phenotypic drug-susceptibility testing (pDST), especially determination of the minimal inhibitory concentration (MIC) is indispensable. Unfortunately, the long turnaround time and the need for primary culture and biosafety level 3 infrastructure complicate pDST and delay results. We aim to validate the ability of thin-layer agar (TLA) testing directly from sputum to establish the MIC for BDQ.

As a first step we evaluated TLA as indirect pDST method, inoculating 40 replicates of the H37Rv reference strain by two operators on different days, with and without CO_2 incubation, along with a panel of *Mycobacterium tuberculosis* isolates with a known pattern of resistance, which were tested 8 times each. Results were compared against the indirect 7H11-polystyrene(PS)-tube testing with 3 weeks incubation.

The pooled MIC for H37Rv ranged from 0.03 to 0.125mg/L with a mode of 0.06mg/L, similar to previously reported 7H11 results, and all below the proposed breakpoint. Regarding the first 6 clinical isolates (Table 1), their MIC values are identical to 7H11-PS in a 81.3% of the cases, with only a 1-dilution difference in the rest, which is considered within acceptable range, and with a better accuracy without the presence of CO_2 (70.8% CO_2 incubator vs 91.7% CO_2-free). TLA results for all isolates were obtained at day 7.

			BDQ MIC (mg/L) by indirect TLA																
			CO_2 incubator									CO_2 free							
Isolate number	Origin	7H11-PS MIC	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2
H37Rv	clinical	0.06				**14**	6							4	**15**	1				
99-01856	clinical	0.25						**3**	1								**4**			
12-00594	clinical	0.06			1	**3**									**4**					
14-01618	clinical	1								**4**								1	**3**	
15-02116	clinical	0.03		1	**1**	2							1	**3**						
15-02119	clinical	0.06				**4**									**4**					
15-02150	clinical	0.06				**2**	2								**4**					

These preliminary results strengthen our hypothesis that TLA could be a fast and reliable technique for pDST for BDQ, accessible to low-resource settings in a biosafety level 2 without CO_2 incubators. The accuracy for direct-on-sputum testing needs to be evaluated.

Proposed workflow model for target elucidation of anti-tuberculous compounds with selective intracellular activity

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The emergence of drug-resistant tuberculosis is a health care threat, which has increased the effort of identifying anti-tuberculous drugs with novel targets. Phenotypic whole-cell screenings, however, often identify drug candidates targeting the same mode of actions. Therefore, an alternative dual screening approach is increasingly exploited not only to identify novel canonical anti-tuberculous compounds but also anti-virulence and host-directed compounds to support antibiotic treatment. The elucidation of the mode of action of the later classes hereby is often very difficult since the gold standard of generating resistant mutants is not applicable.

Here we propose a workflow model for target elucidation of compounds with selective intracellular activity. In a first approach, compounds are subject to investigate carbon source-dependent activity, which might render a compound inactive in *in vitro* or *ex vivo* and ultimately in *in vivo* conditions. Alternatively, discrepancy between *in vitro* and *ex vivo* data could indicate an intracellular pro-drug activation. Such a conversion is quantified by LC-ESI/MS. Subsequently, hit compounds need to be evaluated for anti-virulence or host-directed activity. Multiple assays can be exploited for this task. These include compound-preincubation of *M. tuberculosis* cultures prior to infection of host cells; cytokine analyses of treated and infected host cells; tests for inhibition of important virulence determinants such as the *M. tuberculosis* ESX-1 secretion system for which we developed ELISA based medium-throughput technologies. Finally, omics-based techniques such as RNA-seq, proteomics and metabolomics performed from bacteria or host cells exposed to drugs can be helpful to understand the mechanism of action of these new compounds. The proposed workflow will help to streamline experiments required for successful preclinical development of anti-tuberculous drugs identified in intracellular infection assays.

CanB, a therapeutic validated cellular target of *Mycobacterium tuberculosis*

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Prolonged exposure to drugs due to long-term treatment against tuberculosis (TB) contributes to the selection of *Mycobacterium tuberculosis* strains resistant to currently available anti-TB drugs, representing a serious threat for public health worldwide.

The study of new therapeutic targets against *M. tuberculosis* is needed to develop novel effective drugs to be implemented in clinical practice.

We validated CanB, one of the three *M. tuberculosis* β-carbonic anhydrase (CA) as a possible therapeutic target against TB. CAs are metalloenzymes that catalyse the reversible reaction of CO_2 hydration to form HCO_3^- and H^+. CanB is the β-CA that shows the highest catalytic activity for CO_2 hydration than the other two mycobacterial CAs.

Firstly, we demonstrated the essentiality of CanB for the survival of the pathogen *in vitro* by constructing conditional knock-down (cKD) mutants using the TetR-Pip-OFF system.

In addition, to search for CanB inhibitors active against *M. tuberculosis* growth, conditional *canB* mutants were also constructed using the Pip-ON system. This system is characterised by the presence of the pristinamycin (Pi)-sensitive transcriptional repressor Pip, the presence of Pi in the culture medium allows *canB* transcription in a dose-dependent manner.

Subsequently, by molecular docking and MIC assays we selected molecules that could inhibit both CanB activity and *M. tuberculosis* growth, thus implementing a target-to-drug approach. The identification of three compounds, which appear to effectively target CanB was pursued by target-based screening using the resazurin reduction microplate assay against Pip-ON *canB* mutants. The redout was the degree of growth inhibition which directly correlates with the degree of gene attenuation.

To definitively validate the inhibitory action of these compounds, CanB protein was produced and purified. The enzyme was then characterised and, to obtain information in terms of binding affinity and mechanism of action, the inhibition of CanB due to the three molecules was studied using enzymatic assays and thermal shift analysis. The lead compound also showed a bactericidal activity by time killing assay.

In conclusion, the three compounds identified by the *in silico* screening proved to be not only high-affinity CanB ligands but also endowed with antitubercular activity, demonstrating the validity of the approach used.

In vitro and *in vivo* antimycobacterial activities of novel aroylhydrazone derivatives

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Emergence of multi drug resistant *Mycobacterium tuberculosis* strains requires new efficient anti-tuberculosis (TB) agents. We describe *in vitro* and *in vivo* antimycobacterial activities of novel aroylhydrazone derivatives about their: (i) acute and subacute toxicity in mice; (ii) redox-modulating capacity (iii) pathomorphological observation in differentiated tissue specimens; and (iv) intestinal permeability. The *in vitro* activity was assessed on the laboratory reference strain *M. tuberculosis* H37Rv. The most active compounds were further used for *in vivo* assessment of the toxicity, redox-modulating capacity and *in vitro* intestinal permeability and selection of the spontaneous resistant mutants. The screening identified 3a (MIC=0.0730 µM, cytotoxicity - HEK-293T IC_{50} = 256.7 µM, SI=3516), 3b (MIC=0.3969 µM, cytotoxicity - HEK-293T IC_{50} = 785 µM, SI=1978.83) and 3c (MIC=0.4412 µM, cytotoxicity - HEK-293T IC_{50} = 279.5 µM, SI=633.49) as a new promising hit compounds against *M. tuberculosis* H37Rv. According to the Hodge and Sterner toxicity scale, 3a,b,c are classified as slightly toxic with an LD_{50} > 2000 mg/kg for both oral and intraperitoneal administration. The histological examination proved that the tissue findings do not show toxic changes. Liver findings showed isolated changes without a pathological organ profile. The *in vitro* antioxidant assays confirmed the results found *ex vivo*. The derivatives possess better permeability then INH and the changes of pH does not induce noticeable variations in permeability of compounds. This suggests pH independent permeation through the GIT membranes and is theoretically supported by physicochemical properties of compounds. The mutant positions were presented as mixed wild type and mutant alleles while increasing concentration of the compound lead to semi-proportional and significant increase of mutant alleles. These compounds display promising antitubercular drug-like properties and can be used for development of novel anti-TB drugs. Acknowledgements: This work was supported by the Bulgarian National Science Fund (Grant KP-06-N41/3, 2020).

Contribution of Xpert® MTB-RIF in the surveillance of drug-resistant tuberculosis in new tuberculosis cases and retreatment patients

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Introduction: Despite the various WHO strategies and multi-faceted support to the National Tuberculosis Control Programs (NTCP) to control the tuberculosis endemic, the emergence of MDR-TB (Multi-Drug Resistance Tuberculosis) strains presents itself as a real challenge to the strategy <<end TB by the year 2035>>. The Central African Republic remains to this day one of the 30 countries with a high burden with an incidence of 540 cases/100 inhabitants and a mortality of 91 cases/100,000 inhabitants. Since 2019, the National Tuberculosis Reference Laboratory (NRL-TB) uses according to WHO recommendations the Xpert® MTB/RIF as a first-line diagnostic test for the early diagnosis of drug-resistant tuberculosis. The aim of this study was to evaluate the contribution of the Xpert® MTB-RIF test to the surveillance of rifampicin resistance in new cases of tuberculosis and in patients undergoing retreatment.

Material and methods: Data from different types of patients registered at the TB-NRL on Xpert® MTB-RIF test usage during 2019 were retrospectively analyzed. These patient types were: New cases, treatment failures, relapses, abandon treatment, and TBMR subject contacts.

Results: 1404 patients were registered, the mean age was 39.2 years [2 - 90] and a sex ratio M/F of 1.16. Overall, 32. 3% (454/1404) had TB of which 22.5% (102/454) had rifampicin resistance. While primary resistance was only 9.1% (27/298), secondary resistance was 46.6% (75/161). Treatment failure and relapse were significantly associated with rifampicin resistance ($p<0.005$). However, the OR showed that the risk of occurrence was only 3.06 [1.51 - 6.16] for relapse and 21.15 [11.47 - 38.99] for treatment failure.

Conclusion: The surveillance of resistance to anti-tuberculosis drugs should henceforth be a priority for the NTP and the Ministry of Health in Central African Republic for better control of the spread of TBMR.

Keywords: *Xpert® MTB-RIF, Rifampicin, Surveillance, Bangui.*

OPTIKA, a new high content drug combination *in vitro* kill-kinetic assay to evaluate the efficacy of novel anti-TB drug combinations

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Tuberculosis (TB) is one of the top 10 causes of death worldwide. Recently, FDA approved the combination of bedaquiline, pretomanid and linezolid (BPaL), recently recommended by the WHO for 6 months XDR-TB treatment. Understanding how to develop such regimens remains however a challenge.

The main limitation of the traditional methods used to develop drug combinations such as checkerboard assays (CBA), or DiaMOND, is the use of growth inhibition as a metric of drug activity because it requires secondary validation by time-kill assays (TKA), the gold-standard *in vitro* proxy. Unlike CBA and DiaMOND assays, TKA rely on a cidal parameter (Log_{10}CFU/mL), being one of the most valuable *in vitro* assays, and basis of pharmacometrics modelling of antimicrobial drug action. However, this method requires large culture volumes, and has limited throughput and long readout times, creating a barrier to validate interactions of more than 3 drugs per experiment.

In this context, we developed a new methodology named OPTIKA (Optimized Time Kill Assays) that dramatically increases traditional TKA capacity, and allows for facile and dynamic interrogation of drug interactions with a CFU-free methodology. OPTIKA is based on the CARA assay and replaces the use of CFU with a resazurin-based fluorescence readout in a 96-well plate format. This technique has been optimized to robustly analyse up to 770 unique conditions (in quadruplicate: 3080 samples) at the same time and allows data to be delivered after 10 days, almost 2 weeks earlier than traditional CFU-based methods.

The enhanced throughput capacity allowed the interrogation of all possible 3-way combinations including 17 anti-TB compounds (680 combinations) in three different carbon sources: glucose, cholesterol or fatty acids (total of 2,040 combinations). Favourable triple interactions were identified in all three media (207 for glucose, 48 for cholesterol and 114 for fatty acids) but only eight 3-way combos matched all three conditions. Future *in vivo* and hollow fiber studies will evaluate the proposed combinations.

OPTIKA can play a critical role in initiatives such as the ERA4TB consortium, which aims to evaluate the efficacy of novel anti-TB drug combinations as well as new molecules to progress through the drug development pipeline.

Multicentric *in-vitro* characterization of MPL-204, a new anti-tuberculosis drug candidate, by the ERA4TB collaborative initiative

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The development of new anti-tuberculosis compounds with novel mechanisms of action is still a slow and cost-consuming challenge. One of the goals of the IMI2-funded EUROPEAN REGIMEN ACCELERATOR FOR TUBERCULOSIS (ERA4TB), which aims to boost the development of new therapeutic regimens for the treatment of tuberculosis, is to accelerate this process through collective efforts of various teams with different expertise in drug development.

The *in-vitro* characterization of new tuberculosis drug candidates, combined with pharmacokinetic/pharmacodynamic data generation, is crucial in order to understand the multiparametric mechanisms involved in patients. The development of appropriate assays and models is a pre-requisite to provide valuable information and to predict activity and efficacy *in vivo* as well as in clinical trials, but also to anticipate and understand the response and potential defence mechanisms adopted by drug-resistant strains.

Here, this collaborative effort is illustrated through the *in-vitro* characterization of a novel indole carboxamide drug candidate, developed by TB Alliance, targeting the mycobacterial membrane protein large 3 (MmpL3) transporter, whose central role in cell envelope biogenesis has been validated as a promising therapeutic target. By combining traditional assays and cutting edge tools in drug development, we report comprehensive characterization of this novel candidate with respect to: I) time kill kinetics combined with drug quantification by LC-MS/MS analysis; II) determination of the frequency of resistance using standard *in-vitro* fluctuation analysis followed by whole genome sequencing of resistant mutants; III) assessment of the effect of the drug on the biosynthesis of mycobacterial lipids; IV) study of the impact of the proton motive force as well as single carbon sources on drug activity, and V)

evaluation of the activity of the drug in the novel human e*x-vivo* model of granuloma-like structures during *Mycobacterium tuberculosis* infection. Moreover, the artificial *caseum* model was used to determine the drug penetration properties of this compound.

Altogether, we describe a multicentric collaborative approach that can fuel modelling predictions, and support progression of promising drug candidates and combinations to the clinic.

This work has received support from the Innovative Medicines Initiatives 2 Joint Undertaking (grant No 853989).

A Fish's Take on Tuberculosis: rapid *in vivo* Drug Screening to fuel the Pipeline

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Although the tuberculosis drug discovery efforts have recently increased, the pre-clinical bottlenecks, such as *in vivo* efficacy testing, have limited the number of compounds reaching clinical studies. Thus, the emphasis has been placed on the predictive value of pre-clinical model systems. Our study aimed to incorporate the *in vivo* model at a very early step of the drug discovery route in order to select the compounds with the highest chance of being active in later mammalian models. We utilized the zebrafish-embryo model, which presents a middle ground by providing a highly reproducible *in vivo* infection model for compound evaluation, as well as the ability for medium-throughput screening due to assay miniaturization and process automatization. In this infection model, *Mycobacterium marinum* was used as a surrogate for *Mycobacterium tuberculosis* (Mtb). Using approved antibiotics, we demonstrated that successful waterborne treatment of infected fish with antibiotics is predictive of high oral bioavailability in humans. The testing platform was employed to rapidly screen 240 compounds of an anti-TB hit-library for their toxicity and *in vivo* activity. We identified 14 compounds that significantly reduced the bacterial burden in infected zebrafish embryos. The identified compounds also showed activity against Mtb in culture, and 12 compounds were highly active against intracellular Mtb. One of the most active compounds was the tetracyclic compound TBA161, which was studied in more detail. Analysis of spontaneous resistant mutants revealed point mutations in *aspS* (*rv2572c*), encoding an aspartyl-tRNA synthetase, an essential enzyme within the protein translation pathway. The target was genetically confirmed, and molecular docking studies propose possible binding of TBA161 in a pocket adjacent to the catalytic site. The *in vivo* zebrafish infection model was further used to identify and evaluate synergistic combinations of novel hit compounds with approved antibiotics and compounds in the drug pipeline. Several drug combinations have been tested, and some promising synergistic combinations have been identified, showing more than 100-fold increased activity during infection. Taken together, we showed that the zebrafish-infection model is suitable for rapidly identifying novel drug scaffolds with high *in vivo* activity and synergistic drug combinations.

Tricyclic-spirolactams: potent anti-tuberculosis molecules targeting the electron transport chain

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There is an urgent need to feed the anti-tuberculosis drug pipeline to improve future antibiotic regiments and facilitate the fight against drug resistant tuberculosis. To identify novel anti-tuberculosis leads a synthetic chemical library of structurally unique bioinspired 3-D molecules was assembled and evaluated for anti-tuberculosis activity. This screen identified a rigid, sp^3 rich chiral tricyclic-spirolactam (TriSLa) hit molecule whose enantiomer was inactive. Medicinal chemistry efforts allowed for a 2-log improvement in the potency of the hit to give low nanomolar TriSLa inhibitors. *In vitro* profiling found TriSLa to be *Mycobacterium* specific, with activity against *M. marinum*, *M. avium* and *M. absessus* but not against other Gram negative or Positive bacteria tested. Furthermore, TriSLa were not only active against extracellular replicating *M. tb*, but also non-replicating ss18b, and on intracellular H37Rv. In the case of both replicating and non-replicating, TriSLa exposure led to a time dependent bactericidal activity. Selection and characterisation of TriSLa resistant *M. tb* and *M. marinum* isolates identified mutations in the type II NADH dehydrogenase genes as the cause of resistance. Biochemical validation subsequently confirmed that TriSLa were potent inhibitors of both Ndh and NdhA, and that they acted through an allosteric, non-competitive mechanism. Within *M. tb* TriSLas were confirmed to induce a rapid change in NAD^+/NADH ratio as well as a decrease of bacterial ATP levels. Finally, to gain insight on the efficacy of TriSLas in an *in vivo* infection model, these compounds were evaluated in a *M. marinum* zebrafish infection model and found to protect the Zebrafish from the infection. These findings therefore unveil the discovery and characterization of a novel chemical scaffold that may have the potential to become a new class of anti TB drug.

ESX-5 secretion inhibition as new target for antitubercular drug development

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The increasing incidences of drug-resistant tuberculosis, including multidrug-resistant tuberculosis, pose an urgent need for the discovery and development of novel, effective antitubercular drugs. In this study, we focus on a new potential target, ESX-5 secretion. ESX-5 secretion plays an essential role in nutrient uptake and is also involved in immunomodulation during host cell infection, both in *M. tuberculosis* and its close relative *M. marinum*. We developed a high-throughput screening assay in *M. marinum* to identify potential ESX-5 inhibitors based on the secretion inhibition of a highly abundant lipase, LipY (Rv3097c). To circumvent the lethality of ESX-5, we used a strain that expresses the *M. smegmatis* porin MspA. Screening a library of 32.000 compounds with subsequent validations, macrophage cytotoxicity testing, and *in vitro* testing against *M. tuberculosis* yielded nine compounds. Next, we tested the compounds for their inhibitory activity of ESX-5 secretion using PE-PGRS substrate in *M. marinum*, which resulted in a preferred hit compound, C8. Structure-Activity Relationship studies successfully generated two derivatives with more than 10-times higher antitubercular activity, C8-106 and C8-229. More importantly, both compounds were able to reduce the bacterial burden in infected macrophages and infected zebrafish. To discover the mode of action, resistant *M. tuberculosis* strains against C8 derivatives were selected and their genomes were sequenced. All identified mutations were located within the non-essential monooxygenase *ethA* (*rv3854c*) gene, suggesting C8 is a prodrug activated by EthA. Increased susceptibility of *M. marinum* and *M. tuberculosis* against C8 upon EthA overexpression confirmed this finding. Transcriptomic analysis suggested that C8 elicits a high oxidative stress response in *M. marinum*. To identify the target of activated C8, we investigated the effect of C8 on all essential genes using CRISPRi-sequencing assays in *M. marinum*. These CRISPRi-sequencing data implied that C8 might target biosynthesis pathways downstream of chorismate. Overall, our work demonstrated that C8 is a highly active antitubercular compound potentially targeting ESX-5 secretion system that can progress further in preclinical development.

Structure - antimycobacterial activity relationships of 3,5-dinitrophenyl-containing compounds

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The nitro group is considered to be responsible for higher toxicity such as hepatotoxicity, genotoxicity, mutagenicity. However, its presence in the structure of some drugs (delamanid, macozinone) is crucial for their activity against *Mycobacterium tuberculosis* (*M.tb*).

5-Alkyl/aryl-2-(3,5-dinitrobenzylsulfanyl)-1,3,4-oxadiazoles (**1**) and 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**2**) prepared and studied in our group showed excellent activity against both drug susceptible and drug-resistant *M.tb.* strains with minimum inhibitory concentrations as low as 0.03 μM (0.011-0.026 μg/mL). Furthermore, oxadiazoles **1** were highly effective against non-replicating *M.tb.* SS18b strain. Despite the presence of two nitro groups in the molecules, lead compounds showed low cytotoxicity against various cell lines, including isolated human hepatocytes. [1, 2]

However, the presence of two nitro groups is the main obstacle for further development of these potent antimycobacterial agents. Therefore, in this work we focused on the replacement of one nitro group of 3,5-dinitrophenyl moiety with another (electron-withdrawing) substituents or dealt with the synthesis of compounds where 3,5-dinitrophenyl fragment is substituted for nitro-group containing heterocycle.

As the main disadvantage of lead structure **1** was low metabolic stability because of sulfur oxidation, derivatives with modified linker were also prepared and evaluated for their antimycobacterial activities.

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Novel analogues of *p-Aminosalicylic* acid active against multidrug-resistant mycobacteria

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Spread of drug-resistant *Mycobacterium tuberculosis* (*Mtb.*), together with latent tuberculosis (TB), COVID-19 co-infection and increasing prevalence of non-tuberculous mycobacteria, is a serious threat for public health justifying a strong need for new antimycobacterial agents. Modification of established drugs to obtain derivatives with improved properties represents a viable approach.[1]

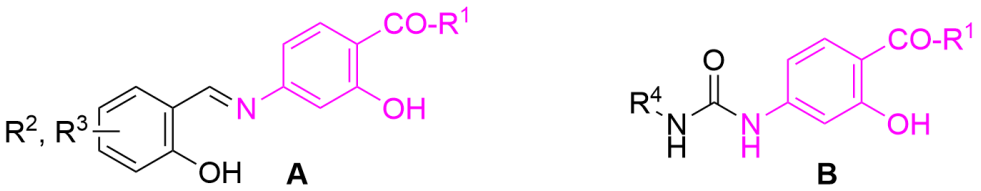
p-Aminosalicylic acid (PAS) is a well-known second-line drug used mainly in the treatment of resistant TB. It is a prodrug targeting folate biosynthesis. Recently, we have published three promising PAS derivatives and their peptide conjugates as antitubercular agents.[1] Lipophilic compounds are of special interest for targeting intracellular mycobacteria and facilitating penetration through mycobacterial waxy cell wall.

Therefore, we have designed and prepared a series of lipophilic imines and ureas based on PAS scaffold. For synthesis of imines (Fig. 1A), halogenated salicylaldehydes with PAS or its methyl ester were treated in boiling methanol. Ureas (Fig. 1B) were obtained from PAS, its methyl ester and various commercially available or in-house prepared aliphatic, alicyclic, and phenylalkyl isocyanates in dichloromethane, with addition of triethylamine in the case of PAS. These reactions gave good yields.

The compounds were evaluated against a panel of mycobacteria (*Mtb.* H_{37}Rv, *M. avium*, *M. kansasii*), drug-resistant *Mtb.*, Gram-positive and negative bacteria, fungi and for their toxicity to identify structure-activity relationships.

Importantly, our PAS analogues inhibited all mycobacterial strains with MIC ranging from 1, 125, and 1 µM for *Mtb.*, *M. avium* and *M. kansasii*, respectively. Importantly, the compounds retained their activity even against multidrug- and extensively resistant TB strains (MIC ≥2 µM). In general, they showed higher potency than the parent PAS. Ureas were more active than imines, favouring *n*-alkyls from C_8 to C_{13}, cycloheptyl and 1-adamantyl.

Moreover, some imine derivatives also showed inhibition of Gram-positive cocci including methicillin-resistant *Staphylococcus aureus* (MIC from 7.81 µM). Importantly, most derivatives lacked cytotoxic or cytostatic effects on eukaryotic cell lines (HepG2, MonoMac6). Their mechanism of action is under investigation.



R^1 = OH, OCH_3
R^2, R^3 = H, halogen
R^4 = *n*-alkyl, cycloalkyl, Ph, Ph-alkyl

Fig. 1. Investigated PAS analogues

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Hollow fiber system for TB: optimization studies with Moxifloxacin

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The Hollow Fiber System for tuberculosis (HFS-TB) is an *in vitro* preclinical tool for drug evaluation. It can mimic pharmacokinetics (PK) profiles with high accuracy to forecast optimal dosing. Guidelines to assemble the system are public, but standard procedures, and reported fixed parameters to ensure reproducibility intra-laboratories are lacking. The objective of this study was to reveal the key parameters that guarantee the accuracy and reproducibility of PK and pharmacodynamics (PD) assays in HFS-TB. For this purpose, we used moxifloxacin (MXF).

For PK evaluation, we studied the suitability of the polysulfone cartridges by mimicking the concentration in human plasma from single oral dose of MXF (400 mg) in the absence of bacteria. Then, we mimicked MXF q.d. oral doses for three days with bacteria. All HFS-ports were sampled and drug quantification was performed by HPLC-MS/MS. Expected PK profiles were achieved in the central compartment but lower concentrations were found from the cartridge ports at Tmax. Traditionally, investigators have reported levels at the central compartment but not from cartridge ports where bacteria are exposed. Our study reveals that it cannot be assumed that drug concentrations at the central compartment will replicate inside the cartridges.

For PD evaluation, we tested three adaptation periods of *M. tuberculosis* H37Ra within cartridges: 24 h, 72 h, and 7 days before MXF administrations. We mimicked 400 mg q.d. oral dose as described above for nine days. Bacterial killing was dependent on the length of the adaptation period, being faster upon shorter adaptation times. This suggests the metabolic stage and initial bacterial load may influence kill rates and drug effectiveness interpretations. Bacterial killing was different than previous publications mimicking the same dose. Variability may rely on cartridge features, broth supplementation, inoculum size and adaptation period.

Our findings contribute to the standardization of the HFS-TB and should be considered when designing protocols to allow reproducibility between laboratories.

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Implementing best practises on data generation and reporting of Mycobacterium tuberculosis time kill assays: a case study of standardized protocol within the ERA4TB consortium

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Tuberculosis (TB) is the deadliest infectious disease after COVID-19. The European Regimen Accelerator for Tuberculosis (ERA4TB) is a public-private partnership of more than 30 different institutions with the objective to progress new anti-TB regimens into the clinic. Thus, robust and replicable results across independent laboratories are essential for reliable interpretation of treatment efficacy. Time-kill assays provide essential input data for pharmacometric model informed translation of single agents and regimens activity from in vitro to in vivo and clinic.

An ERA4TB standardization workgroup was established to unify time-kill assay protocols and data reporting templates. Five conditions were assessed in six independent laboratories using four bacterial plating methods. Baseline bacterial burden varied between laboratories, but variability in net drug effect was limited.

This exercise lays down the foundations of collaborative efforts for robust data generation, reporting and integration within the overarching Antimicrobial Resistance Accelerator programme.

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Intrinsic antibacterial activity of beta-cyclodextrins potentiates their effect as drug nanocarriers against tuberculosis

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Multi-drug resistant tuberculosis (TB) is a major public health problem concerning about half a million cases each year. Patients hardly adhere to the current strict treatment consisting of more than 10,000 tablets over a 2-year period. There is a clear need for efficient and better formulated medications. We have previously shown that cross-linked poly-β-cyclodextrins (pβCD) are efficient vehicles for pulmonary delivery of powerful combinations of anti-TB drugs. Here, we report that in addition to be efficient drug carriers, pβCD nanoparticles are endowed with intrinsic antibacterial properties.

In mouse infected lung, we observed that pβCD nanoparticles are mainly engulfed by alveolar macrophages and modulate host cells properties impairing *M. tuberculosis* (Mtb) establishment. pβCD hamper colonization of macrophages by Mtb by interfering with lipid rafts, without inducing toxicity. Moreover, pβCD provoke macrophage apoptosis leading to depletion of infected cells, thus creating a lung micro-environment detrimental to Mtb persistence. Taken together, our results suggest that pβCD nanoparticles loaded or not with antibiotics have an antibacterial action by their own and could be used as carrier in drug regimen formulations effective against TB. This activity could fit into the emerging and promising concept of anti-TB approaches by host-directed therapy, which aims to empower host immune properties for the elimination of mycobacteria and/or for the reduction of tissue damage induced by the infection.

Screening of novel anti-mycobacterial drugs based on the structure of acetohydroxyacid synthase

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Mycobacteria are aerobic, acid-fast bacilli. *Mycobacterium tuberculosis* (Mtb) is a causative bacterium of tuberculosis (TB). Recent epidemiological studies have shown the incidence of non-tuberculous mycobacterial (NTM) diseases is increasing globally. Despite having a similar genetic structure between Mtb and NTM strains, anti-Mtb drugs are inefficient to treat NTM diseases. Therefore, it is necessary to develop new antibiotics that can inhibit the growth of both Mtb and NTM strains. Mtb acetohydroxyacid synthase (AHAS) has been proposed as a critical target for antibacterial agents. Here, we tested 88 AHAS targeted synthetic chemicals based on the structure of AHAS of NTM strains. Ten of the eighty-eight chemicals are isolated as effective compounds to suppress the growth of *M. avium*, *M. abscessus* and Mtb. We have investigated the synergic effects of the chemical candidates to suppress NTM and Mtb when it was treated as a combination therapy with conventional antimycobacterial drugs. Among 88 chemicals, 6 of them were selected based on its synergic effect to suppress Mtb or NTM strains. KNT2099 was the most effective chemical compound to suppress the growth of NTM and Mtb.

Structure-activity relationships of pyrazolo[1,5-*a*]pyrimidin-7(4*H*)-ones as novel antitubercular agents

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A pyrazolo[1,5-*a*]pyrimidin-7(4*H*)-one was identified through high-throughput whole-cell screening as a potential antituberculosis hit. The core of this scaffold has been identified several times previously and has been associated with various modes of action against *Mycobacterium tuberculosis* (*Mtb*). We explored this scaffold through synthesis of a focused library of analogues and identified key features of the pharmacophore while achieving substantial improvements in antitubercular activity. Our best hits had low cytotoxicity and showed promising activity against *Mtb* within macrophages. The mechanism of action of these compounds was not related to cell-wall biosynthesis, isoprene biosynthesis or iron-uptake as has been found for other compounds sharing this core structure. Resistance to these compounds was conferred by mutation of an FAD-dependent hydroxylase (Rv1751) that promoted compound catabolism by hydroxylation from molecular oxygen. Our results highlight the risks of chemical clustering without establishing mechanistic similarity of chemically related growth inhibitors.

Metabolites tune the antimicrobial susceptibility of Mycobacterium tuberculosis

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There is an urgent need for new approaches to treat tuberculosis (TB), however antibiotic development remains exceptionally costly and viable candidates for TB treatment remain scarce. An alternative approach which is attractive to TB pharmaceutical developers, is adjuvant therapies that potentiate the activity of existing antibiotics and can prevent or even reverse resistance; thereby extending the life of current anti-TB drugs within the clinic. It is now well-established that the metabolic state of bacteria influences their susceptibility to antibiotics, and metabolite directed modulation of metabolism therefore has the potential to improve antibiotic efficacy and achieve this goal. Here we screened the effects of metabolites on anti-TB drug efficacy and identified metabolites that potentiate bactericidal activity, as well as those which have the opposite effect and enhance antibiotic tolerance. This provides us with important insight into the conditions which protect *mycobacteria* from drug treatment and enhance drug tolerance, considered a major impediment to TB treatment and a factor in the development of antibiotic resistance. For those metabolites which enhanced antibiotic killing we used a medicinal chemistry approach to synthesise drug like compounds which induce a similar antibiotic potentiating affect. Our work demonstrates that this is a viable approach for adjuvant TB therapeutics, and therefore an effective strategy to prolong the life of our current anti-TB drugs.

Outer membrane proteins and antibiotic uptake in *Mycobacterium tuberculosis*

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After a lacuna in the drug discovery pipeline for half a century, the discovery of Bedaquiline emerged to be promising and was followed by the identification of novel lead molecules. Few common features emerging from the phenotypic screening of these compounds indicated a potential bias towards relatively hydrophobic molecules that target proteins such as QcrB, MmpL3 and AtpE, in the cell envelope. However, these hydrophobic chemical scaffolds have low bioavailability, highlighting an urgent need to understand how current anti-TB drugs enter the tubercle bacillus. The aim of this study is to identify outer membrane proteins (OMPs) that are involved in the uptake of hydrophilic moieties (better bioavailability) with the ultimate aim of enhancing the uptake and efficacy of current anti-TB drugs.

Using LC/MS-MS based proteomics, we found that octyl-beta-glucoside (OBG) selectively extracts the outer membrane proteins (OMPs) of *Mycobacterium tuberculosis H37Rv*. The outer membrane fraction of cells grown in exponential and stationary phase was subjected to label free quantitative proteomic analysis. The differentially expressed proteins between nutrient replete and deplete conditions can be enriched to give porins that are involved in the nutrient uptake. Further, thorough bioinformatic analysis identified 10 putative OMPs associated with sugar, phosphate, sulphate or glycine-binding. Crucially, we demonstrated the cell surface accessibility of seven of these proteins using immunofluorescence. Moreover, cells overexpressing these proteins showed hypersensitivity for hydrophilic drugs including streptomycin and exhibited higher membrane permeability, probed by EtBr uptake assay. Overall, through this poster, we show the first ever selective extraction of outer membrane proteins in *M. tuberculosis*, which has unrevealed potential outer membrane proteins that are surface-accessible, associated with OM permeability and antibiotic uptake. Notably, these OMPs will also have implications in designing vaccines due to their surface accessibility.

Long alkylamine decorated 2,5-disubstituted 1,3,4-oxadiazoles as potential antituberculotics

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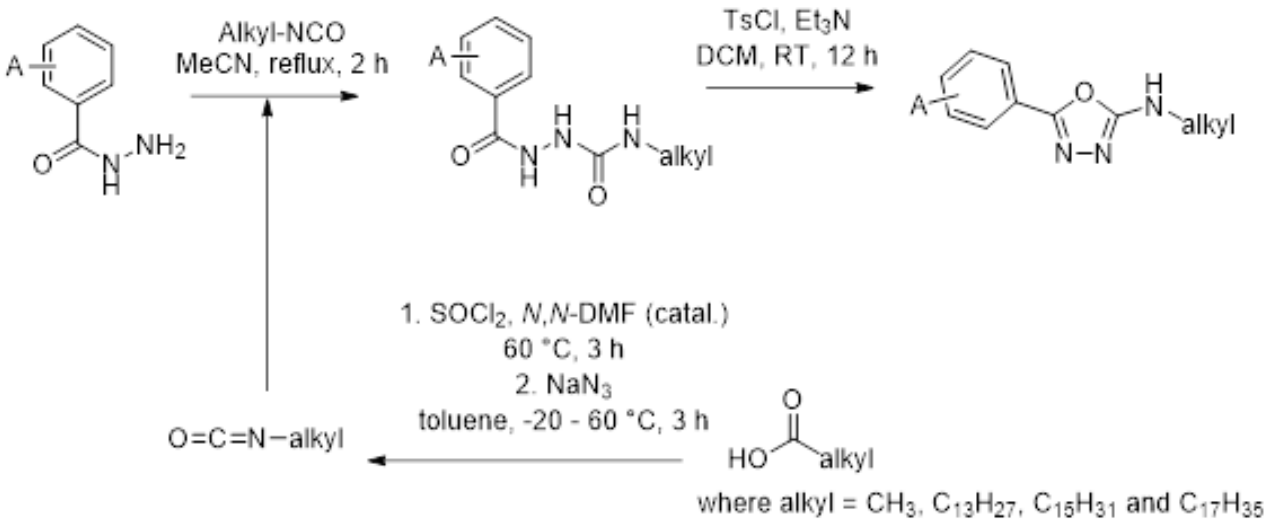
It has previously been shown that appropriately substituted nitrogen heterocycles (e.g., oxadiazoles, tetrazoles, and triazoles) can carry significant antimycobacterial activity [1]. Many compounds with these structural motifs inhibit very effectively the growth of *M. tuberculosis* (*Mtb*.) with various resistance profiles *in vitro*.

Based on our previous knowledge [2], we have designed and investigated a novel series of 2,5-disubstituted 1,3,4-oxadiazoles, many with no structural similarity to any drug developed so far. *N*-Alkyl-5-aryl-1,3,4-oxadiazol-2-amines were obtained predominantly by dehydrative cyclization of 2-arylhydrazine-1-carboxamide precursors with *p*-tosyl chloride in the presence of triethylamine as base. The precursors were available by reaction of hydrazides with isocyanates (commercial or in-house prepared by Curtius rearrangement); **Scheme 1**.

The desired compounds were evaluated for their *in vitro* antimycobacterial activity against drug-susceptible *Mtb*. $H_{37}Rv$ (MIC ≤0.03 μM), selected non-tuberculous mycobacterial strains (*M. avium*, *M. kansasii*) and panel of eight *Mtb*. strains with various resistance profiles including XDR-TB (MIC ≤0.03 μM for each strain). The mechanism of action of the most potent compounds was investigated as well. Preliminary results indicate that some of the presented compounds are highly effective and selective inhibitors of DprE1/DprE2 enzymes involved in the synthesis of mycobacterial cell wall. In addition, no activity against Gram-positive and Gram-negative bacteria, as well as fungal pathogens, was identified. Similarly, no significant cytotoxic activity against mammalian cells was found (HepG2 model). In addition, *in vivo* cytotoxicity assays were also performed on *Danio rerio* embryos.



Scheme 1. - Synthesis of desired compounds

The study was supported by the Czech Science Foundation (reg. No. 20-19638Y), Charles University (SVV 260 547) and "Grant Schemes at CU" (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935).

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Hollow fiber system for tuberculosis setting up at university of Zaragoza

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The Hollow Fiber System for tuberculosis (HFS-TB) is a preclinical tool for drug evaluation qualified by the European Medicines Agency in 2015. The system allows the reproduction of selected drug exposures to infer PK/PD parameters, which will feed *in silico* models able to inform the design of Phase 2/3 clinical trials. Although methodological guidelines for HFS-TB exist, there are no standard procedures for its implementation nor mandatory quality control steps for intra-laboratory reproducibility. In this context, UNIZAR was tasked with the implementation of the HFS-TB in BSL-2 and BSL-3 laboratories within the ERA4TB consortium.

The setting up was divided into two main phases: 1) assessment of experimental needs and internal capacity; and 2) standardization of operational procedures.

First, we tested and selected the more suitable equipment and materials in terms of compatibility, size, and ease to handle, to perform mono and combinatorial assays in BSL-2 and BSL-3 facilities. Capacity assessments were performed including the understanding of economical and personnel cost. Timeframe estimates revealed “protocol design” and “troubleshooting anticipation” as key steps for a successful experimental outcome.

The next phase involved the optimization of different procedures to ensure optimal experimental designs. This included bacterial growth dynamics assessment in several media, different types of cartridges and different inoculum sizes. We also evaluated sampling methods for optimizing PD measurement as well as PK profile verification.

With the info obtained, we stablished the work-flow for HFS studies: an initial study of the fiber compatibility with the drug, confirmation of PK profiles achievement, drug testing of broad conditions in the *M. tuberculosis* H37Ra strain (BSL2 facilities) and finally drug testing of selected conditions in the *M. tuberculosis* H37Rv strain (BSL-3).

In summary, we have successfully implemented HFS-TB at UNIZAR in BSL2 and BSL3 facilities. Our findings contribute to the standardization of the HFS-TB and should be considered when designing protocols to allow reproducibility between laboratories.

This work has received support from the Innovative Medicines Initiatives 2 Joint Undertaking (grant No 853989) and reflects only the author's views. The JU is not responsible for any use that may be made of the information it contains.

Study of the mechanism of action/resistance of antitubercular drug TBAJ-587 and its metabolites

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Bedaquiline (BDQ), a diarylquinoline, is one of the latest drugs introduced in therapy, targeting *atpE* gene, coding for the subunit c (AtpE) of the ATP synthase complex. Quickly, two main mechanisms of BDQ resistance emerged, consisting in mutations in either *atpE* gene, or in *Rv0678*, coding for the repressor of the MmpS5-MmpL5 efflux pump. BDQ is a key player in the new regimen for the treatment of MDR and XDR-TB. The search for next generation diarylquinolines with a superior safety profile for patients was pursued, and led to the discovery of TBAJ-587, a next generation of diarylquinolines. TBAJ-587 is more potent than BDQ, and forms three active metabolites, M2, M12, and M3, the major metabolite.

In a framework of a collaboration of ERA4TB consortium, our aim was the study of the mechanism of action/resistance of the M3 and other metabolites to confirm that AtpE is their cellular target, as for the parent molecule TBAJ-587.

For this purpose, we determined the activity of TBAJ-587 and its metabolites against a panel of *Mycobacterium tuberculosis atpE* and *Rv0678* BDQ resistant mutants available in our laboratory collection. *M. tuberculosis* mutants were resistant to M3 (and to other metabolites) as compared to the parental strains, thus confirming that TBAJ-587 and its metabolites share the same target and the same mechanism of resistance.

Furthermore, to find a new possible mechanism of action/resistance to M3, not related to Rv0678, isolation of spontaneous mutants was performed starting from a *M. tuberculosis Rv0678* mutant. Eighteen M3 resistant mutants were obtained, with different degrees of resistance. Two of them exhibited a 4-fold resistance level higher than the parental strain. In both mutants, the WGS analysis pinpointed a Δ1921 in *mmpL4*, leading to a non-sense mutation. MmpL4 is a non-essential RND transporter, exporting siderophore. Interestingly, *mmpL4* mutants are resistant to M3 and the other metabolites, but not to BDQ and TBAJ-587.The study of the role of MmpL4 in M3 resistance is ongoing.

This work has received support from the Innovative Medicines Initiatives 2 Joint Undertaking (grant No 853989).

When Nature offers the solution: exploiting myco-bacteriophages to develop a precise nano-therapy and fight drug resistance

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Antimicrobial overuse and the rise of (multi)drug-resistant *Mycobacterium tuberculosis* (*Mtb*) strains are nullifying the effects of the existing therapies. We must urgently deploy revised strategies to tackle *Mtb*'s' silent pandemic'. In this framework, we took inspiration from the solutions provided by Nature and exploited the bacterial-restricted lytic properties of bacteriophages to develop an effective nanomedicine-based therapy. All known phages, with no exceptions detected so far, own a very specific set of lytic proteins, namely the endolysin (or lysin). They are highly efficient enzymes binding a very specific substrate, able to swindle cell wall integrity, and, finally, induce the death of the bacteria. Interestingly, mycobacteriophages encode for a second lysin, known as endolysin B (LysB). Contrary to other endolysin, LysB not only bears in its N-terminal the domain responsible for the binding to its target but also recognizes a different substrate represented by the outer layer (*i.e.,* mycolic acids) of the waxy mycobacterial cell wall. This prompted us to hypothesize that the binding properties of lysins, such as LysB, might represent a high-affinity and specific tool that we can exploit to develop a selective antimycobacterial therapy.

Therefore, we undertook a comprehensive study aiming to identify the minimal aminoacidic consensus sequence required for LysB binding to the mycomembrane. In this regard, we generated an inducible fluorescent C-tagged version of both LysB wild-type and of 3 diverse and partly overlapping portions of LysB N-terminal domain. Then, by measuring their binding affinity versus the mycomembrane, we assessed that the 3 truncated LysB forms carry a highest target affinity with respect to the full LysB N-terminal. We now aim to compute site-directed mutagenesis on specific amino acids to increment LysB binding to its target and engineer the most promising LysB mutant to build a mycobacterial-selective targeting tool. The therapy is delivered through super-selective polymeric nanoparticles, which are able to specifically target infected macrophages only, while leaving all the other non-infected cells untouched.

We believe this new bacteriophages-inspired therapy, coupled with the tools offered by nanoparticles delivery, will provide an unprecedent therapeutic approach to Mtb infection and treatment, while also avoiding the rise in antimicrobial resistance.

Novel Series Kills *Mycobacterium tuberculosis* in a pH-Dependent Manner

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Tuberculosis therapy is a unique challenge given the ability of *Mycobacterium tuberculosis* to enter non-replicating states for long periods, causing latent infection and resistance to the host immune system. *M. tuberculosis* as an intracellular pathogen can replicate inside a resting macrophage. However, once the macrophage is activated *M. tuberculosis* is exposed to a series of antimicrobial activities of the immune system including the acidification of the compartment *M. tuberculosis* occupies. The acidification of the macrophage environment can be bactericidal itself or make *M. tuberculosis* vulnerable to other stress. Thus, the ability of *M. tuberculosis* to maintain its pH homeostasis is essential for surviving. We previously identified a series of benzothiazole (BTH) compounds with pH-dependent bactericidal activity against non-replicating bacteria and now we are interested in determining the mode of action and target of this series. The characterization of the mode of action of the series showed that the BTH compounds are only active at pH 4.5, but not at neutral pH. None of the BTH compounds acted as general disrupters of membrane potential. The most active compound of the series showed a minimum bactericidal concentration (MBC) of 0.78 µM at pH 4.5 against non-replicating bacteria. Protein interaction studies identified three transcription regulators (TRs): Rv1556, Rv1685c and Rv2887, as potential targets for the series. As expected, these TRs are not essential at neutral pH. We constructed controllable under-expressing strains using CRISPRi for each of the three TRs in *M. tuberculosis* and determined whether strains could survive at low pH; our preliminary data suggest that transcriptional silencing of *rv1556*, *rv1685c* and *rv2887* does reduce survival, indicating these genes might be essential at pH 4.5. Transcriptional profiling of *M. tuberculosis* treated with the lead compound showed up-regulation of Rv0560c, a methyltransferase which is negatively regulated by the TF Rv2887. These findings implicate inhibition of Rv2887 as the mechanism of action corroborating our pulldown studies results. Further investigation to confirm the mechanism of action is being carried out.

Pyrano[3,2-*b*]indolones affect sulfolipid biosynthesis in *Mycobacterium tuberculosis*

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More than 1.5 million deaths occur every year as a result of *Mycobacterium tuberculosis* infection. An additional challenge concerns latent tuberculosis (TB) with about 1.7 billion estimated to be infected with *M. tuberculosis* without overt symptoms of disease. Searching for new antimicrobials for TB treatment including latent infection presents one of the largest problems in modern drug development. Pyrano[3,2- *b*]indolones were reported to display prominent antibacterial activity for both replicating and non-replicating *M. tuberculosis* [1], however, their exact mode of action has not been elucidated yet.

M. tuberculosis RNA-seq analysis in the presence of pyrano[3,2- *b*]indolone derivative 10a showed significant activation of genes of lipid metabolism. In particular, *mmpL6* encoding membrane protein which is involved in fatty acid transport and takes part in oxidative stress response, and *mmpL8*, which is involved in lipid transport and production of sulfolipid-1 were found significantly upregulated. Pks2 protein, encoding hepta- and octamethylphthioceranic acid synthase which is necessary for synthesis of sulfolipids was also found to be induced. Sulpholipids are required for *M. tuberculosis* virulence, mediating specific pathogen-host interactions during infection. On the contrary, *tgs1* coding for triacylglycerol synthase was found repressed under pyrano[3,2- *b*]indolone 10a exposure. The results of the transcriptome analysis may indicate a disruption of (sulfo)lipids biosynthesis in *M. tuberculosis* and possible decrease in bacterial virulence in the presence of pyrano[3,2- *b*]indolone 10a. Furthermore, compound 10a was found to have a pronounced anti-tuberculosis effect in a model of chronic tuberculosis *in vivo*, significantly reducing the number of bacteria in the lungs and spleen of infected mice after 4 weeks of therapy. Thus, pyrano[3,2-*b*]indolones are a new perspective class of anti-TB compounds which may target *M. tuberculosis* sulfolipid biosynthesis.

This work was financially supported by Russian Foundation of Basic Research (project number 20-04-00798)

Reference:

1. Monakhova et al., Design and Synthesis of Pyrano[3,2-*b*]indolones Showing Antimycobacterial Activity. *ACS Infect Dis*. 2021; 7(1):88-100.

POSTER SESSION 2

Wednesday, September 14, 2022

1.45 pm to 3.15 pm

Development and Characterization of a Robust Foamy Macrophage Assay for use in TB Drug Discovery

S. Sans*[1], P. Bade[1], N. Chappat[1], A. Ray[1], L. Goullieux[1], F. Apparailly[2], I. Duroux-Richard[2], S. Lagrange[1], A. Upton[1], C. Roubert[1]

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The tuberculosis lesion environment is highly complex. Bacteria reside in various niches and their metabolism and other characteristics, including drug tolerance depend on the specific environmental characteristics of those niches. So-called persister bacteria refer to those that difficult to eradicate with drug treatment and that may contribute to the long durations of TB treatment required for cure. Persisters may reside in specific lesion compartments. Hence, many *in vitro* models have been developed in attempts to mimic these niches with the objective of determining potential anti-TB compound activity in models that reflect Mtb's characteristics *in vivo* and perhaps reflect activity against persisters.

Upon Mtb infection, macrophages are the first-line antimicrobial defense and they play a key role by triggering immune responses. However, Mtb is able to circumvent the macrophages' defenses by activating an inappropriate inflammatory response and promoting dysregulation of lipid metabolism, needed for the long-term intramacrophage survival of the bacilli (Russell et al., 2009). Indeed, foamy macrophages are observed in TB pathology, in patient sputum, and also in some rodent models of TB.

Based on Daniel et al., 2011, we developed a foamy macrophage (Foam-M) model, adapting differentiation of THP1 cells to generate lipid-loaded macrophages where Mtb is slowly-replicating or non-replicative. We optimized and validated a robust assay in 96 well plate format, then tested 18 reference TB drugs in this Foam-M assay, as well as our standard M.tb THP-1 macrophage and replicating and non-replicating extracellular Mtb assays. We observe differential activity, across the TB drug panel, between these assays.

Furthermore, using transcriptomics, we quantified 184 inflammatory and host response related genes and transcripts differently modulated by Mtb infection of Foam-M and standard THP-1 macrophages. Our study shows that, upon Mtb infection, the Foam-M inflammatory response is dampened compared to infected THP1 cells. Based on the observed differences between inflammatory responses, Mtb growth rates and drug activities observed, we conclude that the Foam-M and standard THP-1 assays represent differing Mtb intracellular environments. Further work is needed, to better understand the value of the Foam-M assay, taken together with data from other preclinical models, in translation.

Design, synthesis, and evaluation of novel Δ^2- thiazolino 2-pyridone derivatives that potentiate isoniazid activity in isoniazid-resistant *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (*Mtb*) drug resistance poses an alarming threat to global TB control, driving the urgent need to improve treatment regimen with newly developed compounds against both drug-sensitive and resistant strains. In our previous study[1], we discovered that certain substituted ring-fused 2-pyridones (*e.g.* C10) along with inhibition of respiration and biofilm formation, sensitized *Mtb* to stresses encountered during infection and restored activity to the frontline antibiotic isoniazid (INH) in otherwise INH-resistant *Mtb* isolates. In this study, we have further developed and improved the ring-fused 2-pyridone family of compounds by introducing different substituted heterocyclic linkers. The strategy was to design and synthesize common intermediates that allowed subsequent modifications aimed at improved activity and a greater understanding of the structure-activity relationships (SARs) (Figure 1). In this context, we have explored three different heterocyclic spacers (oxadiazole, isoxazole and 1,2,3-triazole) and evaluated their activity against *Mtb*. Two of the new 2-pyridone derivatives with a substituted isoxazole linker were 16-fold more potent than C10 at inhibiting *Mtb* respiration and biofilm formation, as well as potentiating INH activity against an *Mtb* katG mutant. Furthermore, these two compounds exhibited good toxicity profiles in Calu-3 human cells with >60 fold selectivity indexes. One of the enantiomers separated from the racemic mixture of the most active compound showed 5-fold improved activity compared to its racemate. These second generation anti-*Mtb* compounds provide great promise for the future development of therapeutics effective against drug-resistant *Mtb* strains.

1. Flentie et al., “Chemical Disarming of Isoniazid Resistance in Mycobacterium Tuberculosis.”

Photodynamic inactivation of *Mycobacterium tuberculosis*

M. Shleeva*[1], A. Kaprelyants[1], V. Nikitushkin[1], I. Linge[2], A. Apt[2], A. Savitsky[1]
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Mycobacterium tuberculosis (*Mtb*) is able to transit into a dormant state causing the latent state of tuberculosis. Dormant mycobacteria acquire resistance to all known antibacterial drugs, they are also able to survive in the human body for decades and become active, causing the active form of the disease. In order to cure latent tuberculosis new approaches need to be developed. The application of photodynamic inactivation (PDI) using exogenously added photosensitizers was recently discussed as an alternative approach for fighting multidrug-resistant *Mtb*. Earlier we had demonstrated a successful application of PDI for inactivation of dormant *Mycobacterium smegmatis* accumulating a significant amount of endogenous porphyrins.

The aim of this work is to study the accumulation of porphyrins upon transition of active *Mtb* to the dormant state, as well as to verify the effectiveness of PDI for such mycobacteria.

The dormant *Mtb* cells were obtained under gradual acidification in stationary phase in the presence of 5-aminolevulinic acid (ALA). An increase in the concentration of porphyrins in stationary phase correlated with the development of gradual acidification of the culture, the beginning of a decrease in metabolic activity and formation of ovoid dormant forms of the bacteria. Magnesium or zinc had a significant effect on the production of endogenous porphyrins in dormant mycobacteria. Dormant cells were exposed to light with the wavelengths of 532 nm or 565 nm, which correspond to porphyrin's absorption, for 5-60 minutes. Illumination of bacteria by laser beam resulted in inactivation of dormant *Mtb* according to viable bacteria number decrease estimated by MPN assay. We did not observe any PDI effect on active bacteria *in vitro* as these bacteria do not contain unbound porphyrins in substantial amounts. However, after persistence of active *Mtb* cells within macrophages for several days in the presence of ALA, a significant sensitivity of such cells (ca. 99.99%) to light exposure was developed.

For the first time we have demonstrated the successful application of PDI for inactivation of dormant *Mtb* cells and active mycobacteria located in macrophages due to significant accumulation of endogenous porphyrins.

This work was supported by Russian Science Foundation grant 19-15-00324.

Identification and characterization of new compounds targeting the MEP pathway as anti-tuberculotics

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Tuberculosis (TB) is a communicable disease caused by *Mycobacterium tuberculosis* (Mtb) which is one of the top 10 causes of death worldwide. To tackle this epidemic, it is necessary to shorten the TB therapy and find new drug targets. One of the promising drug targets identified is the 2C-Methyl-D-erythritol 4-phosphate (MEP) pathway, which is absent in humans and critically involved in cell wall formation, protein transportation etc. in Mtb. In this project, we focus on newly synthesized inhibitors that target the enzymes of the MEP pathway and evaluate their antimicrobial activity and validate the target using live virulent Mtb.

Forty-nine compounds were initially tested against fluorescent mCherry expressing Mtb at the concentration of 64uM to 1uM. Eight compounds showing 90% inhibition in broth, at the concentration of 64uM or lower, were selected for further evaluation of cytotoxicity and effect on intracellular Mtb growth in primary human macrophages. To validate the gene target of the selected compounds, conditional knockdown mutants of the target genes are being generated using cloning. A comparison of the antibacterial activity of these selected compounds against conditional knockdown mutants and the wild-type strain will validate the target of the compounds. These findings will help identify novel anti TB drugs and confirm the MEP pathway as a drug target for Mtb.

Utilizing evolutionary models for the prediction of resistance in *Mycobacterium tuberculosis* to new compounds

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Although drug resistant *Mycobacterium tuberculosis* complex (Mtbc) strains are emerging worldwide, only three new drugs have been approved for the treatment of tuberculosis (TB) in the last 50 years. To tackle this, various drug development initiatives including partnerships between public and private entities are accelerating bench to bedside pipelines for drug development in the past decade. In this work, through collaborations with TB Alliance and the European Regimen Accelerator for Tuberculosis (era4tb.org) consortium, an evolutionary model to determine drug resistance mechanisms was applied for the new drug candidate TBAJ-587 and its metabolite (M3). TBAJ-587 is a diarylquinoline (like bedaquiline; BDQ) which targets the ATP synthase of Mtbc bacteria.

In a first step, the workflow for the *in vitro* resistance evolution model was optimized and then applied to TBAJ-587 and its major metabolite M3. Mtbc reference strain H37Rv was exposed to sub-inhibitory concentrations of either TBAJ-587 or M3 over five culture passages. The selection of *de novo* resistant mutant clones was achieved by picking bacterial colonies which grew on solid media supplemented with high (>MIC) or low (50% MIC) concentrations of the compounds. The genotypic and phenotypic effect of variants was characterized, and clinical strain databases were scanned for pre-existing resistance.

Altogether, 93 mutant clones were analyzed for genomic variants by next generation sequencing, and phenotypically tested against TBAJ-587, M3, and BDQ. Altogether 33 unique variants were identified, affecting four genes – *atpE, Rv0678, atpB,* and *fadE25*. *Rv0678* and *atpE* variants were the most commonly selected, also known to garner cross-resistance with other diarylquinolines (i.e. BDQ).

A diversity of resistance-associated variants was defined by using the long-term evolution model. Several mutations affect known resistance mechanisms conferring bedaquiline-TBAJ-587 cross-resistance. Mutations in *atpB* and *fadE25* are potential new resistance mechanisms with diarylquinoline cross-resistance; however, their clinical relevance needs to be further investigated. Our data shows, that the model developed here can be used for bolstering genotypic testing even before drugs enter clinical application.

On behalf of the ERA4TB consortium. This work reflects only the author's views, and the JU is not responsible for any use that may be made of the information it contains.

Study of the mechanism of action of new benzothiazolthiazolidine derivative 11726172

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Tuberculosis (TB) is the leading cause of death from a single infectious agent, claiming more than 1.5 million lives per year, and an increasing number of multidrug-resistant *Mycobacterium tuberculosis* strains pose a serious health threat. Recently, notable findings have been made in describing the complexity of TB infection: in particular, the identification of mycobacterial heterogeneity associated with the development of TB disease. Currently, the continuous drug discovery for active and latent TB is critical to address the global health need.

A novel benzothiazolthiazolidine derivative 11726172 (4-nitrobenzo[c][1,2,5]thiadiazol-5-yl thiazolidine-3-carbodithioate) displays antibacterial activity against *M. tuberculosis* H37Rv both *in vitro* (MIC= 0,25 µg/mL), and in vivo. 11726172 also reveals a dose-dependent bactericidal activity against dormant non-culturable (NC) *M. tuberculosis* cells, which are highly tolerant to conventional anti-TB drugs.

The transcriptional analysis of *M. tuberculosis* cells upon 11726172 exposure by RNA-Seq identified several differently expressed genes (DEG) encoding proteins involved in ATP biosynthesis, sulfur and nitrogen metabolism, membrane protein synthesis. Genes involved in mycobacterial stress response and metal metabolism were markedly upregulated in 11726172-treated bacilli, suggesting that 11726172 may affect *M. tuberculosis* metal homeostasis. The search for a possible connection between the 11726172 activity and metal cations revealed that Cu^{2+} gradually reduced the MIC values of 11726172 in the 10-50 µM concentration range by 2-4 times, while Ni^{2+}, Co^{2+} and Zn^{2+} cations did not affect the 11726172 antibacterial activity. Having measured intracellular concentration of Cu^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} in *M. tuberculosis* bacilli, we found no influence of 11726172 on metal accumulation in contrast to several other previously reported copper-dependent antibacterial compounds. However, we cannot exclude that 11726172 may affect one or more *M. tuberculosis* copper-dependent enzymes.

We have identified a new perspective class of anti-TB compounds benzothiazolthiazolidines which are drug candidates for the curing of TB infection, including latent form.

Working Group on New TB Drugs: Global TB Drug Pipeline

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A robust and sustainable pipeline of TB drug candidates and discovery programs is essential for the successful development of new TB drug regimens. With the aim of increasing efficiency and coordination of the global TB drug R&D enterprise through information exchange, the Stop TB Partnership Working Group on New TB Drugs (WGND) conducts an annual survey of the TB drug pipeline including drug candidates from the global community. The WGND has now launched a dynamic web-based database with the aim of gathering comprehensive information and "real-time" updates in the field of TB drug R&D. The interactive pipeline is hosted on the WGND website at www.newtbdrugs.org/pipeline/clinical. We hope to receive maximum participation from the TB drug development community so that this information can serve as a central resource and a reference tool for global TB drug discovery, advocacy, and resource mobilization.

Mycobacterium tuberculosis requires the outer membrane lipid phthiocerol dimycocerosate for starvation-induced antibiotic tolerance

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Tolerance of *Mycobacterium tuberculosis* to antibiotics contributes to the long duration of tuberculosis treatment and to emergence of drug-resistant strains. Nutrient restriction induces *M. tuberculosis* drug tolerance, but the genetic determinants that promote antibiotic tolerance triggered by nutrient limitation have not been comprehensively identified. Here, we show that *M. tuberculosis* requires production of the outer membrane lipid phthiocerol dimycocerosate (PDIM) to tolerate antibiotics under nutrient-limited conditions. We developed an arrayed transposon (Tn) mutant library in *M. tuberculosis* Erdman and used orthogonal pooling and transposon sequencing (Tn-seq) to map the locations of individual mutants in the library. We screened a subset of the library (~1,000 mutants) by Tn-seq and identified 33 and 102 Tn mutants with altered tolerance to antibiotics in stationary phase and phosphate-starved conditions, respectively. Two mutants recovered from the arrayed library, *ppgK*::Tn and *clpS*::Tn, showed increased susceptibility to two different drug combinations in both nutrient-limited conditions, but their phenotypes were not complemented by the Tn-disrupted gene. Whole genome sequencing revealed single nucleotide polymorphisms in both the *ppgK*::Tn and *clpS*::Tn mutants that prevented PDIM production. Complementation of the *clpS*::Tn *ppsDQ291** mutant with *ppsD* restored PDIM production and antibiotic tolerance, demonstrating that loss of PDIM sensitized *M. tuberculosis* to antibiotics. Our data suggest that drugs targeting production of PDIM, a critical *M. tuberculosis* virulence determinant, have the potential to enhance the efficacy of existing antibiotics, thereby shortening tuberculosis treatment and limiting development of drug resistance.

Mutations in PonA1 transpeptidase domain and association with rifampicin resistance in *Mycobacterium tuberculosis*

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Rifampicin-resistance-associated tuberculosis is responsible for 6,000,000 cases worldwide and 240,000 deaths that continue to increase. Resistance to this drug has been associated primarily with mutations in the *rpoB* gene, which prevent rifampicin (RIF) binding. Some RIF-resistant strains harboring mutations in the *rpoB* gene suffer a loss of performance *in vitro*; however, strains have been found with mutations in *rpoB* that do not present these deficiencies but at the same time present mutations in the *rpoA* and *rpoC* genes, which would be generating a compensatory effect.

Based on our analysis of more than 2000 tuberculosis genomes from Peruvian clinical strains, it was found that mutations in *ponA1* were more frequently present in rifampicin-resistant (RIF) strains than in susceptible strains. The aim of our project was to evaluate how mutations in *ponA1* may contribute to the establishment of *rpoB* mutations and the subsequent resistance to RIF.

To explore this hypothesis, the transpeptidase domain of PonA1 was recombinantly expressed in *E coli* and direct interaction with RIF was evaluated by nuclear magnetic resonance-saturation transfer difference (NMR-STD). The affinity range found between the transpeptidase domain of PonA1 and RIF was in the millimolar order, so the generation of resistance to RIF would not be caused by any binding between PonA1 mutants and RIF.

To evaluate whether mutations in *ponA1* can mediate resistance to RIF, we generated a *Mycobacterium marinum* knocked out in the HXW97_06910 locus (*ponA1*'s homologous gene in *Mtb*) by Oligonucleotide-mediated recombineering followed by Bxb1 integrase targeting and complemented with *ponA1* of *Mtb*. We expose this strain with *ponA1* mutation and the wild-type *rpoB* gene to sub-inhibitory concentrations of RIF to induce mutagenesis and evaluate the occurrence of mutations per generation.

Novel synergistic combinations of bedaquiline and antimicrobial peptides kill *Mycobacterium tuberculosis*

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The emergence of antibiotic resistance in tuberculosis remains a global health issue. This is despite novel drugs including bedaquiline (BDQ), which is used to treat multi-drug resistant TB. However, BDQ exhibits severe side-effects in 50% of patients[1,2]. Lowering the dose whilst maintaining the bactericidal activity may result in few side effects. Antimicrobial peptides (AMPs) display a wide-spectrum antimicrobial activity. Lynronne, which were selected from the rumen microbiome, was found to bind to cellular membrane lipids and induce cell lysis in methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii*[3,4].

In this study, a synergistic reduction of the minimum inhibitory concentration (MIC) of BDQ and lynronne-1 and lynronne-3 was noted in the checkerboard assay with *mycobacterium tuberculosis*. Scanning electron microscopy (SEM) revealed variations in cellular structure in bacteria treated between single drugs and the combination. Membrane pores were found in mycobacterial cells treated with BDQ and lynronne-1 which indicates the compromising cell membrane induced by the combination. While with BDQ and lynronne-3, a polar aggregation of cellular contents was observed in mycobacterial cells. From the SEM results, the potential mechanisms of action of BDQ and AMPs might be different and need further investigation.

Results showed BDQ combined with AMPs have a synergistic inhibition and bactericidal effect on mycobacterial cells. The possible synergistic mechanism could be that the cellular membrane lysis induced by AMPs facilitates the uptake of BDQ into cells. However, varied cellular structures indicate the various synergistic mechanisms of BDQ with lynronne-1 or lynronne-3. This study identifies a promising novel synergistic drug combination to further improve the clinical practice of BDQ.

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Rv0140-induced granzyme B, a potential biomarker for antituberculosis treatment monitoring

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The lack of adequate diagnostic tools for patients' management and clinical decision-making during antituberculosis therapy (ATT) is one of the main problems hampering TB control. Thus, the identification of host biomarkers, indicative and predictive of the patient's response to treatment in easily accessible specimens such as blood could be of great help for ATT monitoring.

We previously proposed Rv0140-induced granzyme B as a discriminative biomarker of TB infection versus disease. Herein, we conducted a longitudinal study including 16 pulmonary aTB patients. Based on radiological arguments and smear conversion, our patients were categorized in slow (n=9) versus fast (n=7) responders to ATT. Blood samples were collected before treatment initiation and at 1, 2, 4 and 6 months, we also collected PBMCs from LTBI (n=32). PBMCs secretion of granzyme B and IFNg levels were measured in response to Rv0140 as well as PPD.

Our data showed that there is an increase in granzyme B secretion allowing discrimination of slow responders in comparison to fast responders at M1. This increase is followed by a decline at M2 and then granzyme B levels increased again gradually at M4 and M6 reaching levels observed in LTBI individuals.

Overall, our data suggest that Rv0140-induced granzyme B could be a predictive biomarker of ATT that may allow early stratification (M1) of patients as slow versus fast responders.

Culture-Free Enumeration of *Mycobacterium tuberculosis* in Mouse Tissues Using the Molecular Bacterial Load Assay for Preclinical Drug Development

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The turnaround times for phenotypic tests used to monitor the bacterial load of *Mycobacterium tuberculosis*, in both clinical and preclinical studies, are delayed by the organism's slow growth in culture media. The existence of differentially culturable populations of *M. tuberculosis* may result in an underestimate of the true number. We report here the adaptation of our robust, culture-free assay utilising 16S ribosomal RNA, developed for sputum, to enumerate the number of bacteria present in animal tissues as a tool to improve the read-outs in preclinical drug efficacy studies. We evaluated the utility of the assay, in comparison to bacterial counts estimated using growth assays on solid and liquid media, to accurately inform bacterial load in tissues from *M. tuberculosis* infected mice before and during treatment with a panel of drug combinations. When tested on lung tissues derived from infected mice, the MBL assay produced comparable results to the bacterial counts in solid culture CFU. Notably, under specific drug treatments, the MBL assay was able to detect a significantly higher number of *M. tuberculosis* compared to CFU, likely indicating the presence of bacteria that were unable to produce colonies in solid-based culture. Additionally, growth recovery in liquid media using the most probable number assay was able to account for the discrepancy between the MBL assay and CFU number, suggesting that the MBL assay detects differentially culturable sub-populations. The MBL assay can enumerate the bacterial load in animal tissues in real time without the need to wait for extended periods for cultures to grow. The readout correlates well with CFUs. Importantly, we have shown that the MBL is able to measure specific populations of bacteria not cultured on solid agar. The adaptation of this assay has the potential to decrease the readout time of data acquisition from animal experiments.

Sex Steroids and Sex Chromosome Complement Contribute to Male-Biased Outcomes from Tuberculosis

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Background: Active TB has long been known to be more prevalent in men than women, with a male:female ratio of ~70:30. The female protective effect is observed in both pre- and post-menopausal women, suggesting that sex hormones together with sex chromosomes are the likely determinants of this sex bias. Despite compelling epidemiologic data, studies of the biologic basis of this sex bias have been scarce.

Methods: We employed the 'Four Core Genotypes' (FCG) mouse model in which the sex-determining region Y protein gene (*Sry*) is transposed from the Y chromosome to Chr3. This model yields mice that are XX or XY gonadal females (XX-F and XY-F) and XX or XY gonadal males (XX-M and XY-M), and thus enables a 2x2 comparison of dimorphic phenotypes triggered by sex chromosomes and sex steroids. FCG mice were challenged with a low-dose aerosol infection of Mtb H37Rv; one group was held for time-to-death analysis, and a second group was euthanized at different intervals post-infection for quantification of bacillary burden, cytokine expression, and immunophenotyping by IHC and flow cytometry.

Results: We observed that TB disease progression was significantly higher in gonadal males (XX-M and XY-M) than in gonadal females (XX-F and XY-F), with a median time to death of 20 and 17 weeks vs. 44 and 35 weeks, respectively ($p < 0.001$). Gonadal males also had significantly higher lung bacillary burdens at 4 and 12 weeks post infection (6.9 and 7.4 (XX-M), 7.0 and 7.7 (XY-M) vs. 6.2 and 5.9 (XX-F), 6.3 and 6.1 (XY-F) $\log_{10}$ CFU, respectively). Similar results were observed in their spleen. Quantitative lung histopathology also revealed a higher degree of lung pathology and granuloma formation in gonadal males than gonadal females at both 4 and 12 weeks post-infection. Cytokine analysis in FCG BMDMs revealed that IL-6 levels were highest in XX-F and XY-F while iNOS levels were higher in XX-M and XY-M BMDMs, suggesting IL-6 and iNOS expression is gonadally-driven. These results indicate that the presence of testes and the male hormones they produce are a major determinant of male hyper-susceptibility to TB.

Nevertheless, we found evidence that sex chromosome complement also influences TB outcomes. Comparing XY-F to XX-F, we found a significantly higher spleen Mtb $\log_{10}$ CFU load in XY-F (5.6) than in XX-F (5.2) at 12 weeks post-infection. Indeed, XY-F mice were more susceptible to Mtb infection than XX-F ($p<0.05$). Although no overall difference in survival was observed between XY-M and XX-M, XY-M displayed a striking early mortality compared to XX-M, with an 18 week survival rate of 40% (XY-M) vs. 87% (XX-M) ($p<0.01$). Acute splenomegaly was observed specifically in XY-M and XY-F mice post-infection, signifying a ChrY-mediated control. Of note, XX-F lungs contained significantly higher numbers of $B220^+$ B cell lymphoid

follicles than XY-F and both types of gonadal males.

Conclusion: Altogether, these findings suggest that pulmonary TB progression is primarily influenced by sex hormones, leading to the more rapid progression in FCG gonadal male mice, but that the sex chromosome complement also plays role, with XX genotypes being protective. Further mechanistic studies on the biological factors underpinning the male sex bias towards more progressive TB disease are underway.

DNA Methylation Signatures from Buccal Swabs to Identify Tuberculosis Infection and Exposure

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Tuberculosis (TB) is the largest infectious cause of death worldwide. Pulmonary TB is diagnosed with microscopy detection of *M. tuberculosis* in sputum. This method is time-consuming and requires special laboratory facilities and expertise. There is a need for a time- and recourse-effective diagnostic tool. Developments of non-invasive diagnostic methods have included buccal swabs and face mask sampling to detect *M. tuberculosis* DNA, but with varying sensitivity due to the heterogeneity in levels of bacteria present in the oral cavity and levels of aerosolized *M. tuberculosis*. DNA methylation signatures of the host immune cells are also emerging as diagnostic tools for various diseases, including cancer. For tuberculosis, this has been studied mostly in peripheral blood mononuclear cells and sputum-derived immune cells. In this novel and exploratory pilot study, we show the potential of using buccal swabs to collect host DNA and investigate the DNA methylation signatures as a diagnostic tool. Buccal swabs were collected from pulmonary TB patients (n= 6), TB-exposed household- or occupational contacts (n= 7), and healthy controls (n= 9) at Linköping University Hospital. Using Illumina Infinium 850k EPIC array the DNA methylation status in the buccal swabs was determined. The data were normalized by applying the Subset-quantile Within Array Normalization and standard filtering for CpG sites containing single nucleotide polymorphisms or located in the X and Y chromosomes. A principal component analysis of the data showed a separation of the groups with 70% confidence ellipses. Between the active TB patients and healthy controls 3932 significantly differentially methylated CpG-sites (adjusted p value < 0.05 and $\log_2$ Fold change > 0.2) were identified using linear regression in limma package. In summary, the result from this study shows the potential of using DNA methylation signatures from buccal swabs to develop new diagnostic strategies for TB.

Comparative mRNA expression and alternative splicing profiling of macrophages infected with virulent H37Rv *Mycobacterium tuberculosis* or attenuated H37Ra

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Mycobacterium tuberculosis (Mtb) is the major causative agent of the tuberculosis (TB), which leads to worldwide bacterial cause of death. Although, several cellular responses caused by Mtb infection are well understood, the strain-specific alterations in gene expression by virulent Mtb H37Rv (Rv) or attenuated Mtb H37Ra (Ra) are still unclear. Here, we compared the gene expression changes between H37Rv and H37Ra infected bone marrow derived macrophages (BMDMs). We sorted specific genes with FPKM ≥ 10 and FDR < 0.1%, which are changed by Rv or Ra comparing with that of uninfected control respectively. We found that expression levels of 413 genes were significantly changed by Ra specifically, comparing to uninfected control. Ra specific 262 genes were highly increased, whereas the other 149 genes were decreased. Comparing with uninfected control, Rv infected macrophages showed 131 differentially expressed genes, by which expression levels of 56 genes were decreased and 74 genes were increased. Expression of mRNAs related to endoplasmic reticulum, Golgi apparatus, and cytoplasmic vesicle was more altered in Ra infected cells, whereas mRNAs related to integral components of membrane, such as ATPase and several immune receptors, were more changed by Rv infection. In addition, we analyzed the alternative splicing profile upon Mtb infection and validated the splicing events. 291 genes were alternatively spliced in Rv infected cells and 418 genes by Ra infection. These genes were highly related to cell death process and immune system process. Taken together, our study shed light on the strain specific-gene expression profile in Mtb-infected macrophages, leading to the valuable insights into TB pathogenesis.

Host correlates and determinants of sterilization and relapse in *M. tuberculosis* infection

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Although 5% of tuberculosis (TB) patients relapse after six months of first-line therapy and approximately 20% relapse after four months of short-course therapy, host immune response signatures and biomarkers predictive of relapse to active TB are poorly defined. To address this knowledge gap, our lab developed a model of *M. tuberculosis* (Mtb) infection which aims to recapitulate aspects of human latent TB infection and relapse in mice. We use a genetically engineered Mtb mutant strain that can be cleared from infected mice to levels undetectable by colony-forming unit enumeration (paucibacillary infection), via administration of doxycycline in mouse chow. Months after discontinuation of doxycycline diet, however, about 20-50% of paucibacillary infected mice relapse. Our goals are (i) to identify host immune response signatures and biomarkers predictive of TB relapse by analyzing immune cell populations in the lung and mediastinal lymph nodes – which have been understudied in Mtb infection - during paucibacillary infection, and (ii) to assess the contribution of specific immune cell populations (e.g. NK cells) and host factors (e.g. PD-1 signaling) to control of Mtb infection, via monoclonal antibody therapy during paucibacillary infection. This work will help elucidate aspects of the host immune response during latent TB that either contribute to successful control of infection or lead to TB relapse, and ultimately will help target post-TB screening and care.

A human monoclonal antibody isolated from the IgA repertoire of a chronically exposed, asymptomatic individual mediates protection against *Mycobacterium tuberculosis*

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Tuberculosis (TB) is a life-threatening airborne infectious disease caused by the intracellular pathogen *Mycobacterium tuberculosis* (Mtb). TB has become a global health concern, with a quarter of the global population being latently infected and 10 million active infections annually. The emergence of multidrug-resistant Mtb has developed into a major setback for current antibiotic treatment regimes. Under these circumstances, better vaccination and/or alternative therapeutic approaches are vital to combat the global disease burden. Whilst a functional role for humoral immunity in Mtb protection remains ambiguous, several studies have shown that antibodies play an important role in host defence against this pathogen. Moreover, the potential of antibody therapy/prophylaxis for combating microbes that are resistant to antibiotics represents an interesting alternative approach for targeting this disease. Studying antibody repertoires and identifying natural protective antibodies against Mtb from asymptomatic individuals who remain immune to active disease despite being in a high Mtb exposure setting, would also have significant implications for vaccine developmental strategies. Our aim was to develop fully human monoclonal antibodies against whole-cell Mtb from a cohort of asymptomatic TB health care workers and conduct a thorough characterization of the isolated antibodies. We performed IgG and IgA high-throughput screening from the donor memory B cell repertoire using gamma-irradiated whole-cell Mtb as the target antigen. From the IgA/IgG repertoire, we identified a number of human monoclonal antibodies targeting the Mtb surface antigens. A detailed biophysical and biochemical characterization of their specificity, function and fundamental biology has been studied.

Effect of *in vivo* B-cell depletion during chronic stage of TB-infection in genetically resistant C57BL/6 mice

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It is well established both in humans and in animal models that during tuberculosis (TB) infection B cells migrate into the inflamed lung tissue and form there B-cell follicles (BCFs) in close vicinity of granuloma. The exact role of B cells and BCFs in TB is still under consideration. It was shown previously that B cell-lacking (B-/-) mice display enhanced susceptibility to TB infection and that B-cell depletion before and during the first weeks of infection in non-human primates leads to an altered local T cell and cytokine responses and an increased bacterial burden. Here we demonstrate that whilst a significant number of B cells and BCFs persist up to weeks 25-35 post challenge in the lungs of C57BL/6 (B6) mice, in hyper-susceptible I/St mice BCFs disintegrate and B-cells disappear from the lung tissue as early as 12-16 weeks post infection. This is accompanied with severe lung pathology, diffuse inflammation, formation of necrotic zones and significantly elevated levels of proinflammatory cytokines IL-1, IL-11, IL-17a, and TNF-α as compared to B6 mice. We hypothesized that B cells and BCFs may participate in the control of TB infection and inflammation during chronic stage of infection in resistant B6 mice. To check this hypothesis, we specifically depleted B-cells from B6 mice by administration of anti-CD20 mAbs at week 16 post infection. This intervention had only marginal effect on the level of proinflammatory cytokines, except a slight increase in IL-6 and IL-11 levels at week 4 post B-cell depletion, but resulted in an increase of neutrophil counts, lung mycobacterial burdens and a decreased lifespan of infected animals. Taken together, our data suggest that lung B-cells and BCFs moderately participate in the containment of chronic TB infection.

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LC-MS/MS biotyping as a foundation of high-throughput diagnostics of Mycobacterium infections

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The WHO TB strategy for 2015-2035 is based on three main pillars: diagnostics, therapy and prevention. The major condition for improving the epidemiological situation of tuberculosis is the implementation of fast and reliable diagnostic methods that would enable not only early detection of M.tb infection, but also differentiation of M.tb from nontuberculous mycobacteria (MOTT) and undertake appropriate controlled treatment and monitoring of the progress of the therapy.

Centrum Medycyny Klinicznej Dimedical sp. z o.o. over 2017-2019 conducted research and development works to develop the innovative diagnostic method for accurate detection of M.tb and MOTT based on LC-MS/MS analysis of mycolic acid profiles (MAs). **The solution proposed by our team significantly shortens the time of diagnosis below 24 hours, while increasing the specificity of the test to 95-99,8% and sensitivity below 50 cfu/ml. The obtained results indicate a breakthrough nature of the developed solution due to a significant improvement in the tuberculosis diagnosis process with additional cost reduction in comparison to current diagnostic methods**.

The LC-MS/MS screening methods were based on precursor ion scanning of α-alkylchains from the most frequently occurring microorganisms' MAs in the studied group. The obtained MAs profiles were subjected to PCA analysis. The PCA results allowed for a clear and unambiguous differentiation of selected species and species - specific MAs selection, which can be further used as biomarkers in the biotyping of microorganisms of the Mycobacterium genus.

Since May 2022 our diagnostic method has due registration according to the Directive IVD 98/79/EEC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices.

Our patented medical device comprises of M-Typer software and M-Typer reagents.

M-Typer software has been developed and predefined for comparing the results of mycolic acids (MA) analyzes using tandem mass spectrometry in order to assess similarity between samples (taken from the patient) and the library samples obtained using the same measurement method.

M-Typer reagents are used to isolate mycolic acids from biological material (including sputum) for subsequent analysis, using flow-injection analysis coupled with tandem mass spectrometry.

Differential re-stimulation of *Mycobacterium tuberculosis* specific IgA and IgM mucosal antibodies following an intra-lung challenge in a highly exposed South African cohort

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In order to control the current Tuberculosis (TB) epidemic a highly effective vaccine is essential. Most vaccines have aimed to stimulate a strong cellular immune response, which have shown limited success. Increasing evidence suggests a pivotal role for the humoral immune response in protection, and it is likely that engagement of both arms of the adaptive immune response is essential for protection from infection and disease. New vaccine designs depend on the identification of antigens which stimulate a protective immune response. Protein and lipid microarrays allow for simultaneous assessment of multiple antigens in parallel making it possible to identify antibody correlates of protection.

Individuals previously exposed to *Mycobacterium tuberculosis* (*Mtb*), through household contacts, displaying a spectrum of disease susceptibility, were subjected to a 3-day intra-lung challenge with live bacillus Calmette-Guérin (BCG). Bronchoalveolar lavage (BAL) was collected before and after re-challenge and used to assess the changes in antibody signatures post re-exposure. *Mtb* lipid fractions and various purified glycolipids and lipopolysaccharides were obtained from BEI resources. Nitrocellulose microarray slides were fabricated to create a multiplexed assay to assess multiple antigens in parallel. Binding assays were performed to detect IgM and IgA reactivity to total and purified lipids.

Quantitative data showed differential re-stimulation of antigen specific IgA and IgM antibodies. In a clustering analysis, antibody signatures successfully distinguished between participants with no signs of prior infection and those with one or more cases of active TB. A spectrum of antibody profiles was observed in those with assumed latent TB infection, with participants spread between both the protective and susceptible disease groups.

Identification of 4-markers host protein signature for Tuberculosis diagnosis and treatment monitoring using a multiplex assay

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Background: There is an urgent need for rapid non-sputum-based tests to identify and treat patients infected with *Mycobacterium tuberculosis (Mtb)*. In this study, we aimed to develop and evaluate an in-house multiplex assay for the detection of host biomarkers for both TB triage and treatment monitoring.

Methods: Seven host biomarkers were evaluated in plasma samples obtained from 37 HIV-negative microbiologically confirmed pulmonary TB cases (ATB), at baseline and following 6-months treatment, 23 patients with latent TB infection (LTBI, asymptomatic, positive QFT-P) and 24 healthy donors (HD, asymptomatic, negative QFT-P) from Madagascar. Bio-signatures combinations and performance were evaluated using CombiROC algorithm.

Findings: A total of 120 biomarker combinations have been identified for each comparison group. They were rank by performance. A 4-markers host protein signature (**CLEC3B-ECM1-IP10-SELL**) showed 94.6% of sensitivity and 91.7% specificity to discriminate ATB from HD, reaching WHO target product profile (TPP) for **triage** test. This signature also reached TPP for **treatment monitoring**, by identifying at baseline **fast and slow culture converters** with an AUC=0.87 (sensitivity=83.3%, specificity=84%).

Conclusion: Our data demonstrate that a **CLEC3B-ECM1-IP10-SELL** host biomarker signature is relevant for both TB triage and treatment monitoring, as they reached WHO TPP profile for both purposes.

Purpose	Groups	AUC	SE	SP	CutOff	ACC	TN	TP	FN	FP	NPV	PPV
Triage												
	ATB vs HD	0.958	0.946	0.917	0.412	0.934	22	35	2	2	0.917	0.946
	ATB vs LTBI	0.929	0.892	0.913	0.536	0.90	21	33	4	2	0.840	0.943
	HD vs LTBI	0.741	0.565	0.958	0.564	0.766	23	13	10	1	0.697	0.929
Treatment monitoring												
	Fast vs Slow converters	0.870	0.833	0.840	0.272	0.838	21	10	2	4	0.913	0.714

Distinct antibody response across the clinical spectrum of pediatric tuberculosis

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Tuberculosis, which is caused by Mycobacterium tuberculosis (Mtb), is among the leading cause of death among children worldwide from an infectious agent. Children represent a particularly vulnerable population because of the greater challenges for the diagnosis and the high risk of progression to severe forms of the disease. To study the mechanisms associated with the pathophysiology of pediatric tuberculosis (TB), we focused on the humoral response of infants less than 5 years old living in South Africa, and we comprehensively profiled antibody response against 96 Mtb antigens among healthy children, as well as children with TB. In addition to age-dependent antibody response, mainly involving increased IgG4 levels with age, our results highlighted major biophysical as well as functional differences between healthy children and those with TB. While the control group was associated with higher levels of IgA, correlated with stronger binding to FcαR, children with TB exhibited higher Mtb-specific IgG and IgM titers, as well as stronger antibody binding to Fc receptors. Our results also showed that, among children with TB, higher IgG3 levels were associated with reduced bacterial load, while infants with high bacterial burden had highly functional antibody that strongly induced cellular phagocytosis. Overall, our results highlight the major impact of age on Mtb-specific humoral response and show that Mtb infection in children triggers distinct clinical manifestations that are associated with specific antibody responses.

High levels of *Mycobacterium tuberculosis*-specific IgG and IgM, and moderate IgA in helminth infected TB patients that have dominance of *Ascaris lumbricoides*

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Helminth/tuberculosis (TB)-coinfection can reduce cell-mediated immunity against *Mycobacterium tuberculosis* (Mtb) and increase disease severity, although the effects are highly helminth species dependent. Mtb have long been ranked as the number one single infectious agent claiming the most lives. The only licensed vaccine for TB (BCG) offers highly variable protection against TB, and almost no protection against transmission of Mtb. In recent few years the identification of naturally occurring antibodies in humans that are protective during Mtb infection has reignited the interest in humoral immunity against TB and its possible implementation in novel TB vaccine design. The effects of helminth/TB coinfection on the humoral response against Mtb during active TB are however still unclear, and specifically the effect by the globally prevalent helminth specie *Ascaris lumbricoides*. Plasma samples were used to measure both total and Mtb-specific antibody responses in a Peruvian endemic setting were Ascaris is the dominating helminth infection. Mtb-specific antibodies were detected by indirect ELISA and coating with either purified protein derivative from Mtb (PPD) or a Mtb cell-membrane fraction for more precisely detecting antibodies raised against the surface of Mtb. Ascaris/TB coinfected patients had the highest levels of Mtb-specific IgG (mostly comprised of an IgG1 and IgG2 subclass response against the surface of Mtb) and IgM, whereas TB patients without helminth infection had the highest level of Mtb-specific IgA and almost completely lacked IgM. Ascaris/TB coinfected patients also had a modest but significant increase in Mtb-specific IgG4 altogether suggesting a sustained humoral protection against Mtb with an anti-inflammatory profile. More studies on the species-specific impact of helminths on the adaptive humoral response against Mtb, and in relation to TB disease severity, are needed

Whole blood gene expression signature defines severity of disease in the Cologne cohort of extrapulmonary tuberculosis

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Objectives: With an estimated 10 million new infections worldwide in 2020, tuberculosis (TB) remains a leading cause of mortality and morbidity among infectious agents. Globally, around 15 % of diagnosed cases are extrapulmonary TB (EPTB), and the incidence in some European countries is rising. While there is a plethora of literature on pulmonary TB (PTB), information on EPTB remains scarce. Diagnosis and assessment of therapeutic success are particularly challenging. Our detailed analysis of clinical data in a well-defined cohort will aid in understanding of EPTB and help to improve the treatment of EPTB patients.

Methods: A total of 44 EPTB patients treated at the University Hospital of Cologne, Germany, were enrolled in the EX-TB study. Baseline and longitudinal data were collected including information on the diagnostic process and clinical parameters. In addition, blood and serum samples were collected longitudinally. Global bulk RNAseq was performed to identify gene signatures over time.

Results: Out of 44 patients included in the EPTB cohort, 39% (17) were diagnosed with limited disease (cervical lymph node TB), 61% (27) were diagnosed with extensive disease and 55% (15) of these showed disseminated disease affecting two or more organs. Whole blood RNA sequencing displays disease severity and longitudinal antibiotic treatment success. Further, distinct gene signatures were identified characterizing limited disease and extensive disease. Limited disease signatures are defined by interferon signaling pathways, whereas pro-inflammatory and myeloid cell driven pathways characterize extensive disease signatures.

Conclusion/Outlook: EPTB presents with highly heterogeneous disease manifestations. Whole blood RNA sequencing revealed distinct gene clusters discriminating limited and extensive disease. In the future, these signatures may be used as diagnostic tool for the treatment of EPTB. Single cell RNA sequencing and global multicolor flow cytometry and luminex cytokine arrays will be performed to further decipher the immunological fingerprint of EPTB. A multicenter mEX-TB study will be initiated in 2022 in six German university clinics.

Discovery of a six-marker transcriptomic signature for diagnosis of childhood tuberculosis disease in an african multi-country cohort

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Background: Approximately 1.2 million children develop tuberculosis (TB) disease annually, with an estimated 230,000 deaths. Microbiological diagnosis of TB remains challenging. Gene expression signatures in blood may offer a fast, reliable, non-sputum based diagnostic test. Paediatric data are very limited, therefore most of the TB signatures were discovered in adults, with underperformance in children. We undertook a multi-country gene expression signature discovery study to identify a transcriptomic signature for paediatric TB disease.

Methods: Whole blood was collected from 571 children (<15 years of age) presenting to health care facilities in South Africa, Malawi, Kenya and The Gambia with suspected TB between 2008 and 2018. Cases included pulmonary (PTB) or extra-pulmonary TB (EPTB), with or without HIV-infection; 264 (48%) had microbiologically confirmed TB and 307 (52%) other diseases (OD). RNA extraction and RNA-Sequencing were done on blood samples collected at enrolment. Quality control, batch-correction and normalisation were performed on the data prior to differential expression and feature selection analyses in R.

Results: Differential gene expression analysis identified 178 candidate biomarker genes with log2 fold change over 0.5 or less than -0.5 and adjusted-p-value less than 0.05. A feature selection algorithm with cross-validation (randomly selecting 80% of the data as training set and 20% as test set), run on the 178 genes, selected a 6-gene signature to distinguish TB disease from OD with an AUC of 91.2%, sensitivity of 80.0% and specificity of 92.2% when

maximising the Youden index. When sensitivity was fixed at 72.0%, in line with the minimum WHO requirement for a non-sputum TB test in children, specificity reached 93.1%, meeting WHO specificity criteria. The AUC of the signature was 88.7% in the PTB group, 92.0% in the EPTB group and 94.9% when both PTB and EPTB were present, 87.1% for HIV-infected and 92.1% for HIV-uninfected patients.

Conclusions: This novel 6-gene transcriptomic signature meets the minimum WHO Target Product Profile criteria for a non-sputum-based paediatric TB test. Cross-sample, cross-platform validation using targeted RT-PCR methods is currently underway, aiming at the development of a novel, rapid, blood-based and inexpensive point-of-care test for low and middle-income countries with high TB burden.

The expression of the universal second messenger c-di-AMP is regulated by the PhoPR virulence system and has an impact on safety and efficacy of live tuberculosis vaccines

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c-di-AMP constitute one of the universal second messengers endogenously produced by several pathogens. In fact, its essential synthesis by some bacteria highlights the importance of bacterial c-di-AMP for host-pathogen signalling. The roles of this cyclic (di)nucleotide in *Mycobacterium tuberculosis* immunity and virulence have been largely explored, although its contribution to the safety and efficacy of live tuberculosis vaccines is less understood.

In this study, we demonstrate that the synthesis and secretion of c-di-AMP is negatively regulated by the *M. tuberculosis* PhoPR virulence system. Accordingly, the live attenuated vaccine candidate *M. tuberculosis* vaccine (MTBVAC), based on double *phoP* and *fadD26* deletions, produces more than 25- and 45-fold c-di-AMP levels relative to wild-type *M. tuberculosis* or the current vaccine bacilli Calmette-Guérin (BCG), respectively. These unexpected results of exacerbated amounts of c-di-AMP produced by MTBVAC relative to its parental *M. tuberculosis* MT103 was also confirmed in the *M. tuberculosis phoPR* mutant and a *phoPR*-complemented strain constructed in the H37Rv genetic background. Regarding the secretion of c-di-AMP, it was exclusively detected in MTBVAC but not in *M. tuberculosis* or in BCG. Furthermore, our results suggest a direct correlation between intracellular production and the secretion of this molecule.

We also demonstrate that c-di-AMP synthesis during *in vitro* cultivation of *M. tuberculosis* is a growth-phase- and medium-dependent phenotype.

To uncover the role of this metabolite in the vaccine properties of MTBVAC, we constructed and validated knockout and overproducing/oversecreting derivatives by inactivating the *disA* or *cnpB* gene, respectively. All MTBVAC derivatives elicited superior interleukin-1b (IL-1b) responses compared with BCG during an *in vitro* infection of human macrophages. However, both vaccines failed to elicit interferon b (IFNb) activation in this cellular model, consistent with our hypothesis that a functional ESX-1 system is essential to mount type I IFN responses against *M. tuberculosis*.

We found that increasing c-di-AMP levels remarkably correlated with a safer profile of tuberculosis vaccines in the immunodeficient mouse model.

Finally, we demonstrate that overproduction of c-di-AMP due to *cnpB* inactivation resulted in lower protection of MTBVAC, while the absence of c-di-AMP in the MTBVAC *disA* derivative maintains the protective efficacy of this vaccine in mice.

Exploring new recombinant BCG Vaccines and different Vaccination Routes for better Tuberculosis Protection

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Introduction: The only currently licensed anti-tuberculosis (TB) vaccine, *Mycobacterium bovis* BCG, provides limited protection against pulmonary TB in adolescents and adults. A feature of this attenuated live vaccine strain is the partial deletion of the genomic locus encoding the ESX-1 type VII secretion system, which in the biosafety level 3 (BSL3) pathogen *Mycobacterium tuberculosis* governs phagosomal rupture and cytosolic pattern recognition, key intracellular phenotypes linked to increased immune signaling.

Methods and Previous Findings: To obtain an improved recombinant BCG Pasteur vaccine strain with increased immune signaling but still low virulence, we have previously integrated the extended *esx-1* genomic region of *Mycobacterium marinum*, a BSL2 organism, into a BCG strain of the BCG Pasteur subtype. This recombinant strain named rBCG::ESX-1^{Mmar}, is heterologously expressing ESX-1 functions of *M. marinum* and thereby modulates the host innate immune response via phagosomal rupture-associated induction of type I interferon (IFN) responses and enhanced inflammasome activity. These features result in vaccine-induced higher IL-1b release and higher proportions of CD8$^+$ T cell effectors against mycobacterial antigens and polyfunctional CD4$^+$ Th1 cells specific to ESX-1 antigens. Importantly, rBCG::ESX-1^{Mmar} confers superior protection relative to parental BCG in murine vaccination models (*Gröschel, Sayes et al. Cell Reports, 2017*).

Results: In our most recent studies, we have focused on different routes of vaccination, by using parental BCG Pasteur and rBCG Pasteur::ESX-1^{Mmar}. We found that mice vaccinated via the aerosol route with BCG Pasteur or rBCG Pasteur::ESX-1^{Mmar} yielded higher frequencies of IFN-g-producing CD4$^+$ and CD8$^+$ T effectors in the lungs compared to subcutaneous immunized counterparts. Moreover, only aerosol vaccination was able to elicit Th17 and lung resident memory T cells without severe lung pathology. We show that vaccination of mice with BCG Pasteur or rBCG Pasteur::ESX-1^{Mmar} via the aerosol route leads to improved TB protection and lower lung pathology compared to subcutaneous vaccination.

Conclusion: The attenuated rBCG::ESX-1^{Mmar} vaccine displayed a superior T-cell immunity and TB protection when mice were vaccinated via aerosol or subcutaneous route compared to parental BCG strain and thereby represents an interesting candidate for defining new promising strategies of vaccination against TB.

Investigating the spectrum of virulence and immunogenicity linked to genetic diversity of the *Mycobacterium tuberculosis* complex in human *in vitro* granulomas

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The course and outcome of tuberculosis (TB) is the result of a complex interplay between environmental, host and bacterial traits. Regarding the pathogen's contribution, the human-adapted members of the *Mycobacterium tuberculosis* complex (MTBC) can be classified into seven major lineages (L1-L7). Various epidemiological and experimental observations support that this genetic diversity translates in immunogenicity and virulence heterogeneity. In particular, strains belonging to the Beijing family of L2 have been recurrently associated with a hyper-virulent, hypo-inflammatory phenotype.

A hallmark of the immunopathology of TB is the formation of structurally organized cell aggregates called granulomas. These structures constitute the scene of the crosstalk between the tuberculous bacilli and the host's immune system. To delve into the impact of the mycobacterial genotype on this interaction, we have combined a 3D *in vitro* granuloma model with a collection of strains representative of the global diversity of the MTBC. As proof of concept, we compared four L2 members (encompassing one proto-Beijing, one "ancient" and two "modern" Beijing strains) with the L4 reference strain H37Rv. One of the "modern" Beijing strains showed a significant increase in bacterial burden consistent with the hyper-virulence commonly linked to this family. Moreover, all the L2 strains, with the exception of the "ancient" Beijing, showed a tendency to induce lower levels of pro-inflammatory cytokines when compared with H37Rv. Previous studies have reported an increased expression of the dormancy regulon in L2 strains. We observed variable proportions of bacteria accumulating intracytosolic lipid inclusions, a phenotype associated with dormancy, among the strains before infection. However, these differences were abolished under immune pressure within *in vitro* granulomas.

In summary, these results highlight the relevance of *in vitro* granulomas to dissect host-pathogen interactions in the context of the MTBC's phylogenetic diversity. Our findings may contribute to understanding the mechanisms underlying the diverse virulence and immunogenicity across members of the MTBC.

Investigation of immune biosignatures for cervical tuberculous lyphadenetis diagnosis

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Cervical Tuberculous lymphadenitis (CTL) represents currently a major public health problem in Tunisia. Paucibacillary nature of specimens and mimicking Cervical Non-Tuberculous Lymphadenitis (CNTL) makes its diagnosis challenging. In order to better understand the physiopathology of the disease and identify biomarkers with potential diagnosis, we measured, by RT-PCR, a panel of 14 inflammatory cytokines, anti-inflammatory cytokines and cytotoxic mediators in peripheral blood from patients with CTL (n=17), NCTL (n=14) and healthy controls (HC, n=17) from a prospective clinical study. In CTL group we did describe associations between gene expression levels and different bacillary load based on GeneXpert Ultra results.

Multidimensional analyses showed that *Mycobacterium tuberculosis* (Mtb) infection induced changes in both the expression and correlation profiles of investigated markers. Node analysis of the CTL network in blood indicated that, IL12p35 and CCL5, followed by TGFβ were the most highly connected markers compared to CNTL implying that their regulation may be preferentially more susceptible to modulation by *Mtb* infection. However, at the site of infection, Granulysin and Foxp3 followed by EBi3 and Perforin, were the most highly connected markers compared to CNTL. Surprisingly, we noted that the increase in bacillary load, at the site of infection, do positively correlated with gene expression of TGF-b and negatively correlated with Ganulysin in CTL patients.

Our findings describe local and systemic specific Mtb infection changes in gene expression and correlation profiles in the context of lymphadenitis. In CTL group we described local gene expression signature in association with bacterial load.

Targeting nitrogen metabolism of *Mycobacterium tuberculosis* for identification of novel druggable targets and systems-based whole cell compound screening for anti-TB drug development

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Mycobacterium tuberculosis (Mtb) is a successful human pathogen that uses metabolic adaptations as a strategy to survive in the harsh intracellular host environment. Using a combination of molecular, metabolomic and metabolic flux modelling approaches we measured nitrogen metabolism of Mtb replicating in human macrophages and identified key host-pathogen metabolic cross talk that sustained Mtb's growth, survival, and virulence. We demonstrated that Mtb uses multiple host amino acids including alanine, aspartate, glutamate, glutamine, valine, and glycine as both carbon and nitrogen sources in human macrophages (1). The uptake of these nutrients and assimilation of their nitrogen atoms to other compounds are promising druggable targets.

We recently conducted dual isotopic labelling with ^{13}C-glycerol and ^{15}N-ammonium chloride and developed Bayesian $^{13}C^{15}N$-metabolic flux analysis technique for parallel quantification of *in vivo* carbon and nitrogen metabolic fluxes in Mtb. We quantified all central carbon-nitrogen (CN) fluxes in the system and identified that the highest fluxes were in glutamate and glutamine biosynthesis, demonstrating the importance of this node in sustaining the core metabolism of Mtb (2).

Serine biosynthesis is essential for *in vivo* survival of Mtb as it has no access to this nitrogen source inside macrophages. We demonstrated severe attenuation of Δ*serC* Mtb (serine auxotroph) in macrophages and identified phosphoserine transaminase *serC* (Rv0884c) that catalyzed serine biosynthesis as a druggable target in Mtb. Compounds active against Rv0884c are currently being screened using our novel fluorescent reporter-based whole cell compound assay that targets either *serC* or an enzymatic target that is essential for serine uptake in Mtb.

In summary, our study provides a comprehensive measurement of Mtb's nitrogen metabolism and identified druggable targets for development of new anti-TB drugs.

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Mycobacterium tuberculosis mediated inflammasome inhibition

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Mycobacterium tuberculosis (Mtb) has evolved to evade host innate immunity by interfering with macrophage functions. Interleukin-1β (IL-1β) is secreted by macrophages after the activation of the inflammasome complex and is crucial for host defense against Mtb infections.

In the present study we show that Mtb is able to inhibit host cell NLRP3- and AIM2 inflammasome activation and pyroptosis. We show that the AIM2 inhibition is dependent on a functional ESX-1 secretion system, whereas the NLRP3 inflammasome inhibition is independent of ESX-1. We performed a genetic screen to identify Mtb genes involved in the process of host cell inflammasome inhibition and discovered that the serine/threonine kinase PknF is one protein of Mtb involved in the inflammasome inhibition. The *pknF* deletion mutant of Mtb induces increased production of IL-1β in bone marrow-derived macrophages (BMDMs). The increased production of IL-1β was dependent on NLRP3, the adaptor protein ASC and the protease caspase-1, as revealed by studies performed in BMDMs derived from the corresponding knockout mice. We further demonstrate that infection of BMDMs with the *pknF* deletion mutant results in increased pyroptosis compared to Mtb-infected cells. We detect no significant difference in interleukin-6 (IL-6) production, a marker of inflammation, in BMDMs infected with the Mtb *pknF* mutant strain which suggest that the mutant did not affect the priming step of inflammasome activation. In contrast, the activation step was affected since potassium efflux, chloride efflux and the generation of reactive oxygen species played a significant role in inflammasome activation and subsequent pyroptosis mediated by the Mtb *pknF* mutant strain.

In conclusion, we discovered that Mtb inhibits the host cell inflammasome via several, distinct mechanisms and that the serine/threonine kinase PknF of Mtb, is involved in inhibiting the host cell NLRP3 inflammasome activation pathway.

Characterization of the activation process of the sigma factor σE regulatory network in *Mycobacterium tuberculosis* under stress conditions

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SigE (σ^E) is one of the 13 sigma factors encoded by the *Mycobacterium tuberculosis* chromosome, it is implicated in a very complex regulatory network involving other regulators such as the two component system MprAB, the pleyotropic regulator ClgR and the anti-sigma factor RseA, and its role is of prime importance for virulence.

Through real-time PCR analysis, the dynamic of transcription of several *sigE* dependent genes was studied: *sigE* itself*, sigB* whose transcription is due to SigE both in physiological and under stress conditions*, rseA* and *clp*, encoding a protease responsible for the degradation of the SigE-RseA complex after its phosphorylation by PknB.

The SigE regulatory network was analyzed in mutant strains under different stress conditions that mimic the challenging environments to which tubercular bacilli are exposed during the infection process.

The data strongly support the fundamental role of both MrpAB and ClgR to act out an efficient stress response under surface stress; indeed their presence is necessary to induce the expression of *sigE*. The effect of SigE activity could be seen in the transcriptional dynamic of *sigB* that requires the two-component system and ClgR to be induced and sustained.

In acidic pH, the absence of MprAB and ClgR led to a delayed stress response. In both cases, the anti-sigma factor RseA must be phosphorylated to activate a full *sigE*-mediated response. These results provide a clearer insight into the mechanism of adaptation to specific stress in *M. tuberculosis* and allow a better understanding of the physiology of this powerful pathogen.

A Mycobacterium tuberculosis effector targets mitochondrion, controls energy metabolism and limits cytochrome c exit

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Host metabolism reprogramming is a key feature of Mycobacterium tuberculosis (Mtb) infection that enables the survival of this pathogen within phagocytic cells and modulates the immune response facilitating the spread of the tuberculosis disease. Here, we demonstrate that a previously uncharacterized secreted protein from Mtb, Rv1813c manipulates the host metabolism by targeting mitochondria. When expressed in eukaryotic cells, the protein is delivered to the mitochondrial intermembrane space and enhances host ATP production by boosting the oxidative phosphorylation metabolic pathway. Furthermore, Rv1813c appears to differentially modulate the host cell response to oxidative stress. Expression of Rv1813c in host cells inhibits the release of cytochrome c from mitochondria, an early apoptotic event, in response to short-term oxidative stress. However, Rv1813c ex-pressing cells showed increased sensitivity to prolonged stress. This study reveals a novel class of mitochondria targeting effectors from Mtb and opens new research directions on host metabolic reprogramming and apoptosis control.

Mycobacterium tuberculosis' virulence factor ESAT-6 drives neutrophil necrosis: Implications for a host-directed therapy against tuberculosis

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Rising cases of multi drug-resistant tuberculosis require novel approaches to tackle the global tuberculosis epidemic. Neutrophils represent the main infected cell population in lungs of tuberculosis patients. Here we show that clinical isolates of *M. tuberculosis* from all clades of the *M. tuberculosis* complex induce necrosis of human neutrophils. We identified a single virulence factor being responsible for necrosis induction (ESAT-6). Moreover, after subsequent uptake of infected dead/ dying neutrophils by human macrophages or dendritic cells, previous neutrophil necrosis was a prerequisite for mycobacterial replication in those host cells. After intracellular bacterial growth, macrophages and dendritic cells also succumbed to necrotic cell death, releasing higher numbers of *M. tuberculosis*. Therefore, the bacteria established a vicious circle of necrosis interspersed with periods of replication, a scenario that likely takes place in the lungs of patients suffering from active tuberculosis. This leads to tissue damage and coughing up contagious particles. Importantly, after identification of reactive oxygen species as drivers of the initial neutrophil necrosis, we were able to prevent the necrotic event by pharmacological inhibition of the neutrophil's myeloperoxidase. Infected neutrophils now underwent default apoptosis just like uninfected ones. After uptake of infected apoptotic neutrophils by macrophages, their capability to control mycobacterial growth was restored, an intervention that we recently successfully applied also *in vivo* in mice. Thus, the vicious circle of necrosis and bacterial growth were interrupted. This highlights neutrophil-associated mechanisms as putative targets for host-directed therapies and diagnostic markers in point-of-care testing, which we now aim to stratify to the clinics by investigating patients' sputum with regard to neutrophil-driven signatures.

Tissue- and pathogen-dependent impact of *Prkn* and *Lrrk2* knock-out on the transcriptomic response to mycobacterial infection

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Rationale: *Prkn* and *Lrrk2* are well-known Parkinson's disease genes that are also involved in the response to intracellular pathogens. In the present study, we tested the impact of *Prkn* and *Lrrk2* deletions, as well as *Prkn/Lrrk2* double knock-out (KO), on mycobacterial infections in a mouse model.

Methodology: For that, mice with *Prkn* KO, *Lrrk2* KO, double KO and their wild-type (WT) littermates were inoculated with Bacille Calmette-Guérin (BCG)-Russia via tail vein injection. We performed RNA sequencing from whole lung and spleen tissues in non-infected and BCG-infected mice at 6 weeks post-infection. In addition, *Lrrk2* KO and WT littermates were aerosol infected with *Mycobacterium tuberculosis (Mtb)*-H37Rv. To compare the transcriptomic responses of the KO vs WT mice, we used gene-set enrichment analysis of signatures derived from the gene ontology biological processes.

Results: In the lung, *Prkn* and *Lrrk2* deletions affected the expression of immune-related gene-sets in response to BCG, including overlapping gene-sets between the two KO strains such as monocyte chemotaxis and T-cell differentiation. Interestingly, in lungs from double KO, there was evidence for cross-regulation between the *Prkn* and *Lrrk2* deletion for some BCG response signatures. When response to BCG was analyzed in the spleen, there was a less pronounced impact of the *Prkn* deletion and the effect on molecular signatures was dominated by *Lrrk2* KO, with double KO mice replicating the sets from *Lrrk2* KO. Next, we asked if *Lrrk2* deficiency would present similar effects for human pathogenic *Mtb*. We found that *Lrrk2* deletion impacted in the expression of multiple gene-sets including monocyte chemotaxis, Toll-like receptor signaling and IL1 production in the pulmonary response to both BCG and *Mtb*. However, most of the gene-sets were affected by *Lrrk2* KO exclusively in the response to one of the two pathogens, such as the gene-set for NOD2 signaling in the BCG-response and IL6 or IFN-gamma production in the *Mtb*-response.

Conclusion: Our results show tissue- and pathogen-dependent effects of *Prkn* and *Lrrk2* KO on the transcriptomic response to BCG and *Mtb*.

SigK-regulated proteins may explain *Mycobacterium bovis* and *Mycobacterium orygis* hypervirulence

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Background: *Mycobacterium tuberculosis* (*M. tb)* is the causative agent of human tuberculosis (TB). Closely related members of the *M. tuberculosis* complex (MTBC) are 99.9% identical at the genomic level but have differential gene and protein expression, most clearly evidenced by the cattle associated lineages, *M. bovis* and *M. orygis*. In *M. bovis* and *M. orygis*, the regulator of SigK (*rskA*) has been independently mutated, leading to increased production of the antigenic proteins MPT70 and MPT83. Despite distinct genomic identities, the three most secreted proteins of both *M. bovis* and *M. orygis* are ESAT-6, CFP-10, and MPT70. We hypothesize that the SigK regulon has a role in the infection outcome and pathogenicity of MTBC subspecies.

Results: Canonically, *M. bovis* is more virulent than *M. tb.* Using an experimental aerosol model, we compared the outcome of *M. tb*, *M. bovis* and *M. orygis*. Unlike *M. tb*, where infection is monitored at days 21, 42 and 84, the *M. bovis* and *M. orygis* groups experienced mortality beginning at ~28 days. Further, lung histopathology showed extensive consolidation and airway restriction in both infection groups. To determine whether this increased virulence depends on the production of MPT70 and MPT83, we deleted the genomic region coding for these putative virulence factors (*mpt70_mpt83*). After infection with *M. bovis mpt70_mpt83*, no mortality was observed. Additionally, lung bacterial burden of *M. bovis mpt70_mpt83* infected mice decreased by 4 logs by T= 32 weeks. When *Rag1* deficient mice were infected with *M. bovis mpt70_mpt83* the mortality phenotype was recapitulated, though at a slower rate compared to *M. bovis* infected mice. Aerosol infection of *M. orygis mpt70_mpt83* is currently ongoing.

Significance: We seek to determine if the putative virulence factors MPT70 and MPT83 contribute to the altered pathogenicity of two zoonotic MTBC subspecies compared to *M. tb.* The dependence of *M. bovis* on these 3 genes for full virulence may suggest a role of the SigK regulon in infection outcome and additionally, may explain the differential host ranges of *M. tb, M. bovis* and *M. orygis.*

Identifying a New Vulnerability in *Mycobacterium tuberculosis*

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In all life forms from bacteria to humans, metabolic pathways often involve potentially toxic intermediates, the levels of which are carefully controlled. For example, in *Mycobacterium (M.) tuberculosis* the inappropriate accumulation of endogenous aldehydes makes it highly susceptible to the host defense molecule, nitric oxide. Given that aldehydes are induced as parts of metabolic programs that accompany immune cell activation, we hypothesized that these aldehydes are among the arsenal of effectors needed for pathogen control. While there has been a significant focus on the effects of aldehydes on mammalian physiology, the idea that that they might be purposefully induced to kill pathogens is new. I will discuss evidence supporting the "aldehyde hypothesis", which proposes humans use aldehydes to target *M. tuberculosis.* I will also discuss mechanisms by which aldehydes could weaken bacteria against the antimicrobial transition metal copper, which is also used by mammalian cells to counter infections.

Mycobacterium tuberculosis smallRNAs in Human serum: a bioinformatic characterization of Host-Pathogen interaction

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Among the post-transcriptional regulatory mechanisms, emerging evidence suggests that bacterial smallRNAs (smRNAs) can potentially target host mRNAs, impairing their stability or translation rate, thus hypothesizing that smRNA may have a role in host-pathogen interaction. smRNAs in *Mycobacterium tuberculosis* (MTB), the etiological agent of tuberculosis (TB) are poorly characterized. We investigated if mycobacterial smRNAs can be used as biomarkers of infection and could be eventually involved in pathogenesis by directly controlling host immune response.

Illumina RNA-sequencing (RNAseq) was performed on total RNA extracted from the serum of 93 subjects – 34 Active TB (ATB), 39 TB infection (TBI), and 20 healthy controls (CTRL). We filtered out the human RNAs reads and the remaining were mapped on a list of 600 mycobacterial smRNA candidates previously edited in our lab. We developed a robust *in silico* smRNA-human transcriptome target prediction combining benchmarking of most common target prediction algorithms with a neural network model trained over experimentally validated smRNA-mRNA couples from starBase to minimize the well-known high False positive rates in native target prediction algorithms. After setting up the most accurate model, target prediction analysis was performed considering the total of human protein-coding transcripts. Gene ontology analysis was performed on the *in silico* predicted targets to describe the host-pathogen interactions from a biological point of view.

From RNAseq data, six mycobacterial smRNAs were detected in samples from ATB or TBI and furtherly characterized. Mycobacterial smRNAs were not observed in the sera of CTRL group. The best performing models for target prediction resulted IntaRNA-sTar (AUC= 0.73), and the inner joint prediction of IntaRNA3 and sRNARFTarget (AUC= 0.79). The neural network model reached an accuracy of 65%. The ensuing target prediction model identified a total of 452 hits among human transcripts putatively targeted by the six mycobacterial smRNAs. Gene ontology analysis highlighted 14 significant “terms” associated with immune response processes. *In silico* target prediction suggests a well-organized interacting system between the host and the pathogen. The biological validation of the results is still ongoing and will provide more insights behind this interaction.

Greatly reduced outcomes following oral *Mycobacterium orygis* infection

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Background: *Mycobacterium orygis* is a member of the *Mycobacterium tuberculosis* complex and a cause of zoonotic tuberculosis primarily found in South Asia. The finding that most cases have extrapulmonary presentation has suggested that zoonotic tuberculosis may be caused by drinking unpasteurized milk. Ongoing research in our lab has shown aerosol infection of *M orygis* in mice leads to severe lung disease and early mortality compared to *M. tuberculosis.* We set out to investigate the oral route of infection.

Methods: Since gut microbiota contribute to infection resistance, we tested oral infection with and without 20mg streptomycin pre-treatment. Oligo mediated recombineering was employed to generate a streptomycin-resistant (strep-R) strain of *M. orygis.* C57BL/6 mice were given 10^8 CFU of strep-R *M. orygis*. At 3-weeks post-infection (pi), a portion of infected mice were euthanized and assessed for organ CFUs by plating. The remainder of mice were left as a survival group until 6 months. At this time, the remaining mice and a group of naïve animals were challenged with *M. orygis* infection by aerosol.

Results: At 3-weeks pi, *M. orygis* had poorly colonized the intestine in both groups. However, only mice given streptomycin pre-treatment had *M. orygis* CFUs in their spleen at 10^3-10^4 CFU/g. *M. orygis* CFUs were primarily found within the mesenteric lymph nodes and lungs at 10^1-10^3 CFU/organ and 10^2-10^4 CFU/g respectively. Mice within the survival group maintained a healthy weight and body score until 6-months pi. After *M. orygis* aerosol challenge, 100% of naïve mice but none of the previously gavaged mice reached endpoint by 31-days pi.

Interpretation/Significance: Oral *M. orygis* exposure successfully led to infection, with minor differences following streptomycin pre-treatment. In sharp contrast to aerosol infection, *M. orygis* by gavage did not lead to severe disease or early mortality. However, it did protect mice against future challenge by aerosol. Together, these findings suggest that a naturally virulent zoonotic member of the *M. tuberculosis* complex poses a greatly reduced threat when ingested rather than inhaled.

Inflammasome activation and glycolytic reprogramming are uncoupled in *Mycobacterium tuberculosis* infected macrophages

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The heterogeneous nature of tuberculosis is undoubtfully attributed to host and environmental factors. However, more recent studies have unveiled an important role for the pathogen itself, *Mycobacterium tuberculosis*. We showed that the genetic variability of bacteria impacts host-pathogen interactions and disease outcomes, revealing that clinical isolates from patients with severe forms of tuberculosis evade cytosolic surveillance systems in macrophages and trigger reduced inflammasome activation and interleukin (IL)-1β production. Since the activation of the immune system is very demanding on a bioenergetic level and since IL-1β production and inflammasome activation are commonly associated with glycolytic reprogramming of infected macrophages, we explored glycolytic reprogramming in macrophages infected with genetically variable bacteria. We employed a clinical isolate associated with severe forms of tuberculosis and low IL-1β induction (*M. tuberculosis* 6C4), another one associated with mild disease and high IL-1β induction (*M. tuberculosis* 4I2) and assessed the glycolytic reprogramming of macrophages through different assays. Surprisingly, the ability of these clinical isolates of *M. tuberculosis* to differentially activate the inflammasome and modulate IL-1β release was uncoupled from the glycolytic reprogramming of infected cells. Macrophages infected with either isolate showed a similar shift towards glycolysis, despite downstream differential inflammasome activation. Due to its role in glycolytic reprogramming and IL-1β production, we questioned whether the transcription factor hypoxia-inducible factor (HIF)-1a was modulated in the context of diverse *M. tuberculosis* clinical isolates. Macrophages deficient in HIF-1a showed a decrease in the transcription of genes encoding glycolytic enzymes and IL-1β mRNA, in accordance with previous reports. However, HIF-1a deficiency did not compromise the secretion of bioactive IL-1β. Whereas production of IL-1β required inflammasome activation and the presence of live bacteria, bacillary replication was unchanged in HIF-1a absence, suggesting that HIF-1a may be dispensable for the macrophage activation. We evaluate currently whether distinct *M. tuberculosis* clinical isolates may influence glycolytic reprogramming *in vivo* and thereby potentially modulate the infection outcome.

Acquisition of host-derived lipids by intracellular mycobacteria and its impact on pathogenesis

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Mycobacterium tuberculosis (*Mtb*), the etiological agent of tuberculosis, has the unique ability among intracellular pathogens to hijack the hosts' lipids at its own benefit in order to survive and multiply. The underlying mechanisms remain to be explored, to obtain a comprehensive picture about the impact of host-derived lipids on the intracellular life of mycobacteria. In this context, we use the experimental host model *Dictyostellium discoideum*, an amoeba well-established to study the interactions between intracellular pathogens and cell-autonomous defense mechanism, and the opportunistic human pathogen *M. marinum* widely used as a powerful alternative model for *Mtb* but less constraining to use in laboratory (does not require Biosafety Level 3 laboratory measures). First, using GFP-expressing bacteria, we demonstrated *in vitro* that *M. marinum* WT was able to grow using cholesterol, palmitate or oleate as main carbon sources in a dose-dependent manner. Genetic knock-out of systems involved in lipid import (Mce1/4) and utilization (LipY/Icl1/Facl6) led to growth alterations depending of the lipid and the dose used. These mutations also led to a significant intracellular growth defect in *D. discoideum*, while their initial capacity to infect cells was not altered, suggesting a limited ability to replicate. During their intracellular life, mycobacteria can have access to different lipid sources (such as triacylglycerids, sterols, fatty acids or phospholipids). Their localization inside a niche (called MCV, for mycobacteria-containing vacuole) or in the cytosol can therefore modulate the access. To understand this phenomenon, we initiated microscopy experiments aiming at characterizing the subcellular localization of the *M. marinum* mutants affected in lipid acquisition. Mutants affected in sterol import (Mce4) and related-detoxification (Icl1) appeared to escape the MCV more often at early stages of phagocytosis. These results underline that the use of lipids, particularly sterols, by mycobacteria might represent a crucial step early in the manipulation of the phagocytosis process leading to MCV genesis. As perspectives, we aim to decipher the dynamics of host lipid availability during infection with *M. marinum* WT and various mutants, from the earliest stages of phagocytosis and intravacuolar growth, to escape to the cytosol and finally until their egress and dissemination.

Disclosing the *in vivo* role of IL-1β in *Mycobacterium tuberculosis* infection through pathogen diversity

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Mycobacterium tuberculosis is a pulmonary pathogen that causes over 1,5 million deaths per year. For the first time over a decade, the number of deaths caused by tuberculosis (TB) has increased, partly due to the COVID-19 outbreak. Early immune events in the lung critically define the outcome of TB, with interleukin (IL)-1 playing a relevant role in this process. A recent study from our group showed that *M. tuberculosis* strains isolated from patients with severe TB presentations evaded host macrophage cytosolic surveillance mechanisms, ultimately inducing low levels of IL-1β. In contrast, isolates recovered from patients with mild TB presentations were readily recognized by macrophages, which secreted high levels of IL-1β. Building upon this study, we are now studying how *M. tuberculosis* isolates that evade or not IL-1β responses *in vitro*, may modulate *in vivo* infections. For this, transgenic mice with conditional IL-1R deficiency in specific cell compartments were infected with *M. tuberculosis* isolates 4I2 (mild TB, high IL-1β) or 6C4 (severe TB, low IL-1β). The dynamics of infection in terms of bacterial loads, histopathology, cytokine quantification and analysis of immune cell populations, was evaluated. Recapitulating our clinical data, infection with *M. tuberculosis* isolate 6C4 resulted in higher bacterial loads in the lung, larger and more severe lesions, higher recruitment of immune cells and enhanced expression of genes encoding proinflammatory cytokines, than infection with *M. tuberculosis* 4I2. Moreover, our results show that mice lacking IL-1R in myeloid cells have increased susceptibility to infection only upon infection with the high IL-1β inducer strain, *M. tuberculosis* 4I2. We are now investigating the underlying mechanisms and the impact of IL-1R deficiency in other cellular compartments. Collectively, this work shows that it was possible to recapitulate, in the mouse model, the diverse severities of TB disease caused by different isolates of *M. tuberculosis*, reaffirming the importance of the pathogen diversity for the study of host-pathogen interactions. Moreover, we expect to offer new insights on the mechanisms underlying protection or pathology afforded by IL-1 in the context of TB.

Dissecting physiological effects of genetic knock-out of type III PKSs in *Mycobacterium marinum* and possible role in pathogenesis

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Type III polyketide synthases (PKSs) have been found in bacteria, fungi, and plants. Microbial type III PKSs utilize both malonyl- and methylmalonyl-CoA extender units and biosynthesize methylated alkyl-resorcinol, and methylated acyl-phloroglucinol products, which are essential for mycobacterial survival in stationary biofilms. Similarly, polyketide quinones are produced by type III PKSs using fatty acyl-CoA precursors are responsible for oxygen-deficient niches to maintain cellular bioenergetics. *Mycobacterium marinum* (*M.marinum*) is one of the nontuberculous mycobacteria (NTM), whose genome harbor 34 ORFs homologous to *pks* genes, with four being type III PKSs. Metabolites produced by the PKSs biosynthetic pathways are also involved in forming lipid molecules of the cell envelope of the mycobacterium species, but their essential role in virulence is unidentified. We used CRISPR-Cas9 technology to create knock-out strains to study the pathogenicity of type III PKSs of *M.marinum* in a natural host, zebrafish (*Danio rerio*). Type III PKSs knock-out strains were characterized to see the physiological effects and further studied their roles in pathogenesis. Adult zebrafish were infected with mutant and wild-type *M.marinum* strains and investigated for persistence and virulence. Our infection studies implicate possible roles of type III PKSs in modulating host immune responses that could aid mycobacterial pathogenesis and could provide clues in identifying specific mechanisms mediating host-pathogen interplay.

Dissecting responses of macrophages from tuberculosis patients infected with their matching *Mycobacterium tuberculosis complex* strain *versus* strains representative of other endemic lineages

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Mycobacterium tuberculosis complex (MTBC) species encompasses nine lineages that differ in immunogenicity and virulence and exhibit differences in global distribution; some being geographically restricted while others are widespread. In that context, we hypothesize that TB epidemiology in a given area is shaped by the interaction between specific host genetic traits and the circulating MTBC genotypes. This interaction could contribute to an increased susceptibility to a given MTBC genotype associated to specific human genetic variant in the local population.

To test our hypothesis we recruited adult, active TB patients in the Temeke district of Dar es Salaam, Tanzania. Patient's sputa were processed for MTBC strain isolation and genotyping. We confirmed that MTBC clinical isolates circulating in Dar es Salaam either belong to MTBC lineage 1, 2, 3 or 4. Patients also provided blood for genome sequencing using a customized H3Africa array as well as peripheral blood mononuclear cell isolation and cryopreservation. We are currently isolating monocytes from these PBMCs to derive macrophages and infect them with representative MTBC clinical isolates circulating in Tanzania. Macrophages are therefore either infected with a similar strain that originally infected the patient ("matched infection") or with strains representative of the other endemic lineages ("mismatched infection"). Our read-outs encompass MTBC replication within patients' macrophages as well as macrophage cell death and cytokine and chemokine production in response to infection.

We expect our presented results to shed light on the contribution of host-pathogen genetic interaction at innate immunity level to sustain the observed MTBC lineage distribution in Tanzania.

The ESX-1 substrate PPE68 has a key function in ESX-1 mediated secretion in *Mycobacterium marinum*

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Mycobacteria use specialized type VII secretion systems (T7SSs) to secrete proteins across their diderm cell envelope. One of the T7SS subtypes, named ESX-1, is a major virulence determinant in pathogenic species such as *Mycobacterium tuberculosis* and the fish pathogen *Mycobacterium marinum.* ESX-1 secretes a variety of substrates, called Esx, PE, PPE and Esp proteins, at least some of which as folded heterodimers. Investigations into the functions of these substrates is problematic, because of the intricate network of co-dependent secretion between several ESX-1 substrates. Here, we describe that the ESX-1 substrate PPE68 is essential for secretion of the highly immunogenic substrate EsxA and EspE via the ESX-1 system in *M. marinum*. Surprisingly, PPE68 is processed and degraded upon export to the cell surface by mycobacterial proteases, while nondegraded PPE68 of *M. marinum* and *M. tuberculosis* is present in a cytosolic complex with its PE partner and the $EspG_1$ chaperone. Interfering with the binding of $EspG_1$ to PPE68 blocked its export and the secretion of EsxA and EspE. In contrast, *esxA* is not required for the secretion of PPE68, revealing hierarchy in co-dependent secretion. The final ten residues of PPE68, a negatively charged domain, seem essential for EspE secretion, but not for the export of EsxA and PPE68 itself. This indicates that distinctive domains of PPE68 are involved in secretion of the different ESX-1 substrates. Based on these findings, we propose a mechanistic model, in which PPE68, in complex with its PE partner and EspG chaperone in the cytosol, serves as a reservoir to facilitate secretion of other substrates across the inner and possibly also the outer membrane of mycobacteria.

Understanding pathogenesis and vaccine efficacy of a high, moderate and low transmissible M. tuberculosis strains in mice

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Over the millennium, M. tuberculosis complex strains have branched into several lineages and genotypic variations can determine the virulence and transmissibility of clinical M. tuberculosis (Mtb). However, the mechanism behind the variability in transmission of Mtb remains elusive. Therefore, we investigated understanding the pathogenesis and vaccine efficacy between the high, moderate and low transmission Mtb in mice. The study used three Mtb strains based on their transmission within the Kenyan population - high, moderate and low. The project focused on the characterisation of Mtb strains isolated from Kenyan individuals, BCG vaccine efficacy and pathological analysis in mice against Mtb strains.

Identification of lipid species facilitating the growth of *Mycobacterium tuberculosis* in macrophages upon type I IFN treatment

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Tuberculosis (TB), caused by infection of *Mycobacterium tuberculosis* (Mtb), is a chronic infectious disease leading to numerous deaths annually. There are approximately 2 billion latently Mtb-infected humans worldwide. Multiple factors in terms of bacterial virulence and host susceptibility have been recognized as mainsprings in the progression of active TB. An elevated expression profile of type I interferon (IFN)-inducible gene signatures is a hallmark in active TB patients from latent infection. In addition, a type I IFN signaling prompts mycobacterial growth and aggravates pulmonary inflammation, suggesting type I IFNs are critically involved in TB pathogenesis. However, the mechanisms by which this signaling exacerbates pulmonary inflammatory responses and augments mycobacterial burdens remain unclear. Metabolic status and replication of Mtb are profoundly influenced by the host environment because host metabolites serve as nutrients for the growth of the intracellular pathogen. To identify host metabolites facilitating Mtb growth, we investigated metabolomic profiling upon type I IFN treatment by LC/MS-MS. Notably, significantly altered levels of sphingolipid species were found in Mtb-infected macrophages together with an increase in bacterial burden by treatment with IFN-β, indicating that sphingolipid dynamics might affect the replication of Mtb inside macrophages. Furthermore, it was confirmed that sphingomyelin and ceramide were used as sole carbon sources in Mtb growth on minimal media *in vitro*. Interestingly, exogenous treatment with amitriptyline, an inhibitor of acid sphingomyelinase, markedly reduced the Mtb burden in macrophages when treating with IFN-β. In conclusion, our findings revealed that a type I IFN signaling induced lipid metabolomics by reprogramming intracellular sphingolipid species in Mtb-infected macrophages, and these metabolomic switches provide a favorable environment for the growth of Mtb. Thus, targeting sphingolipid pathways might be an effective host-directed therapy for controlling Mtb infection.

Paradoxical generation of G-CSF-independent permissive neutrophils in GM-CSF deficiency mice during *Mycobacterium tuberculosis* infection

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Mice lacking granulocyte-macrophage colony-stimulating factor (GM-CSF) are extremely susceptible to *Mycobacterium tuberculosis* (Mtb) infection. In humans, the neutralization of GM-CSF by autoantibodies induces increased susceptibility to Mtb infection. In this study, we thus investigated the host pathogenic factors facilitating TB progression by comparing wild-type (WT) and GM-CSF knock-out (KO) mice. We identified an increase in bacterial burden with marked necrotic granulomas in the lungs of GM-CSF KO mice compared to those of WT mice. Interestingly, GM-CSF KO mice exhibited a greater number of neutrophils along with a lower level of Th1-type T-cell response than WT mice in lungs at 4 weeks post-Mtb infection. Depletion of neutrophils by anti-Ly6G monoclonal antibody in Mtb-infected GM-CSF KO mice restored Th1 response with a significant reduction in bacterial burden as well as excessive lung inflammation. Furthermore, we found a significantly increased level of granulocyte colony-stimulating factor (G-CSF), the promoting cytokine for the generation of neutrophils, in GM-CSF KO mice. The neutralization of G-CSF reduced neutrophil numbers, resulted in ameliorated lung inflammation and improved Th1 response while no difference in bacterial burden was observed. Notably, neutrophils from GM-CSF KO mice after neutralization with anti-G-CSF antibody showed a permissive phenotype to Mtb infection and less activated markers but increased phagocytosis-associated markers than GM-CSF KO-intact neutrophils. Taken together, GM-CSF deficiency caused a remarkable increase in not only neutrophils but also G-CSF along with a low number of Th1 cells during Mtb infection. However, targeting G-CSF may not be a beneficial intervention to reduce neutrophils because of shifting the remaining neutrophils to a permissive phenotype harboring more Mtb numbers. We are currently under investigation on the identification of the factors involved in generating permissive neutrophils in absence of both G-CSF and GM-CSF during Mtb infection.

Localized role for B cells in the human TB lung

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There is growing interest in the potential role of antibodies in protective TB immunity. However, little is known about the B cell response to TB in humans, particularly within the lung. To address this knowledge gap, we characterized B cells in TB diseased human lung, lung draining lymph nodes and in matched blood. Histological staining confirmed the association of B cells with TB lung granuloma, both as tertiary lymphoid follicles and lymphocyte cuffs. Flow cytometric analysis of homogenized lung revealed an increased frequency of B cells compared to lungs from non-TB controls. This was not observed in matched blood samples, consistent with an expansion of lung B cells during TB disease. In addition, B cells in lung homogenate were highly enriched for a memory phenotype (CD27+) and expressed the tissue residence marker CD69. This tissue resident memory-like B cell population was highly enriched for CD38-high CD138 expressing long lived plasma cells. To examine the antigen reactivity, we purified immunoglobulins from lung homogenate and matched blood samples using a thiophilic resin and found that TB diseased lungs contain high levels of Mtb-reactive antibodies, and elevated IgM, suggesting a localized specific immunoglobulin production by lung resident B cells. Phenotyping of lung resident B cells revealed a significant expansion of B cells with regulatory function compared to matched blood. However, this was not significantly elevated in TB diseased lungs. Finally, we employed a 3D culture Mtb infection model to investigate the impact of B cells on Mtb growth and found depleting B cells consistently improved Mtb growth (n = 4). Experiments to test the activity of lung purified antibodies on Mtb growth are underway. Together these data support a localized role for B cells in the human TB lung which may involve both antibody-producing plasma cells as well as regulatory elements of the B cell compartment. Importantly, they also highlight the importance of investigating cellular responses within the lung, as these differences were generally not observed in the blood.

Immunogenicity and protective efficacy of RipA/GLA-SE subunit vaccine candidate against *Mycobacterium tuberculosis* in an ultra-low dose aerosol challenge model

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Background: Given that most tuberculosis (TB) subunit vaccine candidates at various stages of clinical trials are designed for disease prevention, one of challenging difficulties for TB control is to prevent reactivation of latent TB towards active disease. Thus, we hypothesized that *Mycobacterium tuberculosis* (Mtb) antigens (Ags) involved in the reactivation process could be the promising vaccine Ag targets. RipA (Rv1477), a peptidoglycan hydrolase required for efficient cell division of Mtb, may be actively associated with this wake-up process.

Methods: Immunologic features of RipA were characterized by investigating interactions with dendritic cells (DCs). In addition, immunogenicity of RipA/GLA-SE subunit vaccine candidate was assessed by focusing on T cell phenotype and antibody responses. Finally, protective efficacy of this vaccine candidate was evaluated in mice aerogenically infected with an ultra-low dose (ULD; ~10 CFUs) of Mtb Korean-Beijing clinical strain K, to reflect the physiologic inoculum of humans.

Results: RipA-treated DCs displayed enhanced MHC-I, MHC-II, CD80 and CD86 expression, indicating that RipA phenotypically induced DC maturation. In addition, RipA-treated DCs remarkably produced pro-inflammatory cytokines (IL-12p70, IL-6, TNF, and IL-1β). Moreover, RipA was recognized by the host immune systems, as evidenced by Ag-specific IFN-γ responses in the lung from Mtb-infected mice at 4 weeks post-infection upon RipA *ex vivo* restimulation. Mice immunized with RipA formulated in GLA-SE adjuvant displayed remarkable generation of Ag-specific polyfunctional $CD4^+$ T-cells in both lung and spleen. Following an ULD challenge with Mtb K, RipA/GLA-SE immunization reduced pulmonary inflammation and bacterial numbers similar to BCG immunization at 16 weeks post-infection compared to Mtb infection only.

Conclusion: Collectively, our findings suggested that RipA/GLA-SE vaccine candidate exhibited a long-term protective efficacy as a single Ag against Mtb K. We are currently under investigation to advance whether this vaccine type has a potential as BCG-prime boost vaccine.

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Characterising the immune responses of *Mustela furo* to experimental infection with *M. bovis*

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Bovine tuberculosis (bTB), or zoonotic tuberculosis, caused by *Mycobacterium bovis* (*M. bovis*) creates biosafety and economical threats to the farming industry and the public. The UK has the highest European bTB prevalence, followed by the Republic of Ireland and Spain. In France, the Officially TB Free (OTB) status is also challenged by a rise of bTB in several regions. BTB eradication is an OIE (World Health Organisation for Animal Health) requirement, but despite comprehensive test and slaughter policies conducted at a high cost for decades, long-term management of transmission from infected wildlife, typically European badgers (*Meles meles)*, is required. Oral vaccination of badgers with the Bacille of Calmette and Guerin (BCG) is an attractive option but developing cost-effective and efficacious BCG-containing baits deployable in the field is an ambitious challenge, human vaccine against TB. Experimental and field studies in captive and wild badgers have already shown that oral BCG is protective but trapping and housing large numbers of wild badgers is not viable for the complete R&D and regulatory programme. We have therefore reverted to ferrets as “clean”, and laboratory-adapted mustelid surrogates for badgers to understand the host/responses interactions and develop quantitative markers of infection suitable for vaccine efficacy studies. The long-term goal of this work is to accelerate the screening of oral BCG formulations and doses. In the present experimental studies, we investigated the pathogenicity and immunogenicity of *M. bovis* in inoculated ferrets by the intra-tracheal route (IT) or exposure by direct contact for 40 weeks. IT infected ferrets' peripheral cellular and serological responses were mostly similar to those seen in infected badgers. In–contact animals presented some responses to mycobacterial antigens but without any visible granuloma observed at post-mortem. Overall, these findings are promising chronic features of bTB, to be further explored for testing the protective efficacy of vaccines.

Constitutive HIF-1 activity confers susceptibility to mycobacterial infection by suppressing mitochondrial metabolism in granuloma macrophages

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The hypoxia responsive transcription factor HIF-1 is induced in the tuberculous granuloma where it is thought to be protective by mediating both metabolic adaptation to hypoxia and interferon-g-dependent cell-mediated immunity. In the zebrafish model of TB, we find that HIF-1 is protective from the innate phase of infection by preventing granuloma necrosis. Paradoxically, however, we find that HIF-1 activity is prominent in multibacillary necrotic granulomas. We show that constitutive activation of the HIF-1 pathway in TB overrides its protective function. Dysregulated HIF-1 activity represses mitochondrial metabolism, thereby sensitizing infected macrophages to the mitotoxic activity of the mycobacterial secreted virulence determinant ESAT-6. The ensuing death of these macrophages leads to granuloma necrosis that promotes mycobacterial growth.

Our findings highlight how a critical mycobacterial virulence factor exploits an adaptive host mechanism in the TB granuloma to enhance pathogenesis.

Developing therapeutic strategies against *Mycobacterium tuberculosis* infection based on manipulation of protease inhibitors

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Tuberculosis (TB) is considered to be a major global health threat mostly due to co-infection with human immunodeficiency virus (HIV), to the emergence of drug resistant strains and lack of an effective vaccine. It is urgent to develop new therapeutic strategies to overcome drug resistance and control TB inflammation. Host-directed strategies could be exploited to boost the host immune responses and improve future vaccination strategies. *Mycobacterium tuberculosis* (Mtb) infects macrophages, manipulating the proteolytic mechanisms, particularly, by decreasing the expression and activity of lysosomal cathepsins. Consequently, Mtb survives and even replicates inside macrophages concomitant with a poor priming of the adaptive immune response. In order to overcome this, we decided to target cystatins (Cst) the natural inhibitors of cathepsins. We found that by silencing cystatin C expression not only significantly improves the intracellular killing of Mtb, but also led to an improved expression of the human leukocyte antigen (HLA) class II and an increased $CD4^+$ T-lymphocyte proliferation along with enhanced IFNγ secretion. In addition to cystatin C we targeted other cystatins by siRNA during infection with laboratory and clinical strains of Mtb with different drug resistant profiles. Our results showed that silencing those cystatins in macrophages significantly improves the intracellular killing of Mtb. After defining a pattern of expression of those relevant cystatins, and how their manipulation provides control of the host immune response against Mtb, we also developed nano-formulations with designed siRNAs as pre-therapeutic approach to target host macrophages infected with Mtb. Their inclusion in nanoparticles targeting macrophages receptors allows their specific delivery to these immune cells and their accumulation in the intracellular milieu. Overall, this approach suggests a novel avenue for the development of potential alternative therapeutic strategies to current antimicrobials against this infectious disease.

Keywords: Cystatins, Cathepsins, Tuberculosis, Nano-formulations, Host-directed therapies

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Potential Role of Ubiquitin Ligase MYCBP2 in Antimicrobial Defense

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While the transcriptional responses that occur in response to *Mycobaterium tuberculosis* (Mtb) infection of macrophages have been well-documented, identification of the cellular pathways controlled by post-translational modifications (PTMs) of macrophage proteins has been more difficult to elucidate. We have used PTM proteomics to globally identify host proteins that are differentially phosphorylated or ubiquitylated in response to Mtb infection. Here we describe studies of a unique E3 ubiquitin ligase, MYCBP2, that is both phosphorylated and ubiquitylated in response to Mtb infection. MYCBP2 is known to regulate synaptic development and response to axon damage in neurons, but its function in immune cells is poorly understood. One study suggests that MYCBP2 inhibits macrophage M2 polarization in response to inflammation, but its role in infection has not been studied**.** Given the post-translational modifications of MYCBP2 in response to Mtb infection and its possible role in macrophage signaling, we asked whether MYCBP2 participates in the host response to Mtb infection. Using CRISPR/Cas9, we found that *Mycbp2*-deficient macrophages were more permissive for Mtb growth. We conducted an aerosol infection with *LysM-cre* conditional *Mycbp2* knockout mice and asked whether MYCBP2 controlled Mtb infection *in vivo.* We found that MYCBP2 did not impact bacterial growth but did appear to impact host survival to Mtb infection. Here we present ongoing investigations of the role in MYCBP2 in Mtb infection and innate immunity.

Impact of natural mutations in the sensor kinase of the PhoPR two-component regulatory system on virulence and transmission of tuberculosis bacilli

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Natural transmission of Tuberculosis (TB) occurs through the production of pathogen-containing droplets by coughing TB patient and inhalation of these particles by naive nearby person. Although phylogenetic lineages of TB bacilli are genetically compact, they exhibit a major variability in their ability to transmit in humans. What are the key functions required by TB bacilli to achieve such an efficient transmission in humans? Understanding these functions may reveal weak spots in the defenses of the pathogen that can be exploited to propose new innovative approaches to block the disease propagation.

Previous results strongly suggest that mutations within genes encoding the two-component regulatory system PhoP/PhoR (PhoPR) required for virulence of TB bacilli modulate aerosol transmissibility in human.

Here we aimed to understand i) how mutations, found in *phoPR* alleles from certain phylogenetic lineages of TB bacilli, do affect the production and secretion of virulence factors and the interaction with host, ii) what is the impact of these mutations on the activity of PhoPR and the response to different microenvironment encountered during infection.

First, we expressed *phoPR* alleles selected from various lineages with low or high transmissibility in humans in mutant strains deleted for their endogenous *phoPR* allele. Using these recombinant strains, we demonstrated that mutations in the *phoPR* genes lead to a deficient expression of virulence factors in several strains of *Mycobacterium canettii* compared to *Mycobacterium tuberculosis* (MTB). We showed also those specific mutations in a strain of *M. canettii*, generate a lower capacity to infect macrophages from human donors, and several mouse models. Second, our results showed that the mutations found in PhoPR variants affect the expression of PhoPR regulon *in vitro* under environment without stress conditions. These mutations impact also their capacity to recognize a signal such as the acid stress encountered during host infection.

We propose that the selection of a specific PhoPR variant helps the tuberculosis ancestor to evolute into human pathogen with high capacity to transmit.

Membrane damage mechanisms during mycobacterial infection: how the pathogen controls its fate

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During infection with *Mycobacterium tuberculosis* (*Mtb*), the establishment of a successful *Mycobacterium*-containing vacuole is driven by cycles of damage and repair events occurring at the delimiting membrane. Despite multiple studies demonstrating the potential bacterial factors involved in damage, the detailed mechanisms of these damage/repair cycles are not well understood, and their functions for the host and the pathogen are still unclear. Here, we focus on the bacterial side, taking advantage of *Mycobacterium marinum*, a close relative of *Mtb* sharing most virulence mechanisms but experimentally easier, making it a powerful model to study tuberculosis at a molecular level. Our aims are to identify the bacterial components required to generate damage at early stages of infection and to understand their mechanisms of action. Our potential candidates have been chosen from the literature. Indeed, ESAT-6, a pore-forming peptide, was the first virulence factor shown to damage membranes two decades ago. However, in the last years, intense studies demonstrated the contribution to damage of other bacterial components including among others PDIMs, methyl-branched cell-wall lipids.

We generated single and double *M. marinum* mutants in genes encoding the type VII secretion system ESX-1, ESAT-6 and/or TesA, an enzyme synthesizing the PDIM/PGL lipids, PGL being the glycosylated form of PDIM mainly absent in *Mtb* except in hypervirulent strains, to evaluate their complex contributions to damage.

Although all the mutants grew well *in vitro*, each of them presented a growth defect during infection of the amoeba *Dictyostelium discoideum,* with a stronger attenuation for the double mutants. The *in vitro* damage activity measured by hemolysis assay showed that ESAT-6/ ESX-1 substrates are the main bacterial agents inducing damage. Surprisingly, in our conditions, the PDIM/PGL lipids do not appear to contribute to hemoglobin release. This unexpected *in vitro* phenotype will be validated during infection by monitoring various reporters of damage/repair such as the recruitment of the ESCRT-III and autophagy machineries, ubiquitination of damaged MCV, and finally Perilipin adsorption on cytosolic *M. marinum*.

Altogether, both ESAT-6 and PDIM/PGL are essential for full virulence during infection of *D. discoideum* but might act at different steps during the infectious cycle.

Identifying single cell phenotypic variants of *Mycobacterium tuberculosis* and their impact on antibiotic activity during infection

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Phenotypic variants of *Mycobacterium tuberculosis* (*Mtb*) can alter growth rates and protein expression patterns to tolerate high doses of front-line drugs and contribute to tuberculosis (TB) therapy failure and relapse of infection[1]. Using a combination of time-lapse microscopy and an inducible fluorescent reporter of translation, we studied the emergence of *Mtb* phenotypic variants in experimental models of increasing complexity. We attempt to delineate spatio-temporal nuances and host mechanisms that influence antibiotic tolerance in *Mtb*.

First, using an optically tractable microfluidic system, we studied the single-cell drug response of axenic *Mtb* cultures by measuring growth rates[2]. We identified non-growing bacterial fractions that were either metabolically active or inactive and traced their lineage within bacterial microcolonies during drug exposure and post-antibiotic regrowth. We observed that maintenance of translational activity and post-antibiotic effects varied in a time-, dose-, and antibiotic-dependent manner. Bedaquiline treatment resulted in delayed growth arrest and cell death, and significant killing even after drug withdrawal. Next, we explored the role of host immunity in driving heterogeneous bacterial responses by infecting a lung-on-chip (LoC) model consisting of macrophages co-cultured with alveolar epithelial and endothelial cells[3]. We found that the proportion of phenotypically heterogeneous *Mtb* generated at the air-liquid interface of the chip varied depending on intra-macrophage localization or growth in typical cord-like structures. Together, these observations suggest that the emergence of phenotypic variants of metabolic activity begins during early infection from the point of first contact between host and pathogen. Lastly, and in contrast to results in early infection, we observed a progressive loss of heterogeneity in *Mtb* translational activity upon recovery from murine lungs, as infection progressed from the acute to the chronic phase. This could be due to the adaptive immune response which modulates multiple stress alleviation pathways and induces a persistence-like state in *Mtb*. Together, these experimental models will fine-tune our knowledge of factors that drive phenotypic heterogeneity which will help develop new approaches to increase the efficacy of TB therapy.

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Investigating the role of the protein MPB70 in mycobacterial-macrophage interactions

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Bovine tuberculosis, caused by *Mycobacterium bovis*, is a threat to animal health and productivity. Transmission of *M. bovis* infection to humans also represents a zoonotic threat. The *M. bovis* genome is 99.95% identical to that of the human pathogen *Mycobacterium tuberculosis*, yet these pathogens have distinct host preference; our hypothesis is that host preference is driven by differences in the expression of key pathogen genes. MPB70, one of the major secreted antigens of *M. bovis*, shows high differential expression between *M. tuberculosis* and *M. bovis*. This protein shows characteristics suggestive of a role played in host-pathogen interaction, yet its role remains to be defined. This study seeks to understand the role played by MPB70 in mycobacterial-host interactions, focusing on the pathogen-macrophage interface. To this end, a model infection assay was built using a virulent wild type strain of *Mycobacterium bovis*, its *mpb70* knock-out mutant and the complemented *M. bovis mpb70* knock-out. Infection assays were performed *in vitro* using bovine alveolar macrophages harvested from calf lungs. Analysis of the infections were performed at 3-, 18, 24-, 30- and 48-hours post-infection. Fluorescence microscopy, CFU counts and macrophage RNA purification for downstream gene expression analysis were performed. Preliminary results showed a differential phenotype between macrophages infected with the *mpb70* knock-out strain compared to the wild-type strain and the *mpb70* knock-out complement, with cell death apparently increased in macrophages infected with the *mpb70* knock-out strain at 48h post-infection compared to the macrophages infected with the two other strains. Current work seeks to determine if this differential phenotype is associated with an altered transcriptomic response of the macrophage to infection in the presence or absence of MPB70 production by the pathogen. Elucidating the role of MPB70 will contribute to a better understanding of *M. bovis* adaptation to the bovine host.

Uncovering the role of Siglec-1 receptor expressed by alveolar macrophages in tuberculosis

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Alveolar macrophages (AM) are the main host cells for *Mycobacterium tuberculosis* (*Mtb*) in the lung and they play a central role in antimycobacterial immunity. AM have been described to survey the alveoli at steady state and to be the first cells infected by *Mtb*. Upon infection, they translocate into the parenchyma, where they initiate the formation of granuloma. These cells express Siglec-1, a lectin involved in antigen presentation and T cell activation in the spleen and lymph nodes, but the role of Siglec-1 in the lung and in the pathogenesis of tuberculosis (TB) remains poorly understood.

We recently published that TB-associated microenvironment increases the cell-surface expression of Siglec-1 on human monocyte-derived macrophages in a type-I interferon-dependent manner. In this context, Siglec-1 localizes on and stabilizes tunneling nanotubes, which are dynamic connections between cells representing a unique route for cell-to-cell communication. We demonstrated that while *Siglec-1*-KO mice show no difference in the *Mtb* burden when compared to controls, they exhibit excessive inflammatory lung lesions. We also reported that Siglec-1^{+} AM become abundant in lung biopsies from TB-infected non-human primates and that their abundance correlates with disease severity. Finally, a null mutation in *Siglec-1* is associated with a higher dissemination of the bacteria in humans. The main objective of this project is to understand whether and how Siglec-1 plays a protective role against TB. We hypothesized that this receptor may play a role in the cell-to-cell communication of AM and thus trigger the activation of the adaptive immune response and the formation of granulomas. To test this hypothesis, we will infect *Siglec-1*-KO mice with *Mtb*. We will use state-of-the-art intravital microscopy in the lung to visualize the dynamics of AM and cell-to-cell interaction at the early stages of *Mtb* infection in the presence or absence of Siglec-1. We also developed an *ex vivo* model of AM to study the molecular mechanisms involved in the identified processes. Our results will bring the first *in vivo* live-imaging of the AM behavior during *Mtb* infection and will characterize the role of Siglec-1 in the AM biology.

Iron-related resistance mechanisms: the role of myeloid H-ferritin during mycobacterial infection

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Iron is a crucial element in host-pathogens interactions. Infectious agents rely on host's iron for proliferation with the consequent establishment of disease. Alterations of iron distribution are an important part of the host defence mechanisms against infection. Within the cells, iron is stored in the protein ferritin, which is composed by two subunits, L- and H-ferritin. Macrophages infected with *Mycobacterium avium* have increased levels of H-ferritin, while L-ferritin remains unaltered. We investigated the role of macrophage H-ferritin in vivo during mycobacterial infections, using mice lacking H-ferritin expression specifically in myeloid cells (*Fth1*$^{-/-}$). Surprisingly, our work showed that *Fth1*$^{-/-}$ mice are more resistant to *M. avium* infection, than wild-type littermates, contrarily to what happens with *M. tuberculosis*. To dissect the mechanisms underlying the differential effect of H-ferritin in the susceptibility/resistance to different mycobacterial infections, we used Ion AmpliSeq technology and performed transcriptomics of lungs from mice infected with *M. avium* or *M. tuberculosis*. Our results showed a very specific signature for each infection, particularly in terms of immune response and iron uptake. On one hand *M. tuberculosis* causes a stronger up-regulation of interleukins, chemokines, and interferon-gamma, while *M. avium* mainly upregulates type 1 interferons. *Ftmt*, the gene coding for mitochondrial ferritin, is upregulated in *M. avium*-infected mice and down-regulated in *M. tuberculosis*-infected mice. This difference may determine intracellular iron distribution and even cell death. These will be crucial for bacterial proliferation and infection outcome. The *Fth1*$^{-/-}$ genotype showed a down-regulation of *Egr1* and *Nr4a1* that are mainly linked to the decrease of the pro-inflammatory response, comparatively with the wild-type. In conclusion, the differential susceptibility to mycobacteria of myeloid H-ferritin-deficient mice may be due to differences in immune responses and on the activation of iron uptake mechanisms by the different mycobacteria species. A detailed characterization of these differences will be the sibject of future work.

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Dimethyl fumarate eliminates “invisible” *Mycobacterium tuberculosis* persisters from infected murine organs

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Tuberculosis (TB) claims nearly 1.5 million lives annually. Current TB treatment requires a combination of several drugs administered for at least 6 months. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, can persist in infected humans and animals for decades. Moreover, during infection Mtb produces “invisible” or differentially culturable bacteria (DCB) that don’t grow in standard media but can be resuscitated in liquid media supplemented with sterile Mtb culture supernatants or recombinant resuscitation promoting factors (Rpf). Here we demonstrate that Mtb DCB are detectable in the lungs after 4 weeks of infection (via intranasal route) and their loads remain largely unchanged during a further 8 weeks. Treatment of the infected mice with dimethyl fumarate (DMF), a known drug with immunomodulatory properties, for 8 weeks eliminates Mtb DCB from the lungs and spleens but does not affect colony-producing Mtb. Standard TB treatment consisting of rifampicin, isoniazid and pyrazinamide for 8 weeks reduces Mtb loads by nearly 4 orders of magnitude but does not eradicate Mtb DCB. Nevertheless, no Mtb DCB can be detected in the lungs and spleens after 8 weeks of treatment with DMF, rifampicin, isoniazid and pyrazinamide. Our data suggest that addition of approved anti-inflammatory drugs to standard treatment regimens may improve TB treatment and open new directions for investigation of molecular mechanisms underlying generation of "invisible" Mtb persisters during infection.

Mycobacterium tuberculosis highjacks c-MYC controlled self-renewal programs for intracellular persistence in macrophages *in vitro*

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Counterintuitively, asymptomatic infection with Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), provides protection against re-infection. This phenomenon is called "concomitant immunity" and requires the pathogen to persists in a host with an otherwise protective immune response. Here, we hypothesize that naive macrophages prior to infection protect the pathogen from the immune response by maintaining a specific niche.

We explored major biological phenotypes (mycobacterial viability, host cell viability, host cell activation) in mouse bone marrow-derived macrophages (BMDM) infected *in vitro* with either H37Rv or the MTB Live/Dead H37Rv reporter strain. Macrophages were stimulated with IFN-γ for 24h either prior or after infection. We analyzed the resulting phenotypes with flow cytometry, plating of bacteria and RNAseq. We inhibited c-MYC signaling was inhibited with a chemical inhibitor (10058-F4) and by transducing the cells with an inducible construct to express a c-MYC inhibitor peptide (omo-MYC).

Activation of macrophages with IFN-γ for 24h prior to infection significantly increased anti-mycobacterial activity of macrophages and decreased the number of live and transcriptionally active bacteria by 45 % (+/- 12 %). In contrast, macrophages activated with IFN-γ for 24 h directly after infection displayed resistance to IFN-γ and allowed bacterial growth similar to naïve macrophages. After phagocytosis of heat killed MTB, macrophages displayed resistance to activation with IFN-γ similar to infected/activated macrophages with live H37Rv. The transcriptional response comparing infected/activated versus activated/infected macrophages showed substantial differences in proinflammatory pathways and transcriptional self-renewal programs including c-MYC regulated transcriptional programs. Inhibition of c-MYC signaling with a chemical inhibitor and omo-MYC expression led to a gain of anti-bactericidal function in macrophages comparable to activation with IFN-γ.

The activation state of the macrophage prior to MTB infection is the key determinant of infection outcomes *in vitro*. Phagocytosis of MTB without pre-activation of macrophages induces c-MYC controlled self-renewal transcriptional programs inhibiting anti-bacterial activity. This effect is also detectable with heat-killed MTB, suggesting that MTB highjacks a cell intrinsic program to allow persistence in the host. The role of c-MYC mediated persistence will be further investigated with *in vivo* experiments in a mouse model.

Small RNAs DrrS and Mcr11 contribute to *Mycobacterium tuberculosis* persistence within host

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Mycobacterium tuberculosis is capable of a long-term survival within host organism resulting in the worldwide spread of latent tuberculosis infection. Small non-coding RNAs regulate gene expression and help bacteria adapt to rapidly changing environment. DrrS and Mcr11 are unique and highly conservative small RNAs for pathogenic bacteria of *M. tuberculosis* complex. They are highly expressed in stationary phase and on dormancy *in vitro*, and also during *in vivo* infection suggesting their role in "host-pathogen" interaction.

RNA-seq analysis of ΔDrrS and ΔMcr11 mutants in log and stationary phase revealed changes in the expression of genes involved in mycobacterial virulence and cell wall modelling. In particular, transcriptome analysis of ΔDrrS strain revealed down-regulation of genes coding for membrane proteins (PE-PGRS, *mce1*, *EspA*, *Esp* etc) that may serve as recognition patterns for immune system of the host. Interestingly, Rv3136 encoding transmembrane protein and possible nutrient importer PPE51 was up-regulated in ΔDrrS and down-regulated in DrrS-overexpressing strain. Deletion of DrrS was found to activate expression of *ahpC* and *ahpD* involved in oxidative stress response.

Transcriptome analysis of ΔMcr11 strain demonstrated down-regulation of genes *kasA* and *ldtB,* involved in mycolic acids and in peptidoglycan synthesis, respectively. Moreover, *pks1*, encoding polyketide synthase*, ppsB* and *ppsA* – phenolpthiocerol and phthiocerol dimycocerosate synthases were also found down-regulated. Some genes coding for enzymes of lipid metabolism were found activated (e.g. *lipU* lipase and *lipQ* carboxylesterase) and some – repressed (lipoproteins *lprP* and *lpqS*) indicating metabolic reconstruction in ΔMcr11 cells.

Remarkably, ΔDrrS and ΔMcr11 mutants had no growth defect *in vitro*, while both strains showed elevated sensitivity to high glycerol concentrations after passage through Black6 mice. Particularly, ΔDrrS and ΔMcr11 were unable to grow on Sauton's medium with 6% of glycerol, while grew normally in 7H9 medium (0,4% of glycerol).

RNA-seq analysis of poly-A fraction isolated from bone-marrow derived macrophages infected with ΔDrrS and ΔMcr11 mutant strains revealed a significant difference in expression of pro-inflammatory mediators indicating differences in host immune response between deletion mutants and wild type indicating probable DrrS and Mcr11 contribution into persistence within macroorganism.

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mTOR-regulated Mitochondrial Metabolism Limits Mycobacterium-induced Cytotoxicity

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Necrosis of macrophages in the tuberculous granuloma represents a major pathogenic event in tuberculosis. Through a zebrafish forward genetic screen, we identified the mTOR kinase, a master regulator of metabolism, as an early host resistance factor in tuberculosis. We found that mTOR complex 1 protects macrophages from mycobacterium-induced death by enabling infection-induced increases in mitochondrial energy metabolism fueled by glycolysis. These metabolic adaptations are required to prevent mitochondrial damage and death caused specifically by ESAT-6, the principal secreted substrate of the specialized mycobacterial secretion system ESX-1, a key virulence mediator. Thus, the host can effectively counter this early critical mycobacterial virulence mechanism simply by regulating energy metabolism, allowing pathogen-specific immune mechanisms time to develop. Our findings may explain why *Mycobacterium tuberculosis*, albeit humanity's most lethal pathogen, is successful in only a minority of infected individuals.

MptpB inhibitors increase the efficacy of antibiotics reducing intracellular burden for *Mycobacterium tuberculosis* and non-tuberculous *Mycobacterium avium*

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Background: *Mycobacterium tuberculosis* and *M. avium* are intracellular pathogens able to arrest phagosomal maturation. Antivirulence drugs are receiving more attention due to the emergence of drug resistance, limited pipeline and high toxicity of long-term antibiotic treatment against *M. tuberculosis* and difficult to treat mycobacteria. One of the approaches is inhibiting a tyrosine phosphatase MptpB, a virulence factor in *M. tuberculosis*.

Aim: We aimed to explore the efficacy of an MptpB inhibitor in reducing intracellular burden of *M. avium* and impact in increasing efficacy of antibiotics rifampicin, bedaquiline, pretomanid or a novel minor groove binding (MGB) compound.

Methods: We first confirmed that MptpB was expressed in *M. avium* and if the inhibitor reduced intracellular burden. Then, we found non-toxic doses of antibiotics and combinations of the inhibitors to test in macrophage infections to assess intracellular burden. We also explored if the inhibitor had any effect on acellular growth, alone or in combination with antibiotics. The best combinations were validated in the *Galleria mellonella* in vivo model of infection. Bioimaging was used to evaluate the effect of the combinations on lysosomal trafficking.

Results: The MptpB inhibitor reduced the intracellular burden not only in *M. tuberculosis* but also in *M. avium*, with no effects on the growth of acellular bacteria, alone or in combination with antibiotics. In vitro infections in macrophages showed a further burden reduction of 25-50% when the inhibitor was added to bedaquiline, rifampicin or MGB compounds. We observed that colocalization with LAMP-1 increased in the presence of this inhibitor, indicating that inhibitor increase the trafficking of the bacteria to lysosomes. When we tested in vivo, a mild increased in survival was observed due to the inhibitor, but *M. avium* load was effectively reduced.

Conclusions: Targeting MptpB is a potential strategy to enhance the efficacy of antibiotics and improve the treatment both of *M. tuberculosis* and *M. avium*.

Distinct DNA methylation patterns in alveolar macrophages and alveolar T-cells induced by Mycobacterium tuberculosis

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Host innate immune cells have been identified as key players in the early eradication of Mycobacterium tuberculosis and in the maintenance of an anti-mycobacterial immune memory, induced through epigenetic reprogramming. Studies on human tuberculosis immunity are dominated by those using peripheral blood as surrogate markers for immunity. We aimed to investigate DNA methylation patterns in immune cells of the lung compartment by obtaining induced sputum from patients with tuberculosis and subjects exposed to M. tuberculosis. Alveolar macrophages and alveolar T cells were isolated from the collected sputum and DNA methylome analyses performed (Illumina Infinium Human Methylation 450k).Multidimensional scaling analysis revealed that DNA methylomes of cells from the patients with tuberculosis, the exposed subjects and controls appeared as separate clusters. The numerous genes that were differentially methylated between the three groups were functionally connected and overlapped with previous findings of trained immunity and tuberculosis. In addition, analysis of the interferon-gamma release assay (IGRA) status of the subjects demonstrated that also the IGRA status was reflected in the DNA methylome by a unique signature data. M. tuberculosis induces epigenetic reprogramming in immune cells, reflected as a specific DNA methylation pattern of immune cells in the lung compartment upon exposure to tuberculosis. The data of this pilot study suggests that DNA methylation-based biosignatures could be considered for further development towards a clinically useful tool for determining tuberculosis exposure in contact tracing and tuberculosis infection status.

Host-pathogen interactions in tuberculosis: the immune response to *Mycobacterium africanum* during infection

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Tuberculosis (TB) affects over 10 million people and, in 2020 alone, killed 1.3 million people. TB is mostly caused by *Mycobacterium tuberculosis* or *Mycobacterium africanum*. In contrast to *M. tuberculosis*, which is geographically widespread, *Mycobacterium africanum* is restricted to West Africa and associates with a slow progressing infection, despite identical transmission rates. We recently showed that infection of mice with a clinical isolate of *M. africanum* is associated with lower bacterial burdens, mild signs of disease and lower tissue pathology, even in immunocompromised animals. Overall, human and experimental data suggest that *M. africanum* may be better controlled by the host immune response than *M. tuberculosis*, over a given period of time. How the immune response to *M. africanum* compares to that triggered in response to *M. tuberculosis* is poorly understood. To address this, we studied a cohort of *M. tuberculosis* and *M. africanum* infections among TB patients in Guinea-Bissau, a known hot spot of *M. africanum*. Through bacterial DNA extraction from sputum samples and subsequent genotyping, we found that around 40% of TB cases resulted from *M. africanum* infections. Whole blood RNA-sequencing of *M. tuberculosis* versus *M. africanum*-infected TB patients suggested a reduced overall response in *M. africanum* infections. In addition, most *M. tuberculosis*-infected individuals presented a transcriptional signature compatible with the nuclear receptor/PKN1 pathway being activated, which was not observed in *M. africanum*-infected counterparts. In agreement, infection of human blood cells or mouse macrophages with either pathogen confirmed a more potent cytokine response induced by *M. tuberculosis* than *M. africanum*. We are currently manipulating the nuclear receptor/PKN1 pathway during *in vitro* infections to directly address its differential contribution to the response generated to *M. tuberculosis* or *M. africanum* infections. Considering the lower virulence of *M. africanum*, understanding the details of its interactions with the host immune system may shed light on novel pathways (such as the nuclear receptor/PKN1) amenable to manipulation to ameliorate TB outcomes.

Inhibition of an Acid-Sensing Two-Component Regulatory System as a Novel Therapeutic Strategy for Tuberculosis

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Tuberculosis (TB) remains a top ten cause of death globally, partly due to its prolonged treatment time, currently comprised of several drugs for 6-12 months. The discovery of novel strategies to decrease treatment time is thus paramount to global TB management. One emerging strategy is the inhibition of two-component regulatory systems of *Mycobacterium tuberculosis*, the etiological agent of TB. In this study, we characterise a two-component system (TCS) of *M. tuberculosis*, TcrXY, comprised of the sensor kinase TcrY and response regulator TcrX, and demonstrate its potential as a therapeutic target for TB.

We demonstrate that TcrXY is an acid-sensing TCS of *M. tuberculosis* that is important for adaptation to environments encountered in the macrophage phagosome, the primary niche of *M. tuberculosis* in the host. The *tcrXY* operon is strongly induced transcriptionally in *M. tuberculosis* during acid stress *in vitro* and during the infection of cultured macrophages. Using transcriptomics, we provide the first description of the TcrXY regulon, comprised of approximately 60 acid-regulated genes. One notable regulon member is a putative protein/peptide translocation system that is expressed during macrophage infection in a TcrXY-dependent manner.

We investigated the role of TcrXY in virulence and the potential of TcrXY targeting to improve treatment efficiency in a murine model of TB. A doxycycline-inducible CRISPR-dCas9 based gene silencing system was employed to knockdown *tcrX* expression in virulent *M. tuberculosis*, H37Rv. Transcriptional silencing of *tcrX* significantly attenuated the persistence of H37Rv in the lungs and spleens of infected mice. Alongside treatment with rifampicin and isoniazid, the knockdown of *tcrX* resulted in a statistically significant 1-log reduction in mycobacterial burden in the lungs of infected mice, as compared to mice in which *tcrX* remained active.

In this work, we have identified, characterised, and validated, an attractive inhibitory target to improve the treatment of TB and potentially shorten therapy duration. Our efforts are now invested toward the discovery of novel small molecule inhibitors of the TcrXY system.

Mechanopathology of biofilm-like *Mycobacterium tuberculosis* cords: new insights from a lung-on-chip model of early tuberculosis infection

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The low minimum infectious dose makes the early stages of tuberculosis difficult to study in animal models. Heterogenous outcomes in the early stages of infection significantly impact the subsequent course of infection. We developed a lung-on-chip model for tuberculosis which recreates the alveolar environment in a programmable and modular manner. In a first report [1], we identified a host-protective role for pulmonary surfactant secreted by alveolar epithelial cells. We recreated a surfactant deficiency that would be lethal *in vivo,* and used long-term time-lapse imaging to show that pulmonary surfactant dramatically reduced Mycobacterium tuberculosis (Mtb) growth. I will outline ongoing work to develop a better understanding of alveolar epithelial cell –macrophage crosstalk using the LoC model, which may provide new targets for host-directed therapies.

More recently, we leveraged the LoC model to understand how Mtb responds to the air-liquid interface by growing into biofilm-like cords [2]. Mtb cords consist of tightly-packed actively dividing bacteria within host cells [3], an observation corroborated by an exhaustive examination of tissue sections obtained from the lungs of infected mice at early stages of infection using confocal microscopy. Using a toolbox of biological and physical approaches we uncover a mechanopathological role for Mtb cords in pathogenesis. *First*, cords restrict antibiotic penetration and are harbour transcriptionally active bacteria in the face of antibiotic treatment. *Second*, intracellular Mtb cords exert mechanical forces on cellular organelles including the nucleus, potentially modulating the host immune response. *Third*, after the death of the “first contact” host cell, cords break cellular tight junctions, and extend between adjacent non-phagocytic cells in a manner that enables host immune evasion. At the same time, cords, are strikingly toxic to and cause rapid cell death of professional phagocytes that attempt to take them up. We propose that cords effectively translate the turgor pressure a slow-growing sessile bacterium into a 3-D architecture that acts as a “wedge”. This hastens the dissemination of Mtb out of the alveoli and into the lung parenchyma where the infection takes hold.

References

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Role of Biotin Protein Ligase in *Mycobacterium tuberculosis* host pathogen interaction

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Tuberculosis treatment strategies have developed into a two-pronged approach involving the development of novel antimicrobials as well as agents that stimulate host responses to the pathogen (host-directed therapies). The presence of complex lipids in *Mycobacterium tuberculosis* (Mtb) cell wall results in a thick waxy coat around Mtb that acts as a structural barrier protecting the bacteria from host factors and rendering the commonly used antibacterial compounds ineffective against Mtb. The cell wall also plays an important role in Mtb interaction with the host immune system with complex lipids involved in modulating the immune response to support bacterial survival throughout the course of infection. Since the cell-wall of *Mycobacterium tuberculosis* (Mtb) is known to be critical for its survival and for elicitation of host inflammatory responses, we hypothesized that the agents that affect the cell-wall integrity have the potential to act as both antimicrobials and immunostimulants. We have previously reported that silencing biotin protein ligase (BPL) expression in Mtb impacts cell integrity leading to a loss of acid fastness and enhanced accumulation of rifampicin inside Mtb cells (PMID: 29695454)

Using mutant Mtb strains that express suboptimal levels of BPL, we demonstrate that these strains elicit robust innate pro-inflammatory responses in murine macrophages by enhanced secretion of proinflammatory cytokines which are known to be critical for the clearance of Mtb *in-vivo.* Our preliminary findings indicate that sub-optimal expression of BPL may augment host responses to Mtb via alteration of cell-wall lipid composition. Based on our studies we hypothesize that targeting BPL for development of new tuberculosis drugs has a bactericidal impact as this enzyme is essential for survival of bacteria. In addition, the cell wall modifications caused by BPL inhibition may play an immunostimulatory role, where an altered Mtb cell membrane aids in eliciting a robust immune response from the host cells.

Extracellular *M. tuberculosis* aggregates induce contact-dependent but uptake-independent macrophage death through PDIM and ESX-1 secreted factors

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Mycobacterium tuberculosis (*Mtb*) infection is initiated by inhalation of small numbers of individual bacteria and implantation in lung alveoli, where they are phagocytosed by resident macrophages. Intracellular replication of *Mtb* leads to death of the infected macrophages, release of bacterial aggregates, and propagation of the infection to newly recruited macrophages.

Here, we show that extracellular *Mtb* aggregates can evade phagocytosis and intracellular host-cell defenses by inducing macrophage death "from the outside" of the cell in a contact-dependent but uptake-independent manner. This effect is not due to bacterial numbers alone, because contact of macrophages with comparable numbers of non-aggregated bacteria does not result in uptake-independent killing. We use single-cell time-lapse fluorescence microscopy to show that contact with extracellular *Mtb* aggregates triggers local plasma membrane perturbation and cytoplasmic calcium accumulation in macrophages. These two processes precede host-cell death by pyroptosis and are dependent on the *Mtb* ESX-1 secretion system and the surface-exposed lipid phthiocerol dimycocerosate (PDIM), respectively. By using a panel of ESX-1 mutant strains, we show that the EsxA/EsxB proteins are required to induce uptake-independent killing. However, when secretion of these proteins is eliminated, EspB can still mediate macrophage killing, highlighting an overlapping role for these ESX-1 secreted proteins in uptake-independent induction of macrophage death.

Our results suggest that bacterial spreading within the lung and formation of necrotic lesions could be suppressed by novel therapies targeting factors such as ESX-1 and PDIM that are required for induction of host-cell death by extracellular *Mtb* aggregates.

POSTER SESSION 3

Thursday, September 15, 2022

1.45 pm to 3.15 pm

Insights into the structural diversity of mycobacterial type VII secretion systems and mechanisms of protein translocation

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The type VII secretion system is required by mycobacteria for the translocation of a range of substrates, that are key for a diverse set of functions including nutrient uptake, DNA transfer and immune modulation. Slow growing, often pathogenic, mycobacteria encode up to five of these systems (ESX-1 to ESX-5). Our recent work solved the high-resolution structure of the ESX-5 system from *Mycobacterium xenopi* using a combination of cryo-EM, X-ray crystallography and crosslinking mass spectrometry studies(*1*). The resulting model along with other structures of the ESX-5 and ESX-3 systems and improved structure prediction methods have paved the way for structural comparison of the different systems.

Structural analysis of the *M. xenopi* ESX-5 pore complex highlighted residues that we hypothesised would play a role in mediating secretion. We probed the role of these residues by generating several site directed mutants and tested the impact of these residues on the structure and function of the ESX-5 complex. Based on these data, we will present our recent insights into the secretion mechanism of the ESX systems and how their structural diversity may mediate their substrate specificity.

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LysX2 is a *Mycobacterium tuberculosis* membrane protein with an extracytoplasmic MprF-like domain

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Aminoacyl-phosphatidylglycerol (aaPG) synthases are bacterial enzymes that usually catalyze transfer of aminoacyl residues to the plasma membrane phospholipid phosphatidylglycerol (PG). The result is introduction of positive charges onto the cytoplasmic membrane, yielding reduced affinity towards cationic antimicrobial peptides, and increased resistance to acidic environments. Therefore, these enzymes represent an important defense mechanism for many pathogens, including *Staphylococcus aureus* and *Mycobacterium tuberculosis* (*Mtb*), which are known to encode for lysyl-(Lys)-PG synthase MprF and LysX, respectively. We used a combination of bioinformatic, genetic and bacteriological methods to characterize a protein encoded by the *Mtb* genome, Rv1619, carrying a domain with high similarity to MprF-like domains, suggesting that this protein could be a new aaPG synthase family member. However, unlike homologous domains of MprF and LysX that are positioned in the cytoplasm, we predicted that the MprF-like domain in LysX2 is in the extracytoplasmic region. Using genetic fusions to the *Escherichia coli* proteins PhoA and LacZ of LysX2, we confirmed this unique membrane topology, as well as LysX and MprF as benchmarks. Expression of *lysX2* in *Mycobacterium smegmatis* increased cell resistance to human β-defensin 2 and sodium nitrite, enhanced cell viability and delayed biofilm formation in acidic pH environment. Remarkably, MtLysX2 significantly reduced the negative charge on the bacterial surface upon exposure to acidic environment. Additionally, we found LysX2 orthologues in major human pathogens and in rapid-growing mycobacteria frequently associated with human infections, but not in environmental and non-pathogenic mycobacteria. Overall, our data suggest that LysX2 is a prototype of a new class within the MprF-like protein family that likely enhances survival of the pathogenic species through its catalytic domain which is exposed to the extracytoplasmic side of the cell membrane and is required to decrease the negative charge on the bacterial surface through a yet uncharacterized mechanism.

Understanding the role of MSMEG_0317 in mycolic acid transport in mycobacteria

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The mycobacterial outer membrane (OM) is distinctively characterized by the presence of mycolic acids (MAs), which in part, render the OM hydrophobic and impervious against various noxious substances, including several antibiotics. While the biosynthesis of MA is well studied, the mechanisms governing its transport from the inner membrane (IM) to the outer membrane (OM) remain largely elusive. Recently, several genes within a conserved genetic locus in mycobacteria and corynebacteria were reported to have putative functions in MA transport. Within this locus, *msmeg_0317* in *Mycobacterium smegmatis* and its homologs in other species have been previously associated with the biosynthesis of lipomannan (LM)/ lipoarabinomannan (LAM). However, this is largely inconsistent with the essentiality of *msmeg_0317* and its homologs, as LM/ LAM are not essential for mycobacterial survival. Furthermore, the *Mycobacterium tuberculosis* homolog of MSMEG_0317, Rv0227c was also recently reported to interact with MmpL3, a key protein involved in MA transportation across the IM. This led us to rethink the function of MSMEG_0317 and its homologs in mycobacteria, specifically in the aspect of MA transportation. To investigate our hypothesis, we generated and characterized a conditional *msmeg_0317* knockout mutant. Here, we show that MSMEG_0317 is essential for mycobacterial growth and required for MA transport from the IM to the OM. We further reveal that MSMEG_0317 exists as dimers and that both its N- and C-terminal helices are important for dimerization *in vitro* and essential for functionality *in vivo*. Taken together, our work offers molecular insights into MA transport and cell envelope biogenesis in mycobacteria.

Elucidation of the role of HtpG in maintenance of mycobacterial proteome

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Proteins are invariably essential macromolecules that are involved in every single physiological process ensuring the precise control of biogenesis, three-dimensional (3D) folding and maintenance of proteins in a functionally-active state, along with their cellular turnover and degradation. In the hostile niche within the host, dysregulation of protein metabolism may fatally impede critical cellular pathways, thus making proteostasis stand as one of the primary and vital arsenals of adaptive response of *M.tuberculosis* (Mtb). Indeed, it may surmise the reason why Mtb has preserved various proteostatic components including array of chaperons and proteolytic machinery, through the course of evolution. Considerable amount of information is available about Mtb chaperons and proteases like DnaK, GroEL/S, ClpB, ClpX, ClpC1 etc. HtpG, encoded by Rv2299c in Mtb and is absent in non-pathogenic mycobacterial species, like *M. smegmatis*; yet little is known about the significant role of Mtb HtpG (mHtpG) and its interacting partners during proteostasis. Taking into consideration the above lacunae, here we aim to biochemically characterize the chaperone activity of mHtpG. This study aims to answer some of the very fundamental questions about the functioning of mHtpG in maintenance of Mtb proteome. This study will not only reveal new aspects of the critical pathways controlled by proteostasis network in Mtb but may also aid in the identification of novel drug targets against tuberculosis, a disease which is prevalent in India, which shares a major part of the global TB burden.

Unveiling the diversity of the mycomembranes in Corynebacteriales

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Corynebacteriales is an order of big importance in human health, as it includes several pathogens like the causative agents of tuberculosis, leprosy, and diphtheria. Compared to the rest of Bacteria, the members of Corynebacteriales have an atypical cell envelope in terms of architecture and composition. This type of envelope is known as mycomembrane and it is composed of a layer of arabinogalactan attached to the peptidoglycan wall, and an outer layer composed of mycolic acids and external lipids. While the pathways involved in the synthesis of arabinogalactan and mycolic acids have been described in the model organisms Mycobacterium tuberculosis and Corynebacterium glutamicum, there are still many open questions on the biosynthesis and assembly of the mycomembrane and its diversity. For example, proteins are an essential component of bacterial envelopes, but only a few mycomembrane proteins have been reported in the literature. In terms of the lipid components of the envelope, the mycomembranes of the different Corynebacteriales have specific complex glycolipids that have not been characterized.

Unveiling the diversity of the mycomembranes is important to understand the processes that led to the origin and evolution of this atypical type of envelope. For this reason, the objective of this work was to characterize the mycomembranes of representative members of all Corynebacteriales diversity. First, we explored the presence or absence of previously reported genes involved in the synthesis of the different layers of the mycomembrane in 244 Corynebacteriales genomes. Then, we identified new potential genes involved in the synthesis and assembly of the mycomembrane, using orthology inference, pattern recognition, genomic context analyses, phylogenetic analyses and protein structure prediction. Finally, we discuss the evolutionary implications of our results in terms of pathogenicity, and the possibility of the identification of new therapeutic targets.

The Rv3432c encoding a glutamate decarboxylase enzyme confers acid tolerance in mycobacteria

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Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis (TB) in humans, is known to survive in active and latent phases intracellularly in its host. Mtb is known to survive against various environmental stress such as oxidative stress, nitrosative stress, and antibiotic stress. However, as soon as the bacilli enters the macrophage, low pH acts as the primary defence of the host. Recently, various studies identified that Mtb survives within acidified compartments of phagosomes and phagolysosomes, suggesting that to survive, the first stress Mtb needs to overcome is acidic stress. The glutamate decarboxylase (Gad) dependent acid resistance mechanism is a well-established system to cope with acidic stress in bacteria such as *E. coli, Listeria monocytogenes, Lactobacillus brevis*. Gad – A pyridoxal 5'-phosphate (PLP) dependent enzyme catalyze formation of Gamma-Aminobutyric acid (*GABA*) from glutamate consuming a proton in the process, which helps in maintaining intracellular pH homeostasis in the bacteria. However, this system has not been explored in mycobacteria. This novel study demonstrates that Mtb codes for *gad* gene and that an active Gad enzyme is present. The *gad* gene was amplified from the Mtb H37Rv genome and cloned in pJAM2 expression vector for purification of the enzyme. The *gad* gene overexpressed in an autologous system, *M. smegmatis* (MS), showed enhanced Gad activity compared to wild type. GABA produced in cell lysate was detected using TLC. With this, we suggest that Gad is playing an essential role in acid tolerance in Mycobacteria. It is interesting that GABA/Glutamate antiporter has not been identified in the genome of Mtb yet. It is proposed that the GABA produced may enter the TCA cycle through the GABA shunt, thus linking acidic tolerance to alteration in metabolism. Mtb is known to switch its metabolism for adaptation in the host, impacting drug tolerance and resistance. Further studies are underway to explore the role of this enzyme in mycobacterial survival in host cells and will be discussed during the meeting.

The sRNA B11 controls virulence-associated phenotypes in *Mycobacteroides abscessus*

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Little is known about the roles of small regulatory RNAs (sRNA) in *Mycobacteroides abscessus*. We found that deletion of the sRNA B11 in a smooth strain caused an intermediate smooth/rough morphology, increased antibiotic resistance, increased virulence in infection models, stronger innate immune activation, and increased transport to lysosomes. We identified several clinical isolates with B11 mutations. We used RNAseq to investigate the effects of B11 on gene expression and test the impact of two clinical mutations. ~230 genes were differentially expressed in ΔB11 compared to a complemented strain. Most of the genes differentially expressed in ΔB11 showed similar expression trends in strains with the clinical mutations, suggesting the clinical mutations caused partial loss-of-function. B11 has two C-rich loops previously found to repress expression in *M. smegmatis* by base-pairing to complementary sequences in ribosome binding sites (RBSs) of mRNAs. Among the genes upregulated in the ΔB11 mutant, there was a strong enrichment for the presence of B11-complementary RBSs. Comparing the proteomes of WT and ΔB11 strains likewise revealed a strong enrichment for B11-complementary RBSs in genes encoding upregulated proteins. Genes upregulated in ΔB11 included components of the virulence-associated ESX-4 secretion system. One of these had a B11-complementary RBS and fusing the RBS to a reporter made the reporter suppressible by B11. Together, our data show that B11 is a negative regulator with pleiotropic effects on gene expression and clinically important phenotypes in *M. abscessus*. To our knowledge, this is the first report of the role of an *M. abscessus* sRNA.

Therapeutic efficacy of antimalarial drugs targeting DosRS signaling in *Mycobacterium abscessus*

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Rapidly-growing nontuberculous mycobacteria (NTM) of the *Mycobacterium abscessus* complex have emerged as important human pathogens globally, and are linked to an increasing number of pulmonary infections among patients with structural lung disease such as chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis. The low cure rate of currently available treatment regimens, in spite of a minimum of 12 months of chemotherapy, notable side effects, and frequent bacterial re-emergence associated with these regimens highlights the need for alternative approaches to treat NTM infections. A search for alternative *M. abscessus* treatments led to our interest in the two-component regulator DosRS, which, in *Mycobacterium tuberculosis*, is required for the bacterium to establish a state of nonreplicating, drug-tolerant persistence in response to a variety of host stresses. We show here that the genetic disruption of *dosRS* impairs the adaptation of *M. abscessus* to hypoxia, resulting in decreased bacterial survival after oxygen depletion, reduced tolerance to a number of antibiotics *in vitro* and *in vivo*, and the inhibition of biofilm formation.

We determined that three antimalarial drugs or drug candidates, artemisinin, OZ277, and OZ439, can target DosS-mediated hypoxic signaling in *M. abscessus* and recapitulate the phenotypic effects of genetically disrupting *dosS*. OZ439 displayed bactericidal activity comparable to standard-of-care antibiotics in chronically infected mice, in addition to potentiating the activity of antibiotics used in combination. The identification of antimalarial drugs as potent inhibitors and adjunct inhibitors of *M. abscessus in vivo* offers repurposing opportunities that could have an immediate impact in the clinic.

Unveiling the biosynthetic pathway for short mycolic acids in Non Tuberculous Mycobacteria

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Mycolic acids, a hallmark of the genus *Mycobacterium*, are unique branched long-chain fatty acids produced by a complex biosynthetic pathway. Although most of the core pathway is comparable between species, subtle structure differences lead to different families delineating the mycolic acid repertoire of tuberculous and non-tuberculous mycobacteria (NTM). Due to their essentiality and involvement in various aspects of mycobacterial pathogenesis, the synthesis of mycolic acids -and therefore the identification of the enzymes involved- is a valuable target for drug development.

We report here the characterization of a *Mycobacterium smegmatis* mutant obtained by chemical mutagenesis, which was defective in the synthesis of the shorter α´- mycolic acids and showed an increased susceptibility to drugs. Whole genome sequencing and bioinformatics analysis identified MSMEG_4301, encoding a fatty acyl-CoA synthetase as the gene responsible for the phenotypes observed and further confirmed by complementation of the mutant strain. We renamed this gene as FadD32S, for its involvement in short mycolates. Subsequent deletion of *Mycobacterium abscessus* ortholog gene, MAB_1915, also abrogated synthesis of α´- mycolic acids, caused growth defects and increased its susceptibility to lipophilic drugs thus identifying a potential target for drug development against this NTM species of increasing medical importance.

A fast and reliable genetic barcoding strategy to confirm antibiotic resistance genotype-phenotypes in *Mycobacterium abscessus*

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Infections caused by nontuberculous mycobacteria are increasing worldwide (Ratnatunga, 2020). Of those, *Mycobacterium abscessus* is the major cause of infections, especially in patients with cystic fibrosis or chronic obstructive disease (Griffith, 2022). Similarly to multidrug-resistant (MDR) tuberculosis, *M. abscessus* diseases are difficult to treat due to the limited therapeutic arsenal and development of drug resistance (Nessar, 2012), resulting in a treatment success rate less than 50% (Victoria, 2021)

A clinical isolate of *M. abscessus* showing bedaquiline resistance was sequenced, and a novel mutation in the *atpE* gene was identified. To establish a direct genotype-phenotype relationship of the D29A mutation, we developed a recombineering-based method (van Kessel, 2008) consisting of the specific replacement of the desired mutation in the chromosome of a *M. abscessus* reference strain. The substrate used in recombineering carried a genetic barcode consisting of silent mutations in codons flanking the *atpE* D29A mutation. After selection of bedaquiline resistant colonies, transformed with substrates, carrying either the sole D29A mutation, or the mutation with the barcode, we obtained similar numbers of bedaquiline resistant transformants. These colonies were confirmed by Sanger sequencing and allele-specific PCR, demonstrating the presence of the genetic barcode in its specific location in the chromosome. All recombinant strains displayed the same profile of bedaquiline resistance compared to the original clinical isolate from which the D29A mutation was identified. We also demonstrated the versatility of this method by confirming the role of an unrelated mutation in the *atpE* gene, A64P, also conferring bedaquiline resistance. To confirm the technical reproducibility, we successfully reproduced results of both mutations using two independent reference strains of *M. abscessus.* Analogs of these two mutations have been reported also in *M. tuberculosis* (Segala, 2012).

Overall, our genetic barcoding strategy represents a useful approach to confirm putative antibiotic resistance mutations identified by current genome sequencing techniques. In addition, the barcoding strategy shortens these experimental results since it allows confirmation of allele replacements without the need of genome sequencing. We envisage a potential extension of this technique to other mycobacteria, and also its application to study virulence, physiology and biological traits of the *Mycobacterium* genus.

Protein-Energy Restriction Advances Disease Progression of Mice Infected with *Mycobacterium avium*

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Nontuberculous mycobacteria (NTM) are ubiquitous and opportunistic bacteria existing in the natural environment, such as water and soil. Pulmonary disease (PD) caused by *Mycobacterium avium* complex (MAC), the most prevalent NTM species, has been globally increasing. Several clinical reports have revealed that patients with MAC-PD tend to have low body weight or low body mass index (BMI), which are associated with persistent positivity of acid-fast smears and poor prognosis. Despite these clinical manifestations, it has not yet been clearly understood how a lower BMI affects MAC-PD progression. Therefore, in this study, using MAC infection-resistant A/J mouse model, we compared MAC-PD progression in protein-energy sufficient and protein-energy restricted groups by providing a standard protein diet [SPD] and low protein diet [LPD], respectively. Five-week after *M. avium* infection, an LPD-fed group was supplemented with SPD to evaluate the effect of protein supplementation on the disease progression. As a consequence, mice fed with an LPD had higher colony-forming units at 10 weeks post-infection, compared to those continued to be fed with an SPD. Interestingly, the protein-supplemented group showed approximately a 10-fold reduction in bacterial loads compared with mice fed with an LPD. When investigating immune responses and metabolic alterations subsequently in lung tissues, $CD45^+$ immune cell populations were significantly reduced in an LPD group. In addition, the number of Th1 and Th17 cells and their representative cytokines, IFN-γ and IL-17A, respectively, also markedly decreased in an LPD group. Importantly, genes related to β-oxidation of fatty acids were significantly up-regulated and five key metabolites associated with the TCA cycle were found at high levels in lung tissues of the LPD group. In particular, these immunocompromised and mitochondrial dysfunctions were restored when switching from LPD to SPD at 5 weeks post-infection. Collectively, our study suggests that the nutritional status of the host affects the immune response to *M. avium* infection through reprogramming lipid metabolism, ultimately resulting in disease progression of MAC-PD from stable status. Our findings may support important clues that sufficient nutrient intake is likely to contribute to preventing disease progression of MAC-PD.

Repurposing β-lactamase inhibitors for *Mycobacterium abscessus* complex

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Infections resulting from *Mycobacterium abscessus* are increasing in prevalence worldwide, with the greatest risk posed to patients with underlying respiratory conditions. Treatment for infections is difficult due to wide ranging intrinsic antimicrobial resistance, which is compounded by the existence of a range of subspecies within the *M. abscessus* complex, each with varying additional antimicrobial resistance profiles. Previously, we have proposed the use of β-lactam/β-lactamase inhibitors within a combination therapy as an effective treatment option for pulmonary *M. abscessus* infections. More recently, we have assessed the *in vitro* efficacy of two non-β-lactam based inhibitors, relebactam and avibactam, as agents against *M. abscessus* with their respective partner drugs imipenem and ceftazidime, as well as in triplicate combinations with additional β-lactam antibiotics against the *M. abscessus* complex. We have shown that the commercially available ratio of imipenem to relebactam is the appropriate ratio for bactericidal activity against *M. abscessus*, whereas the ratio between ceftazidime and avibactam is redundant, due to inactivity of ceftazidime to inhibit the bacteria. We have identified that the use of imipenem and meropenem alongside either relebactam or avibactam yield low minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for each *M. abscessus* subspecies, which are within the therapeutically achievable concentration ranges within the epithelial lining fluid of the lungs. We propose the implementation of imipenem with relebactam in place of stand-alone imipenem into the current treatment regime, alongside meropenem, as a future front-line treatment option for *M. abscessus* complex infections.

Efflux pump activity as a linezolid resistance mechanism in *Mycobacterium abscessus*

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Mycobacterium abscessus is a rapidly growing non-tuberculous mycobacterium that causes a multitude of clinically significant infections. Most first line antibiotics cannot be used to treat *M. abscessus* infection, making therapy arduous and ineffective. For many antibiotics, *M. abscessus* shows resistance despite lacking any genetic polymorphisms that would be expected to confer resistance. This suggests that other mechanisms, such as low permeability to antibiotics or drug efflux, likely contribute to *M. abscessus* recalcitrance to treatment. Our approach focuses on efflux pumps as putative causative factors for resistance to linezolid, an effective antibiotic agent used with success in other mycobacterial infections. However, linezolid is ineffective in many *M. abscessus* isolates, but resistant strains do not display consistent point mutations in linezolid target genes. We identified multiple clinical isolates that show a decrease in linezolid resistance after addition of efflux pump inhibitors. Using transposon insertion sequencing (TnSeq) in a resistant isolate, we searched for conditionally essential genes under linezolid exposure compared to untreated conditions. The results show several efflux pump encoding genes that became essential under antibiotic pressure. To verify our hits and to determine whether efflux pumps in these linezolid resistant strains were responsible for the ineffectiveness of the drug, we expressed a selection of efflux pumps identified by TnSeq into the linezolid susceptible model organism *Mycobacterium smegmatis*. Expression of one efflux pump, MAB_2736c, led to a significant increase in resistance. Moreover, overexpression of MAB_2736c in multiple linezolid sensitive *M. abscessus* strains rendered every strain resistant to linezolid. Together these data suggest efflux pump activity is an underlying mechanism of linezolid resistance for many *M. abscessus* strains, highlighting the therapeutic potential of efflux pump inhibition in combination with linezolid for *M. abscessus* treatment.

Phenotypic and genomic hallmarks of a novel,potentially pathogenic rapidly growing mycobacteria species related to the *Mycobacterium fortuitum* complex

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Introduction: Compelling evidence suggests that Non-Tuberculous Mycobacteria (NTM) might be responsible of respiratory infections in both immunosuppressed and immunocompetent hosts. We describe here a rapidly growing, non photochromogenic mycobacteria species (referred herein to as TNTM28), isolated from the sputum of an apparently immunocompetent young man with an underlying pulmonary disease.

Methods: TNTM28 was unique in that it shows a rough morphoype and a separate branch within the *Mycobacterium fortuitum* complex, based on sequence variability in 16S rRNA, *hsp65*, *sodA* and *rpoB* gene sequences. This has prompted us to sequence its whole genome. De novo annotation, genomic and phylogenmics analyses were proceeded using Prokka, Artemis and Mauve softwares respectively.

Results: The draft genome of TNTM28 consists of 5,526,191 BP circular chromosome with a 67.3% G + C content, and a total of 5193 predicted coding sequences. Phylogenomic analyses revealed a deep-rooting relationship to the Mycobacterium fortuitum complex, thus suggesting a new taxonomic entity. TNTM28 was predicted to be a human pathogen with a probability of 0.804, reflecting the identification of several virulence factors, including export systems (Sec, Tat, and ESX), a nearly complete set of Mce proteins, toxin-antitoxins systems, and an extended range of other genes involved in intramacrophage replication and persistence (*hspX*, *ahpC*, *sodA*, *sodC*, *katG*, *mgtC*, *ClpR*, *virS*, etc.), of which had likely been acquired through horizontal gene transfer. Such an arsenal of potential virulence factors, along with an almost intact ESX-1 locus, might have significantly contributed to TNTM28 pathogenicity, as witnessed by its ability to replicate efficiently in macrophages. Overall, the identification of this new species as a potential human pathogen will help to broaden our understanding of mycobacterial pathogenesis.

Keywords

Nontuberculous mycobacteria, Mycobacterium fortuitum complex, de novo genome sequencing, pulmonary disease, pathogenic NTM species

Drug Susceptibility Distributions of the Prevalent Nontuberculous Mycobacteria Isolated in Israel

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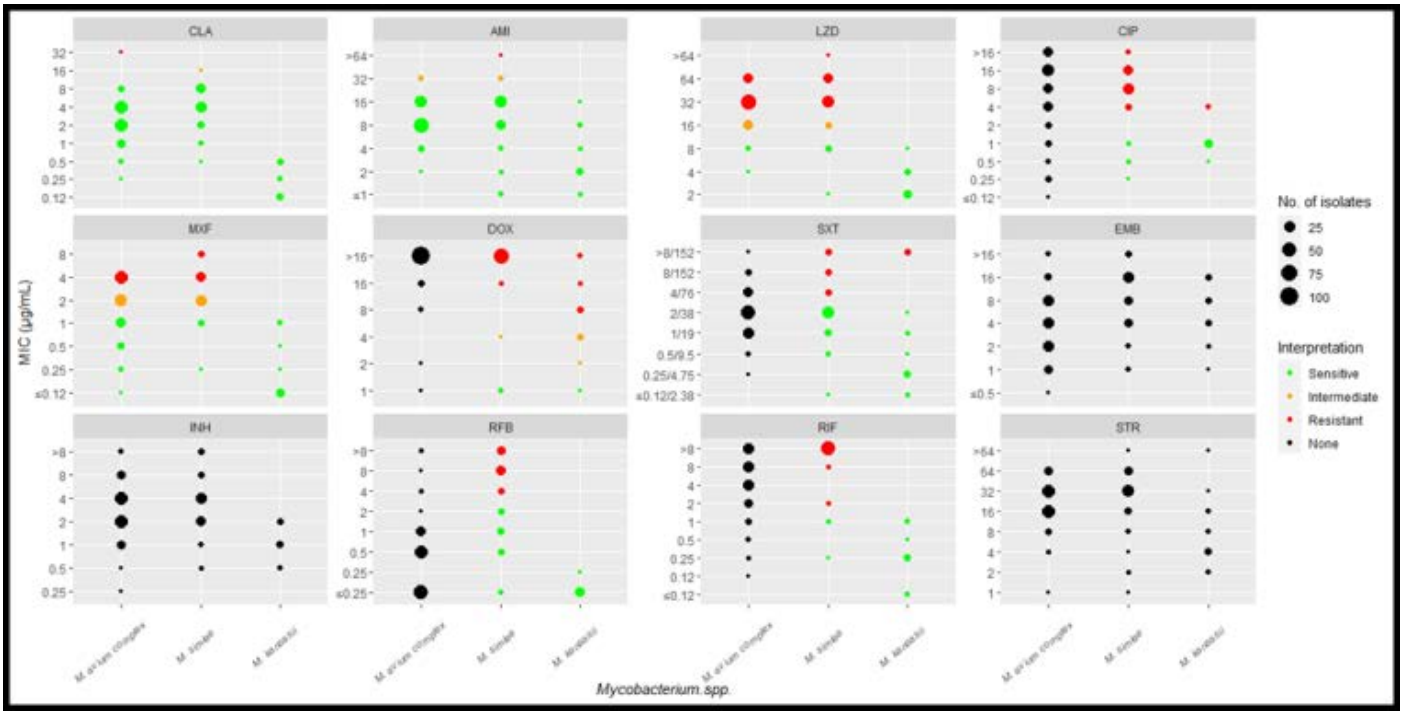
The incidence of nontuberculous mycobacteria (NTM) infections in humans have been steadily increasing globally. Mostly, the treatment of NTM is by combination of antimicrobial, with the treatment regimens selected according to species. However, treatment of NTM can be challenging since resistance to anti-mycobacterial drugs is common. Antimicrobial susceptibility testing (AST) for NTM is essential for selection of an appropriate and effective antimicrobial treatment. In Israel, a few epidemiological studies on the prevalence of NTM species were reported. However, there is lack of data regarding the AST patterns of NTM in Israel. In the current survey, we aimed to investigate the differences in drug susceptibility patterns of the five most prevalent slowly and rapidly growing NTM species. A total of 282 clinical isolates of NTM were investigated in this study: *M. avium* complex n=120, *M. simiae* n=70, *M. kansasii* n=20, *M. abscessus* complex n=52 and *M. fortuitum* n=20. Minimum inhibitory concentrations (MICs) for slowly and rapidly growing for 12 and 11 drugs, respectively, were determined by the microdilution method using the Sensititre assays. MIC_{50} and MIC_{90} values were derived from MIC distribution. The results showed that *M. simiae* isolates had a higher resistance rate and higher MIC_{50} values than *M. avium complex and M. kansasii*. Higher rates of sensitivity to rifabutin compared with rifampicin were seen in slowly growing NTM. For antituberculotic antibiotics, including ethambutol, isoniazid and streptomycin slowly growing NTM demonstrated a poor performance and high heterogeneity between species. *M. abscessus* complex have showed relatively high MIC_{50} rates to fluoroquinolones and tetracycline compared to *M. fortuitum*. In general, resistance to amikacin was rare in both rapid and slowly growing NTM, although antibiotic resistance profiles of other antibiotics varied tremendously among different NTM species. This demonstrated that accurate species identification and AST should be performed in order to guide the usage of these antibiotics.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CIP
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DOX
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EMB
INH
RFB
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50
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100
Interpretation
Sensitive
Intermediate
Resistant
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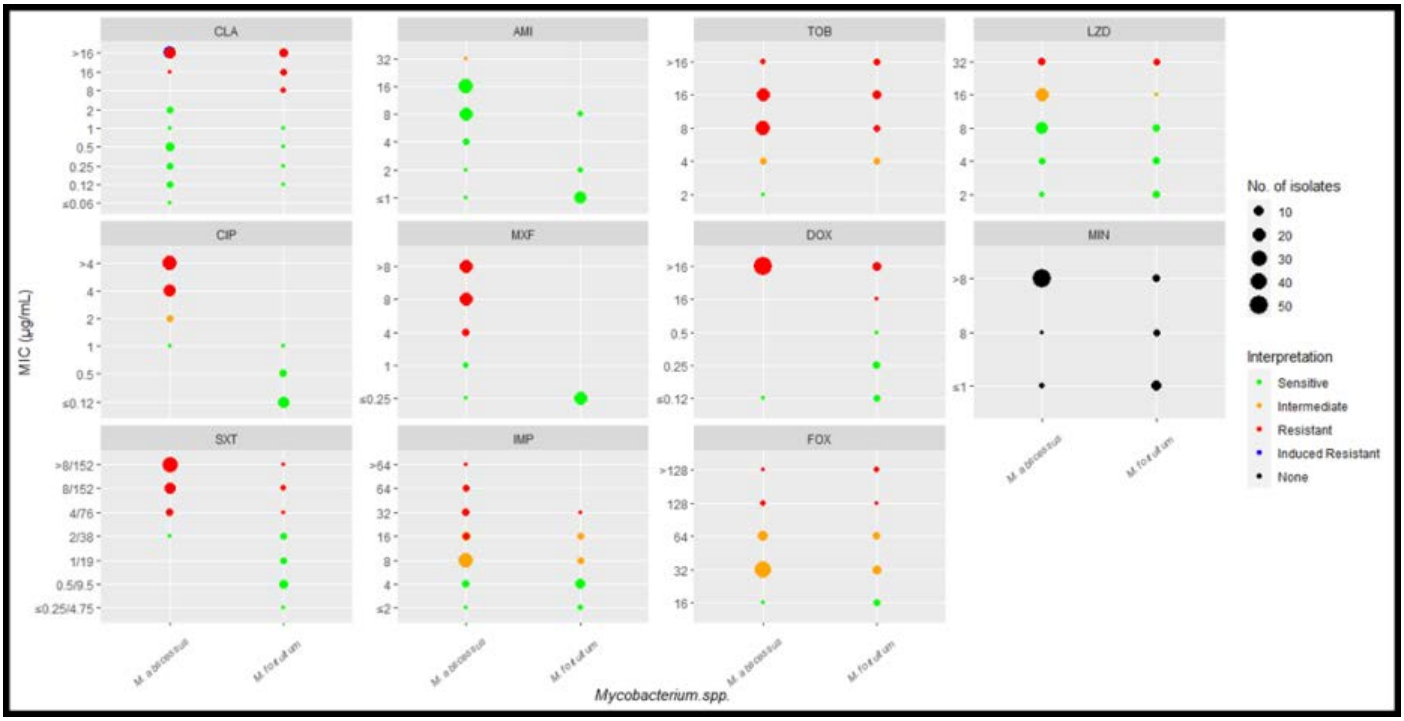
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IMP
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10
20
30
40
50
Interpretation
Sensitive
Intermediate
Resistant
Induced Resistant
None

Inactivation of NucS-dependent non-canonical mismatch repair mediates the rise of antibiotic resistance in *M. abscessus*

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NucS/EndoMS-dependent non-canonical mismatch repair (MMR) ensures the stability of genomic DNA in mycobacteria and acts as a guardian of the genome by preventing the emergence of point mutations. A Δ*nucS* strain was here constructed and explored in the multidrug resistant pathogen *Mycobacterium abscessus* to address whether the inactivation of non-canonical MMR could raise the acquisition of drug resistance by mutation. The deletion of *nucS* resulted in a mutator phenotype with increased acquisition of drug resistance to macrolides and aminoglycosides, the two main groups of antimycobacterial agents for *M. abscessus* treatment, and also to second-line drugs as fluoroquinolones. Inactivation of the non-canonical MMR in *M. abscessus* led to increases of 10 to 22-fold in the appearance of spontaneous mutants resistant to the macrolide clarithromycin and the aminoglycosides amikacin, gentamicin and apramycin, as compared with the wild-type strain. Furthermore, detectable fluoroquinolone (ciprofloxacin) resistance was generated by a *nucS* deficient strain but not by a wild-type *M. abscessus* strain. Acquired drug-resistance to macrolides and aminoglycosides was analyzed through sequencing of the 23S rRNA gene *rrl* and the 16S rRNA gene *rrs* from independent drug-resistant colonies of both strains. Lastly, a dataset of genomic assemblies of *M. abscessus* clinical strains was screened to detect *nucS* naturally occurring polymorphisms. To summarize, *M. abscessus* relies on the NucS-dependent non-canonical MMR to control the rise of drug-resistant isolates by mutation. To our knowledge, this is the first breakthrough that reveals NucS role in an emerging human pathogen, having potential implications for the treatment of *M. abscessus* infections.

In vitro synergy between manuka honey and amikacin against *Mycobacterium abscessus* complex shows potential for nebulisation therapy

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Mycobacterium abscessus is an opportunistic human pathogen of increasing concern, due to its ability to cause aggressive pulmonary infections (especially in cystic fibrosis patients), as well as skin and soft tissue infections. *M. abscessus* is intrinsically drug resistant and treatment regimens are lengthy, consisting of multiple antibiotics with severe side effects and poor patient success rates. New and novel strategies are urgently required to combat these infections. One such strategy thus far overlooked for mycobacteria is manuka honey. For millennia manuka honey has been shown to have wide ranging medicinal properties, which have more recently been identified for its broad spectrum of antimicrobial activity. Here we demonstrate that manuka honey can be used to inhibit *M. abscessus* and a variety of drug resistant clinical isolates *in vitro*. We also demonstrate using a microbroth dilution checkerboard assay that manuka honey works synergistically with amikacin, which is one of the current front line antibiotics used for treatment of *M. abscessus* infections. This was further validated using an *in vitro* inhalation model, where we showed that with the addition of manuka honey, the amikacin dosage can be lowered whilst increasing its efficacy. These findings demonstrate the utility of manuka honey for incorporation into nebulised antibiotic treatment for respiratory infections, in particular *M. abscessus*. These results pave the way for a change of strategy for *M. abscessus* management, offering new therapeutic options for this deadly infection.

Mycothione reductase as a novel target in the fight against *M. abscessus* biofilm-related pulmonary infections

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Background: *Mycobacterium abscessus* (*Mab*) is a multidrug-resistant nontuberculous mycobacteria (NTM) that is increasingly found to cause biofilm-related pulmonary infections in patients with a reduced immunity, such as cystic fibrosis (CF). Unfortunately, *Mab* infections are difficult to treat due to the inherent species related levels of drug-tolerance against most of the conventional antimycobacterial drugs. In order to improve treatment of these infections, new bacterial targets are under investigation that might play key-roles in the survival and biofilm formation of *Mab* within the host. An attractive target to be exploited is mycothione reductase (Mtr), a component of the bacillary redox homeostasis pathway which protects the bacteria against oxidative stress.

Aim: Hence, the aim of this study is to explore Mtr as a drug target in *Mab* by investigating its role during stress conditions and biofilm formation.

Methods: In the present study, an Mtr overexpression *Mab* strain was constructed through stable integration of a recombinant Mtr fragment into the L5 site. After validation of the strain, the phenotypic growth and resistance toward stress conditions were characterized. Next, biofilm formation in relation to Mtr expression was investigated on a polycarbonate membrane for 24H, 48H, 72H and 96H. The biofilm harvested after 24H represents an early stage biofilm while a mature biofilm is represented by the collection after 72H. The collected biofilms were measured by surface size, properties and CFU count.

Result: Unexpectedly, the Mtr overexpressing strain did not show a higher survival during stress conditions or increased ability of forming biofilms.

Conclusion: The obtained results demonstrate that even though Mtr plays a role in protecting *Mab* against oxidative stress, its overexpression does not provide an advantage for bacterial survival and biofilm formation. However, further studies are ongoing to uncover more about the function of Mtr during an infection and its potential as a novel drug target for *Mab*.

RNA sequencing in the hollow-fiber model: a novel method to study the effect of drugs on *M. avium* physiology during treatment

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Background: *Mycobacterium avium* complex (MAC) bacteria are frequent causative agents of nontuberculous mycobacterial pulmonary disease (NTM-PD) worldwide. Rifampicin is recommended for treatment alongside azithromycin and ethambutol, although its efficacy remains debated. We have previously shown in static *in vitro* models that macrolides have the largest effect on bacterial transcription and that the intracellular environment suppresses this response. Here, we employed a novel method of enriching for mycobacterial RNA in samples from the hollow fiber model, thereby allowing for transcriptomic analysis of the bacterial population during treatment to study the differences in adaptation in the presence and absence of rifampicin.

Materials: In a hollow-fibre experiment, THP-1 cells infected with *M. avium* ATCC 700898 were exposed to lung epithelial lining fluid pharmacokinetic profiles of the recommended 3-drug (rifampicin, ethambutol, azithromycin) or a 2-drug (ethambutol, azithromycin) regimen, for 21 days. On day 0, 3, 7, 14 and 21, samples were drawn to determine bacterial load and for RNA sequencing including bacterial enrichment using a custom designed probe-based capture kit (Arbor Biosciences). At day 0 and 21, samples were drawn to generate pharmacokinetic profiles.

Results: The 2- and 3-drug therapies both maintained stasis for 3 days after which regrowth occurred. RNA sequencing shows that the 2- and 3-drug therapies resulted in a similar transcriptional profile that is only minimally influenced by duration of exposure, although the overall effect of treatment on differential gene expression with 2-drugs was slightly larger. GO enrichment showed changes in lipid metabolism in both treatment arms, while enrichment of the NADH dehydrogenase associated with macrolide exposure was seen only in the 2-drug arm.

Conclusions: Rifampicin showed no additive antimycobacterial effect. Rifampicin didn't greatly alter transcription in comparison to the 2-drug regimen, likely because azithromycin and the intracellular environment dominate the stress response. These findings question the role of rifampicin in treatment guidelines. Also, key features of the in vitro response to antibiotics are absent in the HFM model. This highlights the added value of studying bacterial physiology in addition to growth when simulating treatment in preclinical models and the importance of modelling the intracellular environment.

Investigating the key factors responsible for the evolution of *Mycobacterium tuberculosis* as a highly virulent pathogen

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Tuberculosis, caused by *Mycobacterium tuberculosis (Mtb)*, remains one of the deadliest infectious diseases of humankind. *Mtb,* a biosafety level 3 (BSL3) organism, is extensively studied at the biological and genomic levels for years, but some aspects of its evolution are still poorly understood, mainly the outstanding gain of virulence compared to other mycobacteria.

Recently, opportunistic mycobacterial species belonging to biosafety level 1 or 2 organisms (BSL1-2) and named *Mycobacterium decipiens*, *Mycobacterium lacus*, *Mycobacterium riyadhense* and *Mycobacterium shinjukuense* were isolated from human patients. A preliminary study revealed a higher degree of genomic similarity with *Mtb* than previously used comparator species, such as the occasional pathogens *Mycobacterium kansasii* or *Mycobacterium marinum* (BSL2 organisms). These four mycobacterial species, thus represent very interesting study objects, as some of them might represent a missing link between low virulent mycobacterial opportunists and highly virulent specialized pathogens, such as *Mtb*. The comparative study of their genomes and their corresponding phenotypic characteristics will thus help to better understand the molecular factors responsible for the important evolutionary success of *Mtb* as a globally distributed pathogen.

As one example, results show a higher similarity in amino acid sequence identity between *Mtb* and *M. decipiens* than between *Mtb* and *M. kansasii* for the type VII secretion system loci (ESX-1-ESX-5). Moreover, preliminary studies suggest that certain of the four species seem to have a higher virulence than *M. kansasii* in cellular and murine infection models. Another main difference observed is the *in vitro* growth temperature of *M. decipiens,* which does not grow at 37°C but at 35°C. Interestingly, the bacteria are still capable of multiplying during lung infection in mice. According to these preliminary data, some of these novel species might also represent attractive study models as they are genomically and biologically closer related to *Mtb* than *M. kansasii* and *M. marinum,* but still do not require manipulation in BSL3 conditions.

Editing-independent mechanisms to limit toxic norvaline incorporation across the *Mycobacterium abscessus* proteome

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Maintaining the fidelity of the genetic code is critical for correct protein translation and fitness of the cell. Previously, we showed that epetraborole targets the editing domain of LeuRS in *Mycobacterium abscessus*. Epetraborole provided protection to zebrafish from lethal *M. abscessus* infection and decreased *M. abscessus* burden in a mouse model. We also showed that the development of epetraborole resistance by both *M. abscessus* and *M. tuberculosis* was reduced when the bacteria was treated simultaneously with epetraborole and the non-proteinogenic amino acid, norvaline. Furthermore, an epetraborole-resistant mutant of *M. abscessus* enabled non-cognate aminoacylation with norvaline, resulting in norvalination across the proteome and loss of cell viability. In current work, we spontaneously raised two distinct mutants resistant to both epetraborole and norvaline. Mutants were resistant to norvaline as measured by an increased minimum inhibitory concentration of norvaline and by limited incorporation of norvaline across the proteome. Having excluded changes in membrane hydrophobicity and increased efflux activity as mechanisms of resistance, we proceeded to Whole Genome Sequencing (WGS) of our mutants. All mutants had the LeuRS^{D436H} editing domain mutation, characteristic of epetraborole resistance. Low level norvaline resistant mutants manifested a minor growth defect when grown in norvaline and were found to have an additional LeuRS mutation (LeuRS^{L265V}). High level mutants exhibited no growth defect in the presence of epetraborole and norvaline, and sequencing revealed two non-LeuRS mutations (LeuA^{A555V}, and tRNA$^{Leu(GAG)T44C}$). Ongoing work is testing whether the single nucleotide polymorphisms identified by WGS confer resistance to norvaline by recombineering and gene complementation. Our results emphasize the importance of understanding resistance mechanisms for future therapeutic development.

Immunogenicity and whole blood RNA signatures of the adjunctive administration of therapeutic vaccine candidate H56:IC31 in TB disease

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Introduction: Therapeutic TB vaccination represents a Host Direct Therapy strategy which pursues the enhancement of the immune response in order to improve the clinical outcome and shorten its treatment, especially important in multidrug-resistant tuberculosis patients. Here we present the results of TBCOX2 (NCT02503839), a randomized open-label phase I/II clinical trial in which patients received the H56:IC31 vaccine as adjunctive TB treatment.

Sample and methods: Forty TB patients were included in the clinical trial. All patients received standard TB treatment. Twenty-two of them were randomized in the H56:IC31 arm (n= 12) and Control arm (n=10). Five micrograms of H56:IC31 vaccine (Statens Serum Institut; SSI, Valneva Austria GmbH) were administered intramuscularly at day 84 and day 140. Blood samples were collected at different time points and submitted to immunogenic and transcriptomic analysis. To study the vaccine immunogenicity, fluorescence IFNγ/IL-2 immuno-spot assay, whole blood intracellular cytokine staining flow cytometry assay and ELISA quantification of anti-H56 IgG in serum, were performed. For the transcriptomic analysis, RNA was isolated from PAXgenes tubes and longitudinal transcriptomic responses were identified using a targeted gene expression profiling platform (Fluidigm) on 190 immune- and TB-related genes.

Results: H56:IC31 vaccination was not associated with serious adverse events. Increases in both humoral and cellular responses were shown in the vaccine group. There was an increase in anti-H56 IgG titers following the first and second H56:IC31 vaccination. Importantly, a significant increase in T-cell responses in the H56:IC31-group compared to controls was also observed on day 154 (14 days after the second vaccine dose). Differentially expressed genes on days 98, and 154 in the H56:IC31 group versus the Control group were explored. C1QB was significantly upregulated at both time points in the vaccine group. Other genes significantly upregulated at any of the time points selected were FCGR1A_B_CP, SOCS1, RORC, PAX5, GATA3, TBX21, NOD1, MRC1, NCAM1 and FCGR3A.

Conclusion: H56:IC31 vaccination was shown to be safe and immunogenic in TB patients. RNA gene signatures might indicate a beneficial response to H56:IC31 vaccination in TB patients with active disease.

Excessive granulocyte-colony stimulating factor produced in NOX2 deficient mice generates abundant permissive CD11b^{int}Ly6G^{int} neutrophils, exacerbating the pathogenesis of *Mycobacterium tuberculosis* infection

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There are various findings about the roles of host factors in the pathogenesis of tuberculosis (TB) caused by *Mycobacterium tuberculosis* (Mtb). Host factors such as age, sex, immune cells, and cytokines strongly affect anti-or pro-TB immunity. Neutrophils are one of the most crucial host immune cells in TB pathogenesis, as lung neutrophil accumulation or circulating neutrophil counts are highly associated with TB severity and susceptibility. However, the mechanisms associated with excessive lung neutrophil accumulation remain understood incompletely. In order to figure out features and origins of aberrant neutrophils that emerge from Mtb infection, we adapted NADPH oxidase 2 (NOX2) deficient mice, extremely susceptible to Mtb infection, for *in vivo* and *in vitro* experiments. TB susceptible NOX2 deficient mice featured extreme lung inflammation and bacterial burdens at 4 weeks Mtb K post-challenge. Lungs of Mtb-infected NOX2 deficient mice contained excessive neutrophils with CD11b^{int}Ly6G^{int} along with CXCR2^{low}CD62L^{low} phenotypes. The number of granulocyte-monocyte progenitor cells (GMPs) and emergency GMPs (eGMPs) in the bone marrow were also increased. Additionally, various pro-inflammatory cytokines such as IL-1, IL-6, and G-CSF were upregulated in the lungs of NOX2 deficient mice. As G-CSF is known as the core regulator of granulocyte generation, murine bone marrow-derived cells were treated with G-CSF in a dose-dependent manner to investigate whether excessive G-CSF generates aberrant neutrophils. G-CSF treatment on mouse hematopoietic stem cells promoted neutrophil generation in a G-CSF dose-dependent manner, suppressing expression of CD11b and Ly6G. Moreover, those CD11b^{int}Ly6G^{int} neutrophils generated from excessive G-CSF treatment showed permissive phenotype to Mtb infection. Neutralizing G-CSF in Mtb-infected NOX2 deficient mice successfully prevented TB pathogenesis by reversing lung destruction and bacterial burden. Such effects were accompanied by decreased lung neutrophil populations with restored expression of CD11b, Ly6G, CXCR2, and CD62L. G-CSF neutralization also reduced the number of GMPs and eGMPs in bone marrow of Mtb-infected NOX2 deficient mice. These findings suggest that excessive G-CSF production in NOX2 deficient mice following Mtb infection may generate Mtb-permissive CD11b^{int}Ly6G^{int} neutrophils, by altering GMP/eGMP proliferation. Our study may suggest various tactics for host directed TB control through neutrophil/G-CSF axis.

Phase 2b Randomized double-blind, placebo-controlled trial to estimate the potential efficacy and safety of two repurposed drugs, acetylsalicylic acid and ibuprofen, for use as adjunct therapy added to, and compared with, the standard WHO recommended TB regimen (SMA-TB)

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Besides many efforts and achievements, novel therapeutic approaches are still needed to improve tuberculosis (TB) disease outcomes and shorten treatment duration. Host-Directed Therapies (HDT) propose shortening treatment length and improving patients' outcomes while tackling drug resistance.

A multicentre, phase IIB, placebo-controlled, randomized trial (ClinicalTrials.gov Identifier: NCT04575519) started in March 2020 to assess the efficacy and safety of 2 repurposed drugs (acetylsalicylic acid -ASA- and ibuprofen -Ibu), for use as adjunct therapy added to, and compared with, the standard of care (SoC) WHO-recommended TB regimen in drug-sensitive (DS) and multi-drug resistant (MDR) TB patients. The trial will recruit 354 participants with pulmonary TB in 3 sites (2 in South Africa and 1 in Georgia).

TB patients are being enrolled and randomly allocated to receive: 1) SoC TB treatment + placebo; 2) SoC TB treatment + ASA 600mg/day/4 weeks, followed by ASA 300mg/day/4 weeks; and 3) SoC TB treatment + Ibu 800mg/day/4 weeks, followed by Ibu 400mg/day/4 weeks. Patients are being followed-up until 6 months after treatment. Samples are being collected for host and pathogen biomarkers studies (at baseline, weeks 1, 2, 4, 8, end of treatment and 6 months after). Primary outcomes are 1) time to ≥ 67% sustained reduction in the TB score during follow-up and 2) Hazard ratio for time to stable culture conversion (SCC).

Since the CT start, and in spite of a low recruitment rate due to Covid pandemic, 119/354 patients have been enrolled, with a high participant retention rate in all study sites. In general, the investigational medicinal product (IMP) has been well-tolerated. Seven Serious AE have been reported: 3 not related and 4 possibly related to the IMP. No SAE have been considered

probably or definitely related to IMP.

We expect to provide evidence on the potential benefit of anti-inflammatories used as an adjunct to TB treatment as HDT. If proven useful, these enhanced treatment regimens have the potential to result in a better clinical practice, care management, and increased patients' wellbeing.

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Spatially resolved characterization of lung material from tuberculosis patients

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The rise of multidrug-resistant tuberculosis and the dearth of new antibiotic development place an existential strain on successful therapy. Breakthrough strategies that go beyond classical antibiotic mechanisms are needed to combat this looming public health crisis. Reconceptualizing antibiotic therapy in the richer context of host-pathogen interaction is required for innovative solutions. Most studies using patient's material are based on sputum and blood samples which do not reflect the pathological mechanisms occurring in the lung. We assembled a cohort of tuberculosis patients (MDR, XDR, susceptible) who received lung surgery after initial anti-microbial therapy. We analyze spatially resolved transcriptomes of the resected material and generated tissue microarrays of characteristic regions e.g., late and early granuloma, tertiary lymphoid structures, multinucleated cell, T cell, and macrophage areas, diffuse inflammation, and non-affected tissue for multispectral imaging. Spatial transcriptomics and tissue microarrays include lung material of patients from different strata e.g., gender, age, comorbidities, MDR/XDR or susceptible tuberculosis, treatment regimen and duration, etc. Results will provide us with signatures within morphological areas that are linked to a beneficial disease outcome and recapitulate the immunological and metabolomic processes differentiating the patient strata. This may constitute a promising target for supportive host-directed therapies.

Epigenetic governs macrophage phenotypes during Mycobacteria infections

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Mycobacterial infections are very dynamic processes, where alveolar macrophages (AMs) are the first line of the host defense. Because AMs must finely tune the immune response to pathogens and particulates in the lung without disrupting gas exchange, AMs show a pattern of gene expression distinct from other macrophages. They simultaneously express indeed clusters of genes belonging to both classically (M1) and alternatively (M2) activated macrophages. Interestingly, it has been proposed that histone posttranslational modification (PTMs) might chiefly shape AMs transcriptional potential. As a result, histone PTMs, such as the acetylation of H3 lysin 14 (H3K14ac), represent a timely mechanism that provides AMs a temporary, yet long-standing, extra layer of transcriptional flexibility. This suggests that histone H3K14ac might be one of mechanisms driving AMs cell-to-cell phenotypic variation. We hypothesized that such PTMs might result in the coexistence of multiple macrophages subpopulations exhibiting different pro- and/or anti-inflammatory properties, and that epigenetics modification can thus tailor intracellular mycobacterial survival and clearance. Hence, we molecularly probed the effect of H3K14 acetylation in host phenotypic diversity and studied the role played during mycobacterial infections at single-cell level, by combining high-resolution confocal microscopy, image analysis and flow cytometry.

We started our explorative study by analyzing the cell-to-cell printing of H3K14ac within an AMs-like cell line (MPI-2) in steady-state condition. We demonstrated that AMs-like cells exhibit a bimodal distribution already in basal condition. Multi-parametric single-cell analyses identified two coexisting subpopulations having low ($H3K14ac_{low}$) and high ($H3K14ac_{high}$) levels of acetylation. We then further characterize $H3K14ac_{low}$ and $H3K14ac_{high}$ AMs subpopulations bacterial sensing and infection resolving properties, revealing the crucial role of epigenetics in controlling the progression of the infection.

In sum, this project aims to exploit the molecular dynamic governing histone PTMs to propose a novel and alternative host-directed therapy that might enhance the host's ability to resolve mycobacteria infection while avoiding the arising of antimicrobial drug resistance.

High-content imaging for discovery of immunomodulatory compounds effective on intracellular *M. tuberculosis*

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Immune enhancing therapies represent an underexplored opportunity to meet the challenges of treatment failure in tuberculosis (TB). We have shown that the histone deacetylase (HDAC) inhibitor, phenylbutyrate (PBA), can restore *Mycobacterium tuberculosis* (Mtb)-induced impairment of antimicrobial responses in macrophages and enhance clinical recovery of pulmonary TB. Now, we expand our panel of HDAC inhibitors to discover new compounds that can effectively reconstitute immunity in both macrophages and T cells. This involves testing of 21 commercially available HDAC inhibitors with different specificities that are known to modulate inflammation using an in vitro Mtb infection model and high-content live-cell imaging. Human monocyte-derived macrophages or bulk peripheral mononuclear cells (PBMCs) from buffy coat blood were infected with green fluorescent protein (GFP)-expressing Mtb strain H37Rv (virulent) or H37Ra (avirulent) followed by treatment with compounds in the dose range of 0.01 – 10 µM for up to 5 days. IncuCyte® monitors intracellular Mtb growth (GFP-green label) and immune cell viability (Cytotox-red label) over time. From this screen, we have identified several HDAC inhibitors that reduce Mtb growth in macrophages >75% compared to average 40% for PBA, although we observed a spectrum of responses from high efficacy to toxic effects at high doses and HDACi that enhanced intracellular Mtb growth. Sorting of viable Mtb-infected macrophages resulted in around 60% GFP-positive and 40% GFP-negative cells showing an enhanced mRNA expression of pro-inflammatory IL-1β and TNF-α in both populations. Next, we will exploit Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) in combination with RNA-sequencing (RNA-seq) to investigate how open chromatin domains associate with changes in the transcriptome in sorted Mtb-infected macrophages and sorted $CD4^+$ or $CD8^+$ T cells in response to selected HDAC inhibitors. Overall, these results suggest that HDAC inhibitors can restore aberrant immunity induced by Mtb in macrophages and PBMC cultures and enhance intracellular growth inhibition of Mtb.

Phagosomal RNA sensing through TLR8 controls susceptibility to tuberculosis

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Kriztina Hegyi is Deceased Charlotte Maserumule and Charlotte Passemar Contributed equally to the manuscript Genetic determinants of susceptibility to Mycobacterium tuberculosis (Mtb) are poorly understood but could provide insights into critical pathways involved in infection, informing host-directed therapies and enabling risk stratification at individual and population levels. Through a genome-wide forward genetic screen, we identify the Toll-like Receptor 8 (TLR8), as a key regulator of intracellular killing of Mtb. Pharmacological TLR8 activation enhances killing of phylogenetically diverse clinical isolates of drug-susceptible and multidrug-resistant Mtb by macrophages and during in vivo infection in mice. TLR8 is activated by phagosomal mycobacterial RNA released by extracellular membrane vesicles, and enhances xenophagy dependent Mtb killing. We find that the TLR8 variant, M1V, common in far eastern populations, enhances intracellular killing of Mtb through preferential signal-dependent trafficking to phagosomes. TLR8 signalling may therefore both regulate susceptibility to tuberculosis and provide novel drug targets.

Preclinical *in vivo* assessment of replacing linezolid for spectinamide 1599 in the Nix-TB regimen

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Patients diagnosed with drug-resistant tuberculosis (TB) have limited treatment options. Nix-TB trial tested three-oral-drugs, namely Bedaquiline (B), Pretomanid (Pa) and Linezolid (L) (BPaL regimen) against drug-resistant TB. The BPaL regimen shows excellent favorable outcomes, but toxic effects are observed due to the long-term administration of Linezolid. Spectinamide-1599 (1599) is a potent protein inhibitor of *Mycobacterium tuberculosis* (Mtb) without known adverse effects and cross-resistance with other TB drugs. We hypothesized that inhaled 1599 could replace L in the BPaL regimen and provide similar or higher efficacy (BPaS regimen). Therefore, we tested BPaL and BPaS regimens in Balb/c (TB resistant) and C3HeB/FeJ (TB susceptible) mice infected with low-dose Mtb, and evaluated bacterial burden using colony-forming units (CFUs). Compared to untreated mice, CFUs in lungs of BPaL and BPaS treated Balb/c mice were decreased by >1 and 5 logs after 2- and 4-weeks treatment, respectively. In C3HeB/FeJ mice, 4-weeks of BPaL and BPaS treatment decreased CFUs by >3 logs compared to untreated control. However, no statistically significant differences were observed between treatment groups in both the mice strains. Cellular profiling using flow cytometry showed decreased hematopoitic (CD45+) cells and monocytes ($CD11b^{hi}CD14^{hi}CCR2^{hi}$) in the bone marrow of BPaL mice compared to BPaS. Cytokine profiling of the two treatment groups showed enhanced inflammatory response in BPaL mice than BPaS and this elevated immune response could be the cause of myelosuppression as reported for Linezolid. We concluded that inhaled 1599 is a potential replacement for Linezolid if combined as BPaS regimen, and further studies are warranted.

Three-dimensional *ex vivo* granulomas: characterizing protective immune traits across the spectrum of tuberculosis

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An increased understanding of protective immune responses against *Mycobacterium tuberculosis* (*Mtb*), a world leading infectious killer, would aid the development of novel, potentially host-directed, therapeutic approaches for tuberculosis (TB). TB pathology centers around the formation of organized, multicellular structures called granulomas and displays great inter-individual heterogeneity. To reproduce and decipher granulomatous immune responses *ex vivo*, we exploited a peripheral blood mononuclear cell (PBMC)-based 3D granuloma model and a unique collection of cryopreserved specimens from Tanzanian active TB patients (ATB) and *Mtb*-naïve controls (HC). We harnessed immunological (cellular composition and activation, effector molecule responses) and bacterial (colony forming units, metabolic state) read-outs to identify immune cell subsets and their effectors that may confer immune protection against *Mtb*. We found granulomas derived from ATB to control *Mtb* growth *ex vivo* significantly better than those derived from HC did. In addition to well-studied, adaptive mediators (e.g. IFN-γ), our data suggest potent innate responses to contribute to this increased immune control. We are now implementing cytometry by time of flight (CyTOF) technology to dissect the intricate interplay between immune cells and *Mtb* at an advanced level of comprehensiveness. Expanding our cohort to include latently infected individuals and individuals that eliminated *Mtb* upon exposure, we are characterizing granulomatous responses *ex vivo* across the spectrum of TB disease. Dissection of these immune responses at a patient level may pave the way for the design of personalized interventions in TB.

Host-directed antimycobacterial activity of colchicine, an anti-gout drug, via strengthened host innate resistance reinforced by the IL-1β/PGE_2 axis

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Background and purpose: To diversify and expand possible tuberculosis (TB) drug candidates and maximize limited global resources, we investigated the effect of colchicine, an FDA-approved anti-gout drug, against *Mycobacterium tuberculosis* (Mtb) infection because of its immune-modulating effect.

Experimental approach: We evaluated the intracellular anti-Mtb activity of different concentrations of colchicine in murine bone marrow-derived macrophages (BMDMs). To elucidate the underlying mechanism, RNA sequencing, biological and chemical inhibition assays, and Western blot, quantitative real-time PCR, enzyme-linked immunosorbent assay (ELISA) and immunohistochemical analyses were employed. Finally, type I interferon-dependent highly TB-susceptible A/J mice were challenged with virulent Mtb H37Rv, and the host-directed therapeutic effect of oral colchicine administration on bacterial burdens and lung inflammation was assessed 30 days post-infection (2.5 $mg \cdot kg^{-1}$ every two days).

Key results: Colchicine reinforced the anti-Mtb activity of BMDMs without affecting cell viability, indicating that colchicine facilitated macrophage immune activation upon Mtb infection. The results from RNA sequencing, NLRP3 knockout BMDM, IL-1 receptor blockade, and immunohistochemistry analyses revealed that this unexpected intracellular anti-Mtb activity of colchicine was mediated through NLRP3-dependent IL-1β signalling and Cox-2-regulated PGE_2 production in macrophages. Consequently, the TB-susceptible A/J mouse model showed remarkable protection, with decreased bacterial loads in both the lungs and spleens of oral colchicine-treated mice, with significantly elevated Cox-2 expression at infection sites.

Conclusions and implications: The repurposing of colchicine against Mtb infection in this study highlights its unique function in macrophages upon Mtb infection and its novel potential use in treating TB as host-directed or adjunctive therapy.

Role of Herp in *Mycobacterial tuberculosis* infection

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The current treatment for tuberculosis (TB) has many limitations, such as prolonged treatment duration, drug toxicity, and potential risk for the development of drug-resistant strains. There is an urgent need to develop new therapeutic drugs to control TB. In these days, host-directed therapeutics may offer therapeutic options for MDR-TB patients. It has been known that endoplasmic reticulum (ER) stress-mediated apoptosis is important to eliminate intracellular mycobacteria. The accumulation of unfolded proteins in the ER triggers the ER stress response. Homocysteine-inducible ER protein with ubiquitin-like domain 1 (Herp) plays a role in the unfolded protein response (UPR). It inhibits apoptosis by preventing the loss of ER Ca^{2+} and mitochondrial potential during ER stress. However, Herp's function, linkage to apoptosis, and role in ER-associated degradation (ERAD) in macrophages during *Mytobacterium tuberculosis* (Mtb) infection remain largely unknown. Here, we showed that Herp is induced in Mtb-infected macrophages through an activating transcription factor 6 (ATF6)-dependent ER stress response. Although suppression of Herp affects apoptosis, Herp depletion by genetic approaches decreased the production of HRD1 and SEL1L, a conserved branch of mammalian ERAD machinery, and increased the production levels of ER stress-associated molecules, such as IRE1α, BiP and XBP1 after Mtb infection. Herp depleted macrophages also showed increased production of both NADPH oxidase2 (NOX2) and inositol triphosphate receptor (ITPR), which sequentially led to increased reactive oxygen species (ROS) production during Mtb infection. Interestingly, interaction of Herp with HRD1, NOX2, and ITPR was confirmed by proximity ligation assay, which may regulate NOX2 and IRPR during Mtb infection. Herp depletion-mediated ROS increment also not only led to induction of autophagy but also led to suppression of the intracellular survival of mycobacteria in Mtb-infected macrophages. The role of the Herp was further confirmed by blocking this molecule *in vitro* and *in vivo*, which significantly reduced the number of surviving mycobacteria. These findings implicate that Herp mediates the crosstalk between ER stress and ROS-mediated autophagy during Mtb infection, suggesting the potential of Herp as a therapeutic target against Mtb infections.

A selective PPM1A inhibitor activates autophagy to restrict the survival of *Mycobacterium tuberculosis*

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Metal-dependent protein phosphatases (PPMs) have essential roles in a variety of cellular processes, including inflammation, proliferation, differentiation, and stress responses, which are intensively investigated in cancer and metabolic diseases. Targeting PPMs to modulate host immunity in response to pathogens is an ambitious proposition. The feasibility of such a strategy is unproven because development of inhibitors against PPMs is challenging and suffers from poor selectivity. Combining a biomimetic modularization strategy with function-oriented synthesis, we design, synthesize and screen more than 500 pseudo-natural products, resulting in the discovery of a potent, selective, and non-cytotoxic small molecule inhibitor for PPM1A, SMIP-30. Inhibition of PPM1A with SMIP-30 or its genetic ablation (ΔPPM1A) activated autophagy through a mechanism dependent on phosphorylation of p62-SQSTM1, which restricted the intracellular survival of *Mycobacterium tuberculosis* in macrophages and in the lungs of infected mice. SMIP-30 provides proof of concept that PPMs are druggable and promising targets for the development of host-directed therapies against tuberculosis.

Multiple targets in *de novo* pyrimidine synthesis for innovative structure-based antitubercular drug discovery

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Currently available antitubercular drugs target *Mycobacterium tuberculosis* (MTB) proteins involved in essential cellular functions such as cell wall synthesis or energy metabolism. In this context, the *de novo* and salvage synthesis of purine and pyrimidine nucleotides, have been reported as essential for mycobacterial survival, representing a source of promising targets for the development of new drugs against MTB.[1] In bacteria, the *de novo* synthesis of pyrimidines requires a strict regulation of six enzymes involved in a series of consecutive reactions that ultimately lead to the formation of uridine 5'-monophosphate, the common precursor of all pyrimidine nucleotides. Along this enzymatic cascade, the protein dihydroorotate dehydrogenase (DHODH) should be considered as the connecting hub between the nucleotide synthesis and the electron transport chain (ETC). Indeed, *Mycobacterium tuberculosis* DHODH (*Mt*DHODH) catalyses the conversion of dihydroorotate into orotate through FMN cofactor reduction and the electrons transfer to ETC via menaquinone.[2] Nevertheless, *Mt*DHODH is fully unexplored in its biochemical and structural features.

We present here the biochemical characterization of *Mt*DHODH as well as the structural analysis mainly focused on the quinone binding site in order to drive rational drug discovery also by repositioning active molecules tested on DHODH orthologues from other species. Furthermore, we solved the crystal structure of N-terminal truncated version of *Mt*DHODH which lacks of the putative quinone binding site, obtaining structural evidence of the possible discriminating role of N-terminal domain towards different redox molecules.

Future perspectives will include detailed analysis of protein selectivity towards different electron donors to eventually detect cross reactivity with alternative quinones. Indeed, the potential MTB protein activation by the human final electron acceptor ubiquinone, could suggest a possible auxotrophy of MTB towards the host metabolite, discouraging drug design targeting menaquinone synthetic pathway.

Finally, our drug discovery research pipeline aiming at interfering with pyrimidine biosynthesis, also focuses on the *Mt*DHODH downstream enzyme *i.e.* Orotate phosphoribosyltransferase (*Mt*OPRT) for which we propose alternative inhibition strategies based on design of selective molecules mimicking substrate, intermediate state and the product scaffolds respectively.

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Structure and dynamics of a mycobacterial type VII secretion system

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Mycobacterium tuberculosis causes one of the most important infectious diseases in humans, leading to 1.4 million deaths every year. Specialized protein transport systems—known as type VII secretion systems (T7SSs)—are central to the virulence of this pathogen and are also crucial for nutrient and metabolite transport across the mycobacterial cell envelope. Here we present the structure of an intact T7SS inner-membrane complex of *M. tuberculosis*. We show how the 2.32-MDa ESX-5 assembly, which contains 165 transmembrane helices, is restructured and stabilized as a trimer of dimers by the $MycP_5$ protease. A trimer of $MycP_5$ caps a central periplasmic dome-like chamber with the proteolytic sites facing towards the cavity. This chamber suggests a central secretion and processing conduit. Complexes without $MycP_5$ show disruption of the periplasmic assembly and increased flexibility, highlighting the importance of $MycP_5$ for complex integrity. Beneath the periplasmic chamber, dimers of the $EccC_5$ ATPase assemble into three bundles of four transmembrane helices each, sealing the potential central secretion channel. Individual cytoplasmic $EccC_5$ domains adopt two distinctive conformations, probably reflecting different secretion states. Our work suggests a previously undescribed mechanism of protein transport and provides a structural scaffold to aid the development of drugs against this major pathogen.

Understanding mycobacterial “SOS” response to design effective anti-TB strategies

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The “SOS” response is an essential systematic mechanism against DNA damage in bacteria. It is indispensable for its regulatory role in maintaining genome integrity and in gaining fitness advantage by developing useful mutations to tolerate genotoxic stress, leading to the development of antimicrobial resistance. LexA and RecA are the key players regulating the global network of stress-responsive and damage repair genes involved in this pathway. In an era of expanding drug resistance, targeting such non-traditional yet non-compromising pathways can provide useful answers in tackling global health hazards such as tuberculosis.

Despite its importance in "SOS" regulation, LexA from *Mycobacterium tuberculosis* (Mtb) remains poorly characterized and the functional importance of its additional amino acids remained elusive. LexA, under normal conditions, keeps “SOS” inducible genes repressed whereas in response to stress, it autoproteolyses, activating the previously repressed genes to facilitate their action. To target this crucial error-prone mutagenesis pathway, it was essential to first determine the kinetics of Mtb LexA-DNA interaction, which we elucidated through our published study. Mtb LexA was found to bind to different "SOS" boxes, DNA sequences present in the operator regions of damage-inducible genes, with comparable nanomolar affinity. Deletion of 18 amino acids from the linker region of Mtb LexA was found to affect DNA binding unlike the deletion of its unique N-terminal stretch of extra 24 amino acids. The conserved RKG motif was found to be critical for DNA binding. We developed a fluorescent-based reporter system to monitor the activation of mycobacterial “SOS” response in real-time in response to various stressors. This can be used for rapid screening of potential “SOS” pathway inhibitors.

The potential of targeting the “SOS” response is gathering increasing support to strengthen therapeutic efficacy due to its direct involvement in antibiotic resistance. LexA, which does not possess any eukaryotic counterpart, is an attractive drug target that we are putting to test in our studies by screening for its specific inhibitors. Characterizing such inhibitors can be effective in stalling “SOS” induced mutagenesis in mycobacteria and improving the existing arsenal of anti-TB therapeutics.

A conserved membrane protein negatively regulates Mce1 complexes in mycobacteria

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Various virulence factors contribute to the success of *Mycobacterium tuberculosis* as an intracellular pathogen. Among mycobacterial virulence factors, Mce systems, are involved in lipid transport, but the molecular basis for such function was unknown. To understand the composition and architecture of Mce systems, we characterized the putative fatty acid-transporting Mce1 complex. Using affinity purification, we show that the Mce1 system in *Mycobacterium smegmatis* is an ATP-binding cassette transporter complex comprising YrbE1AB as heterodimeric transmembrane domains (TMDs), MceG as nucleotide-binding domains (NBDs), and Mce1A–F as substrate-binding proteins (SBPs) likely forming a heterohexamer. Msmeg_6540, a homolog of Mce1A, can be a functional part of Mce1 complexes in place of Mce1A and may contribute to formation of a distinct SBP heterohexamer. Furthermore, we reveal that Msmeg_0959, herein renamed to Mce1N, is a negative regulator of the Mce1 transporter. Mce1N reduces the amount of MceG accessible to Mce1 by competitive binding to YrbE1B, which outweighs YrbE1A in mediating the TMD-NBD interaction within the complex. The regulatory mechanism is likely conserved across mycobacteria since *M. tuberculosis* Mce1N can also regulate *M. smegmatis* Mce1. Our work provides molecular insights into Mce complexes, shedding light on lipid metabolism and its regulation in mycobacteria.

Towards a mechanistic understanding of mycobacterial siderophore export

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The transport of the essential micronutrient iron is of interest as a target for novel TB drugs. Mycobacterium tuberculosis (Mtb) acquires iron by taking up heme and via siderophores (mycobactins), small iron scavenging molecules. We are interested in the redundant protein complexes that export siderophores through the inner membrane – the mycobacterial membrane protein large (MmpL) transporters 4 and 5 and the periplasmic accessory proteins mycobacterial membrane proteins small (MmpS) 4 and 5. MmpS5-MmpL5 have also been linked to drug resistance against antibiotics including bedaquiline. With the aim to gain insights into the molecular mechanism underlying siderophore and drug resistance, we have established expression and purification protocols for MmpS4-MmpL4. We found that MmpL4 can be expressed in *E. coli* in the context of the *mmpS-mmpL* operon, but not as a solitary protein. We were able to obtain MmpS4-MmpL4 at sufficient quantity and quality to perform single particle cryo-electron microscopy analysis. However, the determination of a high resolution structure has been prevented by the flexibility and degradation of MmpL4's periplasmic domains, observed by SDS-PAGE and by cryo-electron microscopy in 2D classes and 3D models. In an attempt to stabilise MmpS4-MmpL4 we selected synthetic nanobodies (sybodies) against the complex. Thirteen high affinity binders with K_D values in the low nanomolar range were obtained. The sybodies or a legobody (a complex of a sybody, Fab and MBP-PrA/G) were used in cryo-electron microscopy and data processing is currently ongoing. Additionally, the importance of D272, one of the four conserved amino acids containing key carboxylate groups required for the proton translocation that energises transport, was confirmed experimentally in Mtb. When Mtb*Δmmps4-mmpL4* is exposed to increasing concentrations of mycobactin a concentration dependent toxic effect is observed. This phenotype was also present after complementation with MmpS4-MmpL4$_{D272N}$ but not the wild type protein, demonstrating that the carboxylate of the aspartate residue is crucial for the function of MmpL4. Our dual approach combining structural investigation and functional studies is well suited to shed light on the mechanism of siderophore transport by MmpS4-MmpL4.

Structural insights into the activation mechanism of the conserved peptidoglycan hydrolase RipA

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The bacterial cell wall is a multi-layered mesh, both protecting the cell from the environment and containing its inside turgor pressure. Its major component is peptidoglycan, a sugar polymer cross-linked by short peptidic stems. The cell wall is reorganized throughout the cell cycle, especially during cell division where a careful balance of synthesis and degradation is required to allow the separation of two viable daughter cells (septation). The enzymes which degrade peptidoglycan (termed autolysins) therefore need to be precisely coordinated both in time and space to prevent anarchical destruction of the cell wall.

Important species-specific differences in cell wall composition and assembly exist to satisfy different morphologies, growth modes and environments. This is particularly true in *Corynebacteriales*, an order of *Actinobacteria* including important human pathogens such as *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. *Corynebacteriales* have an unusual, complex cell wall surrounded by a mycolic acid layer. *Corynebacteriales* septation is performed through a fast, brutal rupture of the cell walls of the two daughter cells called V-snapping.

We focused here on RipA, a key enzyme for cell separation in *Corynebacteriales*. RipA is an endopeptidase which catalyzes the cleavage of the peptidic cross-links of peptidoglycan and has been shown to be important for virulence of *M. tuberculosis* [1] and *C. diphtheriae* [2]. We characterized the structure of the full-length *Corynebacterium glutamicum* homologue of RipA, which revealed the enzyme in an auto-inhibited conformation mediated by its N-terminal coiled-coil domain. Further work allowed us to show that RipA is activated through specific protein-protein interactions that dissociate the intramolecular complex between the catalytic and the coiled-coil domains, and we are currently trying to understand the cytosolic signals that trigger cell separation. Since depletion of the different proteins involved in cell separation are known to increase antibiotics susceptibility in *Corynebacteriales* [3], we hope this work could lead in the future to the rational design of new drugs which could act in synergy with existing antibiotics.

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Nutrients transport through the outer membrane of mycobacteria

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The transport of substrates across the outer membrane of the devastating pathogen *Mycobacterium tuberculosis* requires porin-like proteins to provide the access to the periplasm. Outer-membrane associated PE/PPE proteins have been recently suggested to be involved in the uptake of nutrients such as heme, glycerol and glucose. Nevertheless, it is still not clear how exactly the PE/PPE proteins transport their substrates, how they look at the structural level and how they reside in the outer membrane. To answer these questions, we focus on PE5/PPE4 proteins, which might be involved in transport of siderophores. These proteins are predicted to have unique structural folds and it is unknown if they function as a heterodimeric complex or require help of other protein partners. We want to purify PE5/PPE4 complex from native membranes to characterize protein roles in potential transport of siderophores using *in vitro* and *in vivo* assays, identify other proteins that interact directly with the PPEs and that might facilitate nutrients uptake, and solve the structure of complex using cryo-EM. Synthetic nanobodies (sybodies) selected against PE5/PPE4 will be generated for an in-depth functional and structural characterization of these transporters, which may provide means for potential drug design. The project is expected to provide novel molecular insights into the largely unresolved question of protein-mediated nutrient transport across the unique outer membrane of this important pathogen.

Cryo-EM-based structural investigation of *Mycobacterium tuberculosis* Nucleotide Excision Repair

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During its entire life cycle, *Mycobacterium tuberculosis* (MTB) deals with toxic agents altering the genomic stability by inducing DNA damage. In particular, MTB bacilli show an excellent capability of surviving the chemicals released by infected macrophages, mounting a DNA repair response in which the Nucleotide Excision Repair (NER) has a key role in counteracting the harmful potential of oxidation and alkylation damages.[1]

NER represents one of the major molecular machineries that control chromosome stability in all living species. In Eubacteria, this pathway includes the three components of the UvrABC excinuclease complex, namely the UvrA, UvrB and UvrC proteins. These proteins act in a multi-step pathway in which the dynamic assembling of protein complexes is required for the lesion sensing and removal activities in an ATP-dependent manner.[2] Specifically, UvrA and UvrB are the first actors of NER and they have been reported as interacting proteins for the recognition of the damage across the DNA double helix. UvrA is thought to scan the DNA searching for the lesion, either alone or in complex with UvrB: interestingly, there are evidences in literature of the formation of UvrA-UvrB complex[3], but both the stoichiometry and the function of the complex in MTB is still under debate.

Here we present a Cryo-EM-based structural investigation of the heterotetrametric complex $UvrA_2UvrB_2$, as well as of the UvrA dimer, both in complex with damaged DNA. Our structural analyses reveal new insights in the DNA binding mode of UvrA protein and an alternative conformation of some crucial regions involved in DNA coordination. Moreover, at supramolecular level, we defined the stoichiometry and the structural determinants of the $UvrA_2UvrB_2$ complex formation, revealing mechanistic details that could also drive a structure-based design of active molecules interfering with DNA repair pathway in MTB.

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Structural basis of atmospheric carbon monoxide oxidation by mycobacteria

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The confinement of *Mycobacterium tuberulcosis* to the granuloma is a hallmark of tuberculosis. Within this immune structure, *M. tuberculosis* is frequently observed in a state of non-replicating persistence (NRP), likely as a result of being cut off from key nutrients and subject to a concentrated immune response. While the cessation of growth and replication makes *M. tuberculosis* appear inactive, minimal metabolic activity continues to maintain cellular electrochemistry and sustain integrity. Flexible metabolism is a widespread trait among the *Mycobacterium* genus that allows the bacteria to satisfy the energetic requirements of NRP and survive otherwise lethal starvation conditions. As organoheterotrophs, mycobacteria depend on organic carbon sources. During carbon starvation, the soil-dwelling *Mycobacterium smegmatis* oxidises the atmospheric trace gas carbon monoxide (CO) to feed its electron donor deficient respiratory chain using the multisubunit enzyme complex Carbon Monoxide Dehydrogenase (CODH). While the role of CO in the pathogenesis of *M. tuberulcosis* remains to be defined, a CODH homolog is present in the reduced genome of the pathogen, CO activates the dormancy regulator DosR, and host macrophages produce CO through a heme-oxygenase upregulated during infection. Therefore, *M. tuberculcosis* may oxidise macrophage derived CO to support persistence in host tissues. While the physiological advantages of mycobacterial CO oxidation have been demonstrated in *M. smegmatis*, the biochemistry that allows CODH to operate at the affinity required to oxidise atmospheric CO and how CO-derived electrons enter the respiratory chain remain unknown.

Here, we present our biochemical and structural characterisation of a mycobacterial CODH. We have natively purified CODH from *M. smegmatis* and determined its structure by Cryo-EM to 1.85 Å resolution. Further, we have determined the kinetic parameters of CO oxidation and have identified its native electron acceptor. Binding and localisation studies are underway to investigate a membrane-associated protein likely involved in electron transfer from the soluble CODH to the respiratory chain. These insights further our understanding of how mycobacteria are able to persist under harsh environmental constraints.

Conserved structural elements essential for siderophore import by the mycobacterial ABC transporter IrtAB

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The mycobacterial ABC transporter IrtAB is responsible for the uptake of iron-charged siderophores called mycobactins. A previously determined inward-facing structure of IrtAB revealed an ABC exporter fold, yet the transporter imports mycobactins across the inner membrane of mycobacteria. By conducting cryo-EM analyses in nanodiscs or detergent under ATP-hydrolyzing conditions, we show here that next to the inward-facing state, IrtAB also adopts a novel outward-occluded conformation featuring a large, enclosed cavity. Transition to the outward-occluded conformation results in a lateral opening of a crevice that may form an access route of mycobactins from the outer membrane leaflet to the internal cavity. We observed lipid and detergent binding at this crevice, and the introduction of bulky amino acids in this region resulted in elevated basal ATPase activity. Conformational changes at the crevice are structurally linked to a highly conserved triple histidine motif donated from two transmembrane helices, which coordinates a transition metal ion. Substitution of these histidines with alanines resulted in a decoupled transporter, which still hydrolyzes ATP, but lost its capacity to import radioactively labelled mycobactin into the cell. Our data support a transport mechanism in which IrtAB imports mycobactins from the outer membrane leaflet by alternating between its outward-occluded and inward-facing conformations in response to ATP binding and hydrolysis.

Oxidation of the mycobacterial redox cofactor mycofactocin by MftG

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The *in-silico* discovery of the mycofactocin (MFT) biosynthetic cluster led to the postulation of a new redox cofactor present mainly in mycobacteria (1). This notion was reinforced by the finding that the MFT system plays a crucial role in ethanol metabolism (2) and has implications on *Mycobacterium tuberculosis* growth in infection models (3). *In-vitro* studies established the role of the *mftABCDE* genes in the new ribosomally synthesized and post-translationally modified peptide (4). *In-vivo* studies using comparative metabolomics and structure elucidation closed further gaps concerning the function of the glycosyl transferase MftF and revealed numerous glycosylated and methylated derivatives of the new cofactor including oxidized (mycofactocinones) and reduced forms (mycofactocinols) produced by *Mycolicibacterium smegmatis* (5). However, the question remained how reduced mycofactocins are re-oxidized in the cell.

The last gene of the cluster (*mftG*) encodes for a putative glucose-methanol-choline oxidoreductase. Here, we revealed an enzymatic function of MftG as a mycofactocin oxidase. Gene deletion showed a similar phenotype as other MFT mutants with the cease of growth on ethanol as sole carbon source. Comparative metabolomics showed an accumulation of the reduced MFT pool suggesting a bottleneck in MFT re-oxidation while duplication of the gene resulted in the contrary effect on the MFT pool. Furthermore, comparing cell free extracts of Δ*mftG* and Δ*mftG*::*His*$_6$-*mftG* in activity assays with MMFT-2H$_2$ as a substrate confirmed the oxidase activity.

These results close an important gap of knowledge concerning the mycofactocin system and pave the way towards the elucidation of MFT's functions in pathogenicity and treatment of mycobacteria.

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Molecular ruler mechanism and interfacial catalysis of the integral membrane acyltransferase PatA

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Glycolipids are prominent components of bacterial membranes that play critical roles not only in maintaining the structural integrity of the cell but also in modulating host-pathogen interactions. PatA is an essential acyltransferase involved in the biosynthesis of phosphatidyl-myo-inositol mannosides (PIMs), key structural elements and virulence factors of *Mycobacterium tuberculosis* (Boldrin et al., 2021). The enzyme transfers a palmitoyl moiety from palmitoyl–CoA to the 6-position of the mannose ring linked to 2-position of inositol in PIM_1/PIM_2 (Albesa-Jové et al., 2016; Tersa et al., 2018). We demonstrate by electron spin resonance spectroscopy and surface plasmon resonance that PatA is an integral membrane acyltransferase tightly anchored to anionic lipid bilayers, using a two-helix structural motif and electrostatic interactions (Anso et al., 2021). PatA dictates the acyl chain composition of the glycolipid by using an acyl chain selectivity "ruler." We established this by a combination of structural biology, enzymatic activity, and binding measurements on chemically synthesized nonhydrolyzable acyl–coenzyme A (CoA) derivatives. We propose an interfacial catalytic mechanism that allows PatA to acylate hydrophobic PIMs anchored in the inner membrane of mycobacteria, through the use of water-soluble acyl-CoA donors. Many structural, chemical, and mechanistic aspects of this type of membrane enzymology remain intriguing and major challenge. From a drug discovery perspective, we hypothesize that the presence of clear and deep pockets might facilitate the discovery of potent inhibitors against the enzyme.

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Pretomanid and its effect on mycolic acids of mycobacteria

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Bicyclic nitroimidazoles, such as pretomanid (PTM), belong to the new generation of antituberculosis drugs that act on both replicating and non-replicating *M. tuberculosis*. PTM is a prodrug, which is activated by mycobacterial deazaflavin dependent nitroreductase Ddn (1). Resistance to PTM can occur through genetic changes that cause loss of function of this activator, enzymes FbiA, FbiB, FbiC and FbiD involved in deazaflavin cofactor F_{420} production or glucose 6-phosphate dehydrogenase Fgd1 catalyzing F_{420} reduction to $F_{420}H_2$ (2). Under anaerobic conditions PTM functions as a respiratory poison following nitric oxide release to kill nonreplicating bacteria (1). Recently it was shown that PTM forms an adduct with NAD that contributes to its bactericidal activity (3). In actively replicating cells of *M. bovis* BCG PTM by yet unknown molecular mechanism affects the synthesis of certain types of mycolic acids, in particular the oxidation of hydroxy-mycolates to keto-mycolates (4). In our work we focus on the elucidation of the effect of PTM on mycolic acids in the cells of pathogenic strain *M. tuberculosis* H37Rv. By ^{14}C metabolic labeling we show the consequences of PTM treatment on mycolic acids modifications in the cells of this strain, as well as of its mutated versions carrying mutations in *ddn* or *fbiC* genes encoding proteins involved in PTM activation. Using PTM we address the question of enzyme involved in the synthesis keto-mycolic acids.

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Mycobacterial monooxygenases: key actors for future antituberculosis strategies

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is the leading cause of death from a single infectious agent worldwide. Moreover, the continuous rise in the number of drug-resistant strains highlights the urgent need for innovative anti-TB drug development. One emerging approach is to potentiate and improve anti-TB compounds with validated mechanisms of action towards more effective and less toxic drug candidates. In the actual TB treatment, many anti-TB drugs require bioactivation by mycobacterial enzymes, notably through oxidation by Flavin-containing monooxygenases (MOs). *Mtb* was shown to have MOs that play roles in lipid metabolism as well as activation of anti-TB prodrugs. However, despite their emerging importance, their distribution, structural and functional features in mycobacteria remain enigmatic.

We applied a comprehensive bioinformatics analysis among selected actinobacteria, including mycobacteria and confirmed the presence of six monooxygenases in *Mtb.* We used *in silico* sequence/structure/function characterization and *in vitro* validation to examine these enzymes further and provide evidence that they contain characteristic motifs. We confirmed their activity and outlined their substrate preference.

These data emphasize the selectivity and questions the potential redundant role played by these class of enzymes in mycobacterial physiology and infection. Further biochemical and structural knowledge will allow modulating Mtb resistance to prodrugs, opening therefore avenues to innovative anti-TB strategies.

Keywords: *Mycobacterium tuberculosis* - monooxygenase - bioactivation - enzyme activity – molecular modeling.

Mycobacterial DNA replication initiation: biochemical insights into the replicative helicase loading mechanism

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Among the different aspects of *Mycobacterium tuberculosis* (MTB) biology, the biochemical processes underlying DNA replication are essential steps of the mycobacterial infection cycle. Key studies provided new insights into the dynamics of MTB replisome as well as on its hierarchical organization that is functional to the faithful duplication of genetic material. However, MTB replisome is relatively poorly characterized, and the working model of the multi-step DNA replication cascade has been assumed through comparison with model organisms. By considering the process as described for the reference bacteria, after the recognition of the origin of replication (oriC) by the DNA initiator protein (DnaA) and the exposure of single-stranded DNA AT-rich region, the bacterial replicative helicase DnaB is recruited. Despite the absence of a canonical helicase loader, it was recently demonstrated that MTB possesses an ancestral replicative operator named DciA, which displays the features of the replicative helicase-operating proteins associated with the replication initiation. Even though the loading of the replicative helicase onto DNA is a key step of the DNA synthesis, structural and kinetic details of this process in mycobacteria have so far not been investigated. In order to be definitively validated as a helicase loader, MtDciA should at least: i) interact with the replicative helicase, ii) sustain DnaB loading onto ssDNA, and iii) repress DnaB ATPase activity to prevent the initiation of unforeseen replication. Here, we present preliminary data on the ability of MtDciA to engage with the replicative helicase via the pull-down assay, on its capability to bind the DNA and on its peculiar domain architecture analyzed through the native mass spectrometry and the limited proteolysis, respectively. Moreover, Surface Plasmon Resonance analyses allowed us to describe the kinetic of the interaction and the role of the N-terminal and C-terminal domains of DciA in the binding to the replicative helicase. The final aim of the project is to define the mechanistic details of the replicative helicase loading in MTB as well as to exploit the structural information for a rational design of molecules interfering with a key aspect of mycobacterial DNA replication.

Looking at mycobacterial galactan biosynthesis

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Galactan polymer is a prominent component of the mycobacterial cell wall core. Its biogenesis starts at the cytoplasmic side of the plasma membrane by build-up of the linker disaccharide [rhamnosyl (Rha) – N-acetyl-glucosaminyl (GlcNAc) phosphate] on the decaprenyl-phosphate carrier. This decaprenyl-P-P-GlcNAc-Rha intermediate is extended by two bifunctional galactosyl transferases, GlfT1 and GlfT2 (1) and then translocated to the periplasmic space by an ABC transporter Wzm-Wzt (2). Cell wall core synthesis is then finalized by the action of an array of arabinosyl transferases, mycolyl transferases and ligases that catalyse an attachment of the arabinogalactan polymer to peptidoglycan through the linker. Based on visualization of the GlfT2 enzyme fused with fluorescent tags it was proposed, that galactan poylmerization takes place in a specific compartment of the mycobacterial cell envelope, so-called pMF domain (pure membrane free of cell wall components), which localises to the polar region of the mycobacterium (3). It this work, we examined the activity of the galactan-producing cellular machine in the enzyme fractions (cell-envelope and membrane) from *Mycobacterium smegmatis* in the cell free system using radioactively labelled substrate (UDP-^{14}C-Galactose). We found out that despite a high abundance of GlfT2 in both tested fractions, galactan is produced only in the reaction mixtures containing the cell envelope fraction. Next, we attempted to visualize galactan biosynthesis in permeabilized *Mycobacterium smegmatis* by UDP-^{13}C-Galactose coupled to nano-scale secondary ion mass spectrometry, but this approach needs more optimisation.

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Enzymatic lysis of mycobacteria using newly-discovered glycoside hydrloases

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The mycobacterial cell envelope provides a physical barrier between the bacterium and its external environment. This includes stressors ranging from chemicals such as antibiotics, to biotic threats such as phage and predatory bacteria. This protective barrier also hinders high-throughput -omics approaches to mycobacterial study and diagnostics. While enzymatic reagents have been developed for most bacteria, mycobacterial lysis typically requires some form of mechanical means, either by bead-beating or sonication. By mining the human gut microbiome, we have recently discovered several new classes of glycoside hydrolases which when combined with known enzymes can completely solubilise the mycobacterial cell wall. We will describe the biochemical characterisation of these enzymes and discuss their conservation amongst bacteria. We will also discuss the x-ray crystal structure of several of these families of enzymes and identify unique features governing their activity. Finally, we will show that when combined as a gentle mycobacterial lysis reagent we can use them to isolate high-quality genomic DNA. This has allowed us to close mycobacterial genomes with as few as 3 contigs, highlighting their utility to the mycobacterial research and diagnostics communities.

When division meets elongation in Corynebacteriales

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How bacterial cell division occurs is an old question. While many cell division genes were identified in model systems such as *Bacillus subtilis* or *Escherichia coli*, and an interaction network starts to be well drawn, the actual molecular mechanisms underlying bacterial cell division remain to be understood, especially in bacteria that display different modes of growth or morphologies. In Corynebacteriales, an order including important human pathogens such as *Mycobacterium tuberculosis* or *Corynebacterium diphtheriae*, many of the well-studied interactors of the divisome are missing from the genomes, and how the elongasome is assembled and controlled remains mysterious. The tubulin-like FtsZ and the scaffolding protein DivIVA (Wag31 in *Corynebacteriales*) direct the assembly of the divisome at mid-cell and the elongasome at the cell poles respectively. Since in these polar growing rod-shaped bacteria the division site will become the new poles of the daughter cells, some crosstalk between the divisome and elongasome machinery is expected but the physical and dynamic link between these two large multi-enzyme machineries remains unknown.

In this work we have discovered, using an integrative approach, a novel interaction network of the corynebacterial divisome, directly linking division (via FtsZ) to elongation (via Wag31). We show that such a link is provided by a stable complex of two proteins which are part of the early divisome: Sip1, that interacts with the conserved C-terminal domain of FtsZ, and Sip2, a transmembrane protein that acts as a Sip1 membrane receptor and also directly interacts with Wag31. Our cellular studies show that both proteins mediate the regulation of cell elongation possibly via the control of Wag31 network assembly at the division site. Sip1 and Sip2 could thus serve as regulators of the divisome-elongasome transition, through mechanisms which remain to be understood.

Understanding the eukaryotic pathways modulated by mycobacterial phosphatases: *Mtb*-PtpA interaction and activity on human TFPα, a key enzyme of host-lipid metabolism

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The virulence factor PtpA, belonging to the Protein Tyrosine Phosphatase (PTP) family, is delivered into the macrophage during *Mtb* infection. It represents an ideal target for drug design because potential inhibitors do not need to cross the mycobacterial envelope. Numerous groups, including our, identified PtpA inhibitors, but these are targeted at the conserved active site within PTPs family. Thus, identifying less conserved secondary sites continues to be a challenge for drug design. During infection, PtpA interacts with numerous eukaryotic proteins modulating several cell signaling pathways as phagosome maturation, innate immune response, apoptosis, and also host-lipid metabolism as suggested by our group. We identified as a *bona fide* PtpA substrate the human trifunctional enzyme subunit alpha (*h*TFPα), a key enzyme in beta-oxidation of long chain fatty acid. This pointed out PtpA as a bacterial factor related to host-lipid metabolism which is relevant to bacterial persistence. As most mitochondrial proteins, *h*TFP is synthesized in the cytosol and then translocated to this organelle. *h*TFP crystallographic structure was recently resolved, showing the relevance of the *h*TFPα helix-10 for its anchorage to the inner mitochondrial membrane and its activity. In addition, it has been reported that *h*TFPα is no longer detected in mitochondria of macrophages infected with the virulent *Mtb* H37Rv. In order to characterize the PtpA role on *h*TFPα, using bioinformatics tools, we identified the P-Tyr-271 as the potential PtpA target, a residue located in the *h*TFPα hélix-10 reported as relevant for its localization and activity. We also studied the characteristics of the mycobacterial PtpA interaction with eukaryotic TFPα and ubiquitin, a PtpA activator that interacts with this phosphatase through a site different from the active one. Furthermore, we evaluated the impact of PtpA phosphatase activity on human monocyte bioenergetics, in the absence and presence of different inhibitors and/or an uncoupling of the respiratory chain. The results showed that PtpA activity is associated with a decrease in the ability to acidify the extracellular medium of human monocytes. Further studies to characterize the relationship between these biochemical changes and lipid metabolism modifications during infection are planned.

Systemic influenza infection impairs whole blood control of mycobacteria in a human challenge study

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Background: Of the millions of people infected with *Mycobacterium tuberculosis* (Mtb) each year, only a small proportion will develop TB disease, with most immunologically containing or clearing Mtb. Factors influencing TB progression risk are incompletely understood. Co-infections, including influenza, have been proposed as a risk factor for TB progression via disruption of anti-Mtb immune responses. We employed a human influenza challenge study to investigate the effect of systemic influenza infection on host mycobacterial control.

Methods: A whole blood (WB) mycobacterial growth inhibition assay was utilised to compare mycobacterial growth and anti-mycobacterial immune responses before and after influenza infection. Thirty adults were per-nasally inoculated with H3N2 influenza virus (Day [D] 0). Influenza PCR assay of D4 nasal swab confirmed infection. WB, collected pre- (D0, "pre-influenza") and post-inoculation (D6, "post-influenza"), was infected with *Mycobacterium bovis* Bacille Calmette Guerin (BCG)-*lux*, a reporter gene-tagged BCG. WB was incubated for 72 hours (h) and WB mycobacterial growth (expressed as a growth ratio [GR]) measured. In parallel, BCG-*lux*-infected and uninfected blood aliquots were incubated for 0, 6, 24 and 72 h and measurements of cytokines (Meso Scale Discovery U-plex) and gene expression (RNA-Sequencing) were undertaken. Comparisons between pre- and post-influenza infection blood samples were made.

Results: In 22 influenza PCR-positive (+) subjects, median GR was significantly higher in post- (1.69) vs pre-influenza samples (1.03, p=0.0016) with no significant difference in the PCR-negative subjects. In PCR+ subjects, significant differences in BCG-*lux*-stimulated cytokine production were observed including higher interleukin-1β, tumour necrosis factor-α and interleukin-10 production after 6 h and 24 h incubation with BCG-*lux* in the post- vs pre-influenza samples (p<0.01). Longitudinal differential expression analysis revealed significantly different transcriptomic responses to BCG-*lux* infection over time in the post- vs pre-influenza samples. Significantly differentially expressed genes mapped to pathways related to TB susceptibility and supported the experimental cytokine observations.

Discussion: Systemic influenza infection reduces whole blood control of mycobacteria through modulation of anti-mycobacterial immune responses. Cytokine differences may contribute. These novel findings suggest that influenza may be a risk factor for TB disease and influenza vaccine could have a non-specific effect on TB immunity.

Genetic basis of resistance to infection by *Mycobacterium tuberculosis*

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Despite intense exposure to *Mycobacterium tuberculosis* (*Mtb)*, some individuals seem resistant to infection, as inferred by tuberculin skin test (TST) or interferon-gamma release assays (IGRAs). Few studies have attempted to unravel the genetic factors underlying resistance to *Mtb* infection and the polymorphisms identified have only a modest effect. We performed a whole genome sequencing study of individuals with extreme phenotypes of resistance who remained TST and IGRA negative (so-called resisters) despite high vulnerability due to HIV infection and high levels of exposure to *Mtb*. We enrolled 55 resisters and 100 *Mtb* infected HIV+ individuals from South Africa and 66 resisters and 57 *Mtb* infected HIV+ individuals from Haiti. Single variant analysis identified a locus on chromosome 12q15 associated with resistance to infection in South Africa with replication in Haiti, leading to a combined *P value* of 1.1×10^{-6} (OR[95%CI]=0.23[0.12–0.44]). The lead SNP, rs11286051, is an eQTL for *LYZ* which encodes the antimicrobial enzyme lysozyme. The allele associated with resistance to infection is associated with an increased expression of *LYZ* in whole blood and lung in the GTEx dataset. Gene-based analysis for rare (minor allele frequency<5%) nonsynonymous variants identified *PPM1H,* a promising candidate gene with 17.4% of carriers among resisters vs. 4.5% among infected individuals in the combined sample ($P=1.7 \times 10^{-4}$, OR=0.20[0.08–0.50]). *PPM1H* encodes a phosphatase which counteracts LRRK2, an inhibitor of BCG-triggered apoptosis and a negative regulator of *Mtb* phagosome maturation in macrophages. Our results will guide the understanding of the molecular mechanisms involved in resistance to *Mtb* infection.

A cost-benefit algorithm for rapid diagnosis of Tuberculosis and Rifampicin resistance detection during mass screening campaigns

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Background: Active tuberculosis (TB) case finding is important as it helps detect pulmonary TB cases missed by the other active screening methods. It requires periodic mass screening in risk population groups such as prisoners and refugees. Unfortunately, in these risk population groups periodic mass screening can be challenging due to lengthy turnaround time (TAT), cost and implementation constraints. The aim of this study was to evaluate a diagnostic algorithm that can reduce the TAT and cost for TB and Rifampicin resistance **(**RR) detection. The algorithm involves testing with TB-LAMP followed by Xpert MTB/RIF for positive TB-LAMP cases to diagnose TB during mass campaigns in prisons and refugee camps.

Methods: The National Tuberculosis Control Program (NTCP) organized routine TB mass-screening campaigns in 34 prisons and 3 villages with refugees camps in Cameroon in 2019. TB LAMP was used for initial TB diagnosis and all TB-LAMP positive cases tested with the Xpert MTB/RIF assay to determine RR. TAT and cost benefits analysis of the combined use of TB-LAMP and Xpert MTB/RIF assays was determined and compared to the Xpert MTB/RIF assay when used only.

Results: A total of 4075 sputum samples were collected from TB presumptive, 3,672 cases in 34 prisons and 403 samples in 3 villages. Of the 4,075 samples screened with TB-LAMP, 135 were TB positive (3.31%) and run on the Xpert MTB/RIF. Of the 135 positives cases, Xpert MTB/RIF revealed 3 were RR (2.22%). The use of TB-LAMP followed by testing with Xpert MTB/RIF for TB and RR detection reduced the TAT by 73.23% in prisons and 74.92% in villages. In addition to a reduced TAT, the two molecular tests used in synergy is cost benefit from year 2 onwards.

Conclusion: This study demonstrates the advantages of a diagnostic algorithm based on an initial testing with TB-LAMP followed by testing with Xpert MTB/RIF for TB diagnosis. This approach improved early and rapid TB detection with an added advantage of providing RR status. The proposed algorithm is effective and less costly from the second year of implementation and should be used by TB control programs.

Use of nucleic acid amplification tests on stool for rapid detection of intrathoracic tuberculosis in low-income countries in Children under 5 years

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Introduction: Tuberculosis (TB) is among the top ten causes of death in children, and it remains under-reported in low-income countries for a variety of reasons. Nucleic acid amplification tests are strongly suggested for rapid and quality bacteriological TB diagnostic. But in most cases, it is done on pulmonary samples which are not easy to get from children notably gastric aspirates.

Objective: Analyse detection of Mycobacterium tuberculosis Complex (MTC) DNA in gastric aspirates and stools of presumptive intrathoracic TB cases in children under 5 years

Material and methods: TB-Kids is a multicentric prospective study started on March 2016 in three African big cities (Abidjan, Antananarivo, and Yaoundé). After obtaining the consent from parents of children fulfilling the inclusion criteria, a standardized questionnaire was administered to the family. Then, gastric aspirates or spontaneous sputum was collected depending on children age. Stool and nasopharyngeal secretions were taken to all children. GeneXpert MTB/RIF and culture (liquid and solid medium) analyses were performed on all samples. Each enrolled child also received a tuberculin test, a chest X-ray and blood samples for haematological (blood count) biochemical (AST-ALT), serological (HIV), and virological (viral load).

Results: A total of 407 children with presumptive intrathoracic tuberculosis were enrolled in Côte d'Ivoire including 210 aged <5 years, and 145 < 2 years. Thirty out of 210 children were confirmed as TB and treated accordingly with first line regimen. Among them, 17 had positive skin test and 11 were bacteriological confirmed MTBC DNA. Five out of 11 MTBC DNA were also confirmed on stool samples. Detection of MTC DNA in gastric aspirate was 5.2% $CI_{95\%}$ [2.2%; 8,2%] and 3.3% $CI_{95\%}$ [0.9% ; 5,7%] in the stools. Sensitivity and specificity of MTC DNA detection in stool were 45,4%, $CI_{95\%}$ [16,7%; 76,6%] and 98,9%, $CI_{95\%}$ [97,4% ; 1%] respectively.

Conclusion: Our results showed that detection of MTC DNA in stool samples is lower than in Gastric aspirate for intrathoracic TB confirmation but it's possible.

Evaluation of Xpert MTB/RIF assay on nasopharyngeal aspirates and stool for the diagnosis of pediatric intra-thoracic tuberculosis in three sub-Saharan African cities: Abidjan, Yaoundé and Antananarivo (TB KIDS study)

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Introduction: Diagnosis of pulmonary tuberculosis (TB) in children is often a challenge due to frequent non-specific clinical and radiological manifestations. Besides, bacteriology which is realized on gastric aspirates (GA) is often negative. The Xpert MTB/RIFmolecular test, recommended by WHO in 2012 for the biological diagnosis of TB in children has been evaluated on gastric aspirate (GA) or sputum in areas with high TB prevalence but on small sample size. Alternatively sample collection methods, less invasive such as nasopharyngeal aspirate (NPA) or stool collection could yield the same performance.

Methods: We conducted a multicentric, diagnostic study in pediatric hospitals in three African cities: Abidjan, Antananarivo, and Yaoundé. We assessed the diagnostic performance of Xpert MTB/RIF test on two alternative samples, NPA and stools, for the diagnosis of intrathoracic TB in children aged 15 years and below suspected of having TB. We used the classic performance estimation method and also a latent class model. For the classic performance estimation, we used the Graham classification as reference standard.

Results: We recruited 1165 children (500 in Madagascar, 394 in Ivory Coast, 271 in Cameroon) from March 2016 to December 2018. The median age was 3.7 years (interquartile range [1.3-8.7]) and 12.2% were HIV-positive. Of the included, 25.8% had a positive TST and 31.7% had radiological images consistent with intra-thoracic TB children, 31.6% received anti-TB treatment, and 11.7%. died. Sensitivity of the Xpert MTB/RIF test on NPA or NPA/stool was almost similar to that of culture, respectively 58.3% (95% CI 49.4-66.8) and 63.2% (95% CI: 54.8-71.1) using the classical method and 74.7% (95% CI: 66-82.7) with the latent class model. Although slightly low on stool, specificities were high (>90%), compared to data reported elsewhere in other studies.

Conclusion: Our results showed that the Xpert MTB/RIF test performed on NPA and stool are an alternative that can be used for the diagnosis of TB in children. The feasibility of collecting these samples at the health facility level should be explored.

Assessing the test-and-treat strategy for incident, active tuberculosis among HIV+ patients in the Swiss HIV Cohort Study

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Patients infected with Human Immunodeficiency virus -1 (HIV-1) (PLWH) are often coinfected with latent tuberculosis (LTBI) and at high risk for progressing to active tuberculosis (ATB). Two immune assays to determine mycobacterial infection status and predict ATB risk are in clinical use: the tuberculin skin test (TST) and the interferon gamma release assay (IGRA). Preventive treatment is recommended in case of a positive test result. The efficacy of this commonly recommended test and treat preventive approach remains unclear.

Here, we use data from the Swiss HIV Cohort Study (SHCS) to assess the efficacy of the test and treat approach from 1990 to 2022. The outcome, i.e., incident ATB, was defined as active TB at least 6 months after SHCS inclusion. We used time updated hazard regression considering the following variables: preventive LTB treatment (intake of either Isoniazid, Rifampicin), Calendar time, age, RNA cps/ml, CD4+ cells/ml, BMI, ethnicity, and HIV transmission route (includes sex). We modeled the potential impact of better physician adherence guidelines and increased test sensitivity on ATB incidence.

A total of 12`534 patients with an immune assay result (TST or IGRA) were included, 69 of which had subsequent incident ATB. Of those, 42/2 had a negative TST/IGRA and 20/5 a positive TST/IGRA resulting in overall 64% false negative LTBI tests. 475 patients out of 995 (47.74%) with a positive test received preventive treatment. 1.89% (n=9/475) of preventively treated and 3.08% (n=16/520) of untreated patients with a positive immune assay result developed ATB. Risk to develop active TB was associated with a positive TST-/IGRA-test when compared to negative TST/IGRA (HR 10 [5.6, 17.85]). Low CD4+ T cell counts (HR 9.63 [4.19, 22.13]) and detectable HIV-1 viral load were independently associated with development of ATB (HR 2.11 [1.16, 3.82]), whereas preventive treatment was protective (HR 0.4 [0.18, 0.92]). Modelling suggests that increasing the sensitivity of LTBI testing has profound effects on reducing ATB in PLWH.

Immune assay positive PLWH have an increased risk to develop ATB, which is significantly reduced by preventive treatment. However, negative immune assays precede most ATB cases impairing the efficacy of the test-and-treat approach.

Shortening Buruli ulcer treatment: the BLMs4BU clinical trial

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Buruli ulcer (BU) is a neglected tropical skin disease, caused by *Mycobacterium ulcerans (Mul),* that affects mainly children under the age of 15 years in Africa. Current WHO-recommended treatment requires 8-weeks of daily rifampicin and clarithromycin, wound care and, sometimes, tissue grafting and surgery. Healing can take up to one year and may pose an unbearable financial burden to the household. Recent repurposing studies demonstrated that beta-lactams combined with rifampicin and clarithromycin are synergistic *in vitro* against *Mul* (PMID: 30689630) leading to the hypothesis that the inclusion of amoxicillin/clavulanate may improve and shorten BU therapy.

The aim of the BLMs4BU clinical trial is to evaluate whether co-administration of amoxicillin/clavulanate with rifampicin/clarithromycin can shorten BU treatment from 8 to 4 weeks. A randomized, controlled open label non-inferiority Phase II, multi-centre trial started in Benin in December 2021 (*ClinicalTrials.gov Identifier: NCT05169554*). A Phase III multi-centre trial in Ghana, Togo and Côte d'Ivoire is planned to start in December 2022. Patients are stratified according to BU category lesions and randomized to two regimens: (i) standard: rifampicin/clarithromycin (RC) for 8 weeks; and (ii) investigational: standard RC plus amoxicillin/clavulanate for 4 weeks. Patients will be followed-up for 12 months and managed according to standard clinical procedures. Decision for excision surgery will be made at week 14 after treatment initiation by an independent clinical expert panel. The primary efficacy outcome is cure (i.e., lesion healing without recurrence) without excision surgery 12 months after start of treatment.

If successful, this study will create a new paradigm for BU treatment, which could lead to a change in WHO policy and practice for this disease. A shorter, highly effective, all-oral treatment will improve the care of BU patients, adherence to treatment and will lead to a decrease in direct and indirect costs. This trial may also provide information on treatment shortening strategies for other mycobacterial infections, such as tuberculosis or leprosy, where rifampicin is the cornerstone drug.

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Lipobiotin-capture magnetic bead assay for isolation, enrichment and detection of *Mycobacterium tuberculosis* from saliva

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Background: Pulmonary Tuberculosis (TB) is diagnosed through sputum samples. As sputum sampling is challenging in children and cachexic patients, the development of diagnostic tests using saliva appears promising but has been discouraged due to low bacterial load and the poor sensitivity. We present a novel and rapid method to enrich Mycobacterium tuberculosis (Mtb) from saliva, which may serve as a basis for a diagnostic saliva test.

Methods: Lipobiotin-functionalized magnetic beads (LMBs) were incubated with Mtb-spiked PBS and saliva from healthy donors as well as with saliva from TB patients. Flow cytometry was used to evaluate the capacity of the beads to bind Mtb, while real-time quantitative polymerase chain reaction (qPCR) was utilized to detect Mtb and estimate the amount of mycobacterial DNA in different sample types.

Results: We found that LMBs bind Mtb efficiently when compared to non-functionalized beads. The development of an qPCR assay based on the use of LMBs (LMB assay) allowed us to enrich mycobacterial DNA in spiked sample types, including PBS and saliva from healthy donors (enrichment of up to 8.7 fold). In Mtb-spiked saliva samples, we found that the LMB assay improved the detection rate of 10^2 bacteria in a volume of 5 ml from 0 out of 15 (0%) to 6 out of 15 (40%). Consistent with that, the LMB assay increased the rate of correctly identified saliva samples from TB patients in two independent cohorts.

Conclusions: Implementation of the principle of the LMB-based assay may improve the sensitivity of existing diagnostic techniques, e.g. by functionalizing materials that facilitate Mtb sampling from the oral cavity.

Rapid nanomotion-based prediction of *Mycobacterium tuberculosis* susceptibility towards the first-line treatment antibiotics Isoniazid and Rifampicin

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Backgrounds & Objectives: Drug-resistant tuberculosis (DR-TB) is a major global public health problem and reinforces the need for rapid and reliable antibiotic susceptibility testing (AST) to choose effective anti-TB regimen. Conventional phenotypic ASTs are retarded by the slow growth rate of Mycobacterium tuberculosis (MTB). Nanomotion-based AST is growth-independent, recording the fluctuations of bacteria attached to a nanomechanical microcantilever; differences in the fluctuations in response to antibiotic exposure can distinguish susceptible from resistant bacteria. We applied this novel approach for rapid AST for MTB towards the first-line drugs Isoniazid (INH) and Rifampicin (RIF).

Methods: We developed a nanomotion-based AST protocol for MTB suitable for BSL-3 laboratory work. For its validation, we analysed a set of clinical isolates susceptible or resistant to INH and RIF and used a leave-one-out cross-validation (LOOCV) machine learning approach to identify the best features for phenotype prediction.

Results: The nanomotion-based AST protocol for MTB that takes 21 hours was applied to 36 clinical isolates. When considering each recording independently for the LOOCV the maximum sensitivity and specificity were 97.4% and 100% respectively for INH and 100% and 100% for RIF. When using a voting approach for the triplicate of each strain, for the LOOCV both the sensitivity and specificity reached 100% for INH and RIF.

Conclusions & perspectives: By combining nanomotion recording and a machine learning approach we were able to predict the phenotype of clinical isolates for INH and RIF. In comparison to current AST that takes several weeks, the total time for MTB nanomotion AST was 21 hours. This rapid and versatile approach could be extended to other anti-TB drugs to constitute a novel rapid biomarker of drug potency to guide correct treatment of TB.

Key words: Atomic force microscopy, Diagnostic microbiology, Machine learning, Mycobacteria, Nanomechanical sensor, Rapid AST, Tuberculosis

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