

Dependency on the host vitamin B12 has shaped the Mycobacterium tuberculosis Complex evolution

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Biological Sciences - Article

Keywords: Cobalamin, pernicious anemia, Mycobacterium tuberculosis Complex, *M. canettii*, non-tuberculous mycobacteria, host-pathogen crosstalk

Posted Date: March 1st, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2487911/v1>

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Additional Declarations: There is **NO** Competing Interest.

Version of Record: A version of this preprint was published at Nature Communications on March 9th, 2024. See the published version at <https://doi.org/10.1038/s41467-024-46449-8>.

Abstract

Human and animal tuberculosis is caused by the *Mycobacterium tuberculosis* Complex, which have evolved a genomic decay of cobalamin (vitamin B12) biosynthetic genes. Accordingly, and in sharp contrast to environmental, opportunistic and ancestor mycobacteria; we demonstrate that *M. tuberculosis* (*Mtb*), *M. africanum*, and animal-adapted lineages, lack endogenous production of cobalamin, yet they retain the capacity for exogenous uptake. A B12 anemic model in immunocompromised and immunocompetent mice, demonstrates improved survival, and lower bacteria in organs, in anemic animals infected with *Mtb* relative to non-anemic controls. Conversely, no differences were observed between mice groups infected with *M. canettii*, an ancestor mycobacterium which retains cobalamin biosynthesis. Interrogation of the B12 transcriptome in three MTBC strains defined L-methionine synthesis by *metE* and *metH* genes as a key phenotype. Expression of *metE* is repressed by a cobalamin riboswitch, while MetH requires the cobalamin cofactor. Thus, deletion of *metE* predominantly attenuates *Mtb* in anemic mice; although inactivation of *metH* exclusively causes attenuation in non-anemic controls. These phenotypes are specific of *Mtb*, which unlike *M. canettii* and environmental mycobacteria, is unable to consume exogenous L-methionine. Here we show how suboptimal host B12 antagonizes *Mtb* virulence, and describe a host-pathogen cross-talk with implications for B12 anemic populations.

Introduction

Vitamin B12 (B12), historically known as the “anti-pernicious anemia factor” by its ability to cure pernicious anemia, was first documented in 1926 ¹. It has the largest and most complex chemical structure of all the vitamins and biological cofactors, whose intricate structure was solved in 1956 by X-ray crystallography ². The term B12 or cobalamin (Cbl) refers to a group of water soluble, cobalt-containing corrinoid molecules, which are required in a wide range of metabolic processes both in prokaryotes and animals. Specifically, B12 in animals is essential for carbohydrate, fat and protein metabolism, and the formation and regeneration of red blood cells, as well as the maintenance of the central nervous system. Despite most prokaryotes and animals possess enzymes that require B12 as cofactor, only some bacteria and archaea are able to synthesize B12 *de novo* ³. However, even though some colonic bacteria produce Cbl, mammals are not able to uptake Cbl produced at this location, since the small intestine is the sole site of absorption. Therefore, animals, including humans, must assimilate B12 by dietary intake. Indeed, some mammalian herbivores (including rabbits, mice, rats and primates) practice coprophagy to obtain Cbl as they fail to obtain sufficient vitamin from vegetarian dietary sources. In humans, to obtain full benefits from vegan and vegetarian diets, these individuals should ingest fortified foods and/or B12 supplements ⁴.

The presence of B12-dependent enzymes in bacteria and animals makes this vitamin an attractive candidate for host-pathogen cross-talk. Indeed, it has been proposed a role for B12 in shaping the ecological niche of some bacterial genus as *Salmonella* or *Yersinia*. While non-typhoidal *Salmonellae* retain an intact B12 pathway, this is inactivated in the typhoidal human pathogens ⁵. Similarly, although

Y. enterocolitica and environmental *Yersinia* species retain all the genes for B12 synthesis, the human-pathogens *Y. pseudotuberculosis* and *Y. pestis* have lost most of them ⁶. In other words, those serotypes or species maintaining a functional production of endogenous B12 are adapted to cause intestinal infections; while those pathogens lacking B12 biosynthesis are associated with disseminated diseases. A plausible explanation for maintaining the B12 biosynthesis pathway in enteric *Yersinia* and *Salmonella* is that it is required for utilization of ethanolamine, a metabolite released from enterocytes during inflammation ⁷. Thus, the presence of B12-dependent ethanolamine respiration would enable enteropathogenic Enterobacteriaceae to utilize nutrients in the anaerobic environment of the gut and to outcompete other microorganisms from the microbiota. In this latter context, B12 is also necessary for *Salmonella* utilization of 1,2-propanediol, a catabolite produced by gut microbes fermenting fucose or rhamnose, common constituents of plant cell walls and intestinal epithelial cells lining the gut ⁵. Together, maintaining the B12 biosynthesis in enteric pathogens provides a metabolic advantage to outcompete their intestinal microbiota counterparts in an inflamed gut; while loss of B12 synthesis might represent a signature of invasive pathogens for the change of the intestinal niche and a movement away to a more systemic infectious cycle ⁶.

A solid molecular evidence for host-pathogen signalling mediated by B12 is provided by the intracellular pathogen *Listeria monocytogenes*. In this bacterium, a B12 riboswitch regulates the expression of a noncoding regulatory RNA, which in turns controls the expression of enzymes involved in ethanolamine utilization, that also require B12 as a cofactor. In fact, defects in ethanolamine utilization or in its regulation by the noncoding RNA attenuated *Listeria* virulence in mice ⁸. Another example is the presence of a B12 riboswitch in *L. monocytogenes* which controls transcription of a noncoding RNA involved in the regulation of the antisense gene *pocR*. In the presence of B12, this regulatory mechanism allows the PocR transcription factor to activate the expression of genes which mediate propanediol catabolism and are involved in pathogenesis. ⁹. Together, both mechanisms integrate a way to sense host B12 to regulate the virulence of this intracellular pathogen.

In this study, we will focus on the role of the host-derived B12 in the virulence of *Mycobacterium*, a genus comprising relevant intracellular pathogens as *M. tuberculosis*, which still causes 1.6 millions of tuberculosis (TB) deaths each year ¹⁰. The *M. tuberculosis* Complex (MTBC) comprises a group of closely related heterotypic synonyms of *M. tuberculosis*, adapted to cause TB in humans and animals ¹¹. The human-adapted variants include 6 lineages (L) of *M. tuberculosis* (L1 to L4, L7 and L8), and 3 lineages of *M. africanum* (L5, L6 and L9), while the animal-adapted variants are grouped into 4 animal clades (A1-A4). *M. canettii* is considered the progenitor and the putative common ancestor of MTBC members and it is thought that *M. canettii* diverged from the tubercle bacilli before the clonal expansion of the MTBC ¹². The genus *Mycobacterium* also includes non-tuberculous, and environmental, mycobacteria which eventually cause disease in immunocompromised, and chronic obstructive lung disease patients. The existing literature about B12 synthesis in the *Mycobacterium* genus, and more specifically in *M. tuberculosis*, is ambiguous. On the one hand, it has been documented that mycobacteria and related actinomycetes generally retain the ability to synthesize B12, predominantly by the aerobic pathway, and

the genome sequence of *M. tuberculosis* H37Rv reveals the presence of multiple *cob* genes predicted to function in the *de novo* biosynthesis of B12¹³. In another study, it was suggested that Cbl biosynthesis pathway is functional and subjected to purifying selection in *M. tuberculosis*¹⁴. Conversely, on the other hand, our closer inspection of *cob* genes of *M. tuberculosis* and related TB-causing bacteria reveals deletions, insertions, and polymorphisms which may ablate B12 biosynthesis. Here, we aim to disentangle this controversy, and to decipher the role of B12 in the host-pathogen cross-talk of the MTBC.

Results

Bacteria causing TB in humans and animals retain the ability to capture exogenous B12 despite lacking biosynthetic capacity

In spite of being considered highly clonal, the MTBC contains genetic polymorphisms which hypothetically have contributed to shape its evolution and host preference. Specifically, of the 16 *cob* genes putatively involved in Cbl synthesis in *Mycobacterium*¹⁵, we found non-synonymous mutations, insertions and deletions in 10 *cob* genes, which affect different branches of the MTBC (Fig. 1A). We confirmed genomic data by Sanger sequencing of specific clinical isolates belonging to the different MTBC lineages affected by *cob* mutations (Fig. 1A and S1). Of these polymorphisms, it is remarkable the presence of *cobF* and RD9 genomic deletions since they are large polymorphisms affecting several arms of the MTBC. The *cobF* gene is absent in all MTBC lineages, except L8¹⁶, and consequently animal-adapted species, *M. africanum* and the most widespread lineages of *M. tuberculosis* carry this deletion. RD9, which eliminates the 5'-terminus of the *cobL* gene, is absent in *M. africanum* and animal-adapted mycobacteria (Fig. 1A).

Overall, these data indicate that the MTBC have evolved a genomic decadence in B12 synthesis genes. However, being aware that non-synonymous mutations do not necessarily abrogate enzyme activity, and that gene deletions could be compensated by accessory genes, we assayed B12 production in representative *Mycobacterium* strains. None of the MTBC strains tested, which include *M. tuberculosis* L2 and L4; *M. africanum* L5 and L6; and *M. bovis*, produced detectable levels of B12 (Fig. 1B). In contrast, the MTBC progenitor, *M. canettii* showed an optimal B12 endogenous synthesis (Fig. 1B). Further, we also demonstrate that environmental (*M. smegmatis*), and opportunistic (*M. avium*, *M. abscessus*, *M. fortuitum*, *M. goodii*, *M. mucogenicum*, *M. xenopii*) mycobacteria produce variable B12 levels (Fig. 1B). This latter result is in agreement with a recent study demonstrating B12 production in various non-tuberculous mycobacteria¹⁷.

Once demonstrated that B12 synthesis is abrogated in the MTBC, we reasoned that the presence of B12-dependent enzymes in these pathogens¹⁵ necessarily imply the existence of a B12 uptake mechanism. Therefore, upon incubation of *M. tuberculosis*, *M. africanum* and *M. bovis* with exogenous B12, we confirmed the capacity of these bacteria to internalize the molecule (Fig. 1C). All MTBC species tested were able to uptake two B12 isoforms, namely cyano-Cbl and adenosyl-Cbl, in either exponential (Fig. 1C), and stationary growth phases (Figure S2). The evolutionary preservation of a mechanism for B12

transport, irrespective of a deleterious B12 biosynthetic pathway, emphasizes the relevance to scavenge this molecule for the physiology of the MTBC.

M. tuberculosis exhibits reduced virulence in B12 anemic mouse models

Our observation that MTBC members have retained the ability to scavenge exogenous B12, despite lacking endogenous synthesis, led us to investigate whether B12 could represent a host factor which modulate *M. tuberculosis* virulence. To prove this hypothesis, we first established B12 anemic mouse models based on feeding rodents during 8 weeks with a B12-deficient diet¹⁸. We confirmed that immunocompetent C57BL/6, and immunocompromised SCID mice fed without B12 showed 6.5- and 3.4-fold less B12 serum levels, respectively, than their counterparts fed with a normal diet (Fig. 1D). Rodents fed with both diets showed signs of animal welfare, which is in agreement with previous findings that a moderate depletion in B12 serum levels does not impact on the physiological status of mice¹⁹. Then, we infected B12-defective, and control mice, by the intranasal route with *M. tuberculosis* H37Rv and we evaluated either SCID mice survival times, or bacterial loads in C57BL/6 organs (Fig. 1E). We observed that anemic SCID mice survived significantly better after the *M. tuberculosis* infection than the control group (Fig. 1F and Figure S6). This denotes that, in the absence of adaptive immunity, the virulence of *M. tuberculosis* is reduced under suboptimal B12 serum levels, and *viceversa*.

This observation was confirmed after examination of bacterial loads in lungs and spleen of C57BL/6 immunocompetent animals. To rule out that differences in bacterial loads were due to an *in vitro* B12-dependent fitness, we first confirmed equivalent *M. tuberculosis* growth rates in laboratory media supplemented with or without B12 (Figure S3). Depleted B12 serum levels in C57BL/6 mice resulted in significantly lower bacterial replication in the lungs, and lower dissemination to the spleen, relative to the control group (Fig. 1G); reinforcing the hypothesis that *M. tuberculosis* needs optimal host B12 serum levels to develop a complete virulence. Then, we tested a *M. canettii* infection in our mouse anemic models, because unlike *M. tuberculosis*, this ancestor mycobacterium is able to endogenously produce its own B12 (Fig. 1B). First, we optimized the inoculum of *M. canettii* since this bacterium is known to be less persistent and less virulent than *M. tuberculosis*¹². In order to obtain equivalent survival in SCID mice, it was required from 100- to 1000-fold higher *M. canettii* inoculum relative to *M. tuberculosis* (Figure S4). Once established the infection conditions, we found equivalent survival times in SCID mice infected with *M. canettii*, independently of the B12 serum levels of mice (Fig. 1F and Figure S6). Further supporting the independence of *M. canettii* virulence and host B12 status, non-significant differences in lungs and spleen bacterial loads were observed in C57BL/6 mice fed with normal or B12-depleted diets upon infection with the ancestor *M. canettii* (Fig. 1G). *Post-mortem* analysis of *M. tuberculosis*- and *M. canettii*-infected mice confirmed that B12 serum levels remained low in anemic animals relative to control groups throughout the course of the experiments (Figure S5). To confirm the link between *M. canettii* virulence and its intrinsic ability to produce B12, we constructed a *M. canettii* $\Delta cobMK$ mutant unable to synthesize this molecule (Figures S7 and S12). This *M. canettii* B12 mutant was used to infect B12-

deficient mice and we found that, unlike the wild type, the mutant resulted strongly attenuated as measured by longer SCID survival, and significantly lower bacteria in C57BL/6 organs (Figure S7). Together, results indicate that host B12 levels directly correlate with *M. tuberculosis* virulence, but this association does not apply to the ancestor of the MTBC due to its specific ability to synthesize B12.

B12 supplementation of anemic mice recovers *M. tuberculosis* virulence

Results described above led us to speculate that restoring healthy B12 serum levels in anemic mice might impact on the virulence of *M. tuberculosis* in infected animals. Accordingly, 4 groups of SCID mice with different B12 treatments were established: a standard diet control, 2 groups fed with independent B12-deficient diets, and a final group receiving weekly subcutaneous B12 supplementation after being fed with a B12-deficient diet (Fig. 2A). Following each B12 treatment, all animal groups were intranasally infected with *M. tuberculosis* and we interrogated survival times and B12 serum levels at sacrifice. We found that both B12-defective diets resulted in significantly reduced serum B12 levels relative to the control diet. Further, supplementation with B12 resulted in efficient restoration of serum levels of this vitamin, comparable to those of the control mice (Fig. 2B). Survival times of each mice group remarkably correlated to their respective B12 serum levels. Interestingly, the gradual decrease in B12 serum levels observed after treatment with the different diets translated into longer survival times and consequently into reduced *M. tuberculosis* virulence (Figs. 2B and 2C). Confirming this observation, restoration of healthy B12 levels in anemic mice by subcutaneous supplementation resulted in a *M. tuberculosis* virulence indistinguishable from the mice group fed with a standard B12-containing diet (Fig. 2C). Together, this confirms that attenuation of *M. tuberculosis* in mice is precisely due to sub-physiological levels of B12, and the importance to assimilate B12 from the host for this pathogen.

The core B12-dependent transcriptome in *Mycobacterium* establishes a link with methionine metabolism

Having demonstrated that MTBC bacteria scavenge B12 from the host to modulate virulence, we sought to identify the molecular mechanism(s) responsible for this phenotype. We studied bacteria from the most representative lineages causing TB in humans and animals. Specifically, *M. tuberculosis* GC1237 and H37Rv strains were selected as representative of L2 (Beijing sublineage) and L4, respectively; and *M. bovis* (strain AF2122) was selected as the most representative animal-adapted variant. Replicates from each strain were grown in the presence, or absence, of exogenous B12, bacterial RNA was sequenced by RNA-seq, and differential gene expression between both conditions was interrogated (Tables S1 to S6). This analysis produced three differential expression sets, one per each MTBC strain analyzed, which were merged to gain insight into the “core B12 regulon” in the MTBC (Fig. 3A). We identified 7 genes organized into 2 genome clusters, which showed a robust downregulation in the presence of B12 (Fig. 3B and Figure S8). All these genes exhibited B12-dependent repression across replicates in all *Mycobacterium* strains tested, which probes that the identified B12 core regulon is reproducible and extensive to the MTBC (Figs. 3A, 3B, S8 and Tables S1 to S6). To additionally confirm the B12 regulation of the identified genes,

we corroborated downregulated expression of *Rv1129c*, *prpD*, *metE*, *PPE2*, and *cobQ1* measured by quantitative-PCR in *M. tuberculosis* H37Rv cultures incubated with B12 relative to controls (Fig. 3C and Figure S8). Further, we conducted a Selected Reaction Monitoring (SRM) proteomic approach to measure PrpD, PrpC and MetE protein levels in *M. tuberculosis* GC1237 and H37Rv cultures treated with or without B12. Results demonstrated a complete absence of PrpD and PrpC, and an average 9.8-fold reduction of MetE protein, when B12 is added to the cultures (Fig. 3D and Figure S9).

We then focused on the genome cluster containing 5 out of 7 genes of the B12 regulon. This cluster encompasses *Rv1129c* (PrpR), the *prpC-prpD-Rv1132* operon, and the *metE* gene (Fig. 3B). PrpR acts as a transcription factor of *prpDC*²⁰, which encode a methylcitrate dehydratase and a methylcitrate synthase, both involved in the methylcitrate cycle required for optimal *in vitro* growth on propionate as carbon source²¹. In addition, PrpR or PrpDC, are essential for optimal growth of *M. tuberculosis* in macrophages, which is probably attributable to the inability of the mutants to metabolize propionate derived from the host cholesterol²². However, a *prpDC* mutant failed to produce differences in pathology, or changes in organ bacterial loads, or persistence, relative to the wild type in a mouse infection model²¹. This absence of differential phenotypes *in vivo* for the *prpDC* mutant is probably attributable to the metabolic rescue of the methylcitrate cycle in the presence of B12²²⁻²⁴. The great versatility of carbon metabolism in *M. tuberculosis* allows this pathogen to utilize two complementary pathways for propionate detoxification: the B12-independent methylcitrate cycle, and the B12-dependent methylmalonate pathway. Thus, abrogation of the methylcitrate cycle in the *prpDC* mutant provokes deficiencies in *in vitro* or *ex vivo* growth due to the absence of regular B12 supplementation in these experimental conditions. However, the presence of B12 in mice fed with routine diets allows the activation of the alternative B12 methylmalonate pathway *in vivo*. Hence, provided that the role of PrpR, and PrpDC, has been studied in detail, we decided to focus our study in the B12-regulated *metE* gene, whose implications in the virulence of *M. tuberculosis* are less understood.

A *M. tuberculosis metE* mutant is predominantly attenuated in anemic mice

The final step of methionine synthesis in *M. tuberculosis* is catalyzed by two complementary methionine synthases: MetE or MetH. The activity of both proteins is regulated by B12, albeit by different mechanisms. Expression of *metE* is regulated by a B12 riboswitch located upstream the *metE* coding sequence, while MetH requires B12 as cofactor²⁵. Our transcriptomic and proteomic results demonstrate decreased *metE* mRNA and protein expression when B12 is present (Figs. 3B, 3C and 3D), which is in agreement with the regulatory mechanism exerted by the riboswitch, and with previous observations²⁵. We constructed a $\Delta metE$ mutant in *M. tuberculosis* H37Rv (Figure S12) to understand the role of this gene in virulence modulation by B12 (Fig. 3E). This $\Delta metE$ mutant is still able to synthesize methionine by the B12-dependent MetH, and consequently the $\Delta metE$ mutant is predicted to strongly depend on B12 supplementation (Fig. 3E). We confirmed a total absence of *in vitro* growth, in liquid and solid media, of the $\Delta metE$ mutant relative to *M. tuberculosis* H37Rv wild type in the absence of B12 (Figs. 3F and 3G). In

addition, we wondered whether *M. tuberculosis* might scavenge L-methionine from the environment. Cultivation of the *M. tuberculosis* $\Delta metE$ mutant in L-methionine-containing plates without B12 neither resulted in appreciable growth (Fig. 3G), indicative of lack of exogenous L-methionine foraging. To further confirm these phenotypes, we also generated $\Delta metE$ mutants in wild type *M. canettii* and in a *M. smegmatis* strain defective in endogenous B12 synthesis by deletion of the *cobLMK* operon (Figure S12). Both mutants exhibited the strong dependency on exogenous B12 to restore *in vitro* growth to wild type levels previously observed in *M. tuberculosis* (Figure S10), indicative that methionine synthesis by MetH is evolutionary conserved in *Mycobacterium*. Surprisingly, the growth defect in the absence of B12 showed by $\Delta metE$ mutants of *M. canettii* and B12-deficient *M. smegmatis*, was successfully rescued in the presence of exogenous L-methionine (Figure S10). This indicates that ancestor and environmental mycobacteria have retained a mechanism for L-methionine transport, which is lost in *M. tuberculosis*.

Next, we investigated the *in vivo* phenotype of the *M. tuberculosis* $\Delta metE$ mutant. We found a significant attenuation of the mutant measured by improved survival times (Fig. 3H), and lower bacterial loads (Fig. 3I), in SCID and C57BL/6 mice fed with a conventional diet, respectively. This attenuation was much more noticeable upon infection of anemic mice, according to the B12 requirements of the *M. tuberculosis* $\Delta metE$ strain. Comparatively, median survival times of the mutant were 25.5 days longer than the wild type in SCID anemic mice compared to 8 days in non-anemic mice fed with routine diet (Fig. 3J). With regards to lung bacterial loads in C57BL/6 mice, the mutant showed a 7.7-fold CFU reduction relative to the wild type in B12 anemic mice, with respect to a 2.7-fold CFU reduction in the control mouse model (Fig. 3K). In the absence of exogenous methionine uptake (Fig. 3G), these results support the strong dependency of exogenous B12 for L-methionine synthesis mediated by MetH in *M. tuberculosis*. In addition, the remnant attenuation of the *M. tuberculosis* $\Delta metE$ mutant in mice fed with a conventional B12 diet (Figs. 3H and 3I), might indicate that *in vivo* L-methionine synthesis by MetH is far from optimal, due to either insufficient B12 scavenging from the host, or to inadequate enzymatic activity of MetH.

Suppressive mutations in the B12-dependent *metE* riboswitch relieve the attenuation of a *M. tuberculosis* *metH* mutant in non-anemic mice

For a complete understanding of the implications of the B12-dependent methionine metabolism in *M. tuberculosis*, we also studied the phenotype of a *M. tuberculosis* $\Delta metH$ mutant (Figure S12). This mutant still maintains L-methionine production by MetE, and accordingly, it is expected to have growth defects in the presence of B12 due to the inhibition of the *metE* riboswitch (Fig. 4A)²⁵. Once constructed, the *M. tuberculosis* $\Delta metH$ mutant was inoculated in laboratory media with or without B12 confirming absent, or reduced growth, in liquid and solid media, respectively, when B12 is present (Figs. 4B and 4C). We also used this mutant to confirm the absence of exogenous L-methionine uptake in *M. tuberculosis*, since supplementation of B12 plates with L-methionine did not restore bacterial growth to wild type levels (Fig. 4B and 4C). In contrast, a $\Delta metH$ mutant constructed in *M. canettii* (Figure S12), grew equivalently to its parent strain in media supplemented with B12 and L-methionine (Figure S10), confirming that L-methionine uptake is evolutionarily maintained in ancestor mycobacteria, but not in *M. tuberculosis*. *In vivo* evaluation of the *M. tuberculosis* $\Delta metH$ mutant showed reduced virulence in SCID mice (Fig. 4D),

and reduced lung bacterial loads in C57BL/6 mice (Fig. 4E) under a conventional diet. In contrast, no differences in bacterial virulence were observed when animals were foraged with a B12 deficient diet (Figs. 4F and 4G), indicating that suboptimal B12 serum levels are insufficient to repress the *metE* riboswitch. In this latter context, we also wondered why optimal B12 serum levels did not result in a more severe attenuation of the *M. tuberculosis* Δ *metH* mutant. Surprisingly, when we sequenced the riboswitch region from *M. tuberculosis* Δ *metH* colonies grown in the presence of B12, we found that 15 of the 17 colonies analyzed contained mutations at this location (Fig. 4H and Figure S11). Mapping of the mutations to the predicted structure of the riboswitch demonstrated that these polymorphisms were regularly distributed, and different polymorphisms affecting invariant residues of B12 riboswitches²⁶ arose independently in independent colonies (Fig. 4I). This result suggests that suppressor mutations in the B12 riboswitch could alleviate the B12 repression of the *metE* gene and favour the appearance of *M. tuberculosis* Δ *metH* escape mutants when B12 is present either *in vitro* or *in vivo*.

This finding led us to hypothesize that introduction of a B12-independent *metE* gene into the *M. tuberculosis* Δ *metH* mutant should rescue the B12 growth defects of this strain without the need to evolve suppressor mutations in the *metE* riboswitch (Fig. 5A). We placed the Ag85A promoter (Pr-Ag85A) immediately upstream of the *metE* coding region, and this construction was introduced in the chromosome of the *M. tuberculosis* Δ *metH* (Fig. 5A and Figure S12). Improved expression of the Pr-Ag85A-controlled copy of *metE* was confirmed with respect to *M. tuberculosis* wild type and its *metH* mutant (Fig. 5B). Then, we confirmed that the Pr-Ag85A-*metE* copy enabled the *M. tuberculosis* Δ *metH* to grow in liquid and solid media containing B12, in contrast to the parent *M. tuberculosis* Δ *metH* strain (Fig. 5C). The Pr-Ag85A-*metE* complemented strain was tested *in vivo* in mice fed with a B12 containing diet, to confirm that survival in SCID mice and bacterial loads in C57BL/6 lungs were equivalent to those of the wild type strain (Figs. 5D and 5E). This later result was also reproduced in animals fed with a B12-deficient diet (Figs. 5F and 5G). Together, our results confirm the role of B12 suppressor mutations in the *metE* riboswitch to relieve the pressure imposed by B12 over the *M. tuberculosis* Δ *metH* strain *in vivo*.

Discussion

Humans have evolved through different dietary scenarios which might have shaped not only their nutritional requirements, but their susceptibility to infections. Through ages, the human evolution has circumvented the need to supply our bodies with dietary B12. As an example, primates, reminiscent of our ancestor hominids, present satisfactory B12 serum levels in their natural habitats²⁷, which might be obtained from either coprophagy, occasional carnivory, or foraging insects. On the other hand, present-day tribal societies which resemble our hunters-gatherers pre-Neolithic ancestors, show normal B12 serum levels²⁸, indicative that sporadic feeding on hunted meat could represent an optimal supply of B12. Therefore, it is appealing to speculate that during the long co-evolution of humans and pathogens, these latter have developed mechanisms to detect B12 from their hosts in order to sense the intracellular environment and to modulate pathogenesis. Here, we propose the host-derived B12 as such a signaling molecule employed by *M. tuberculosis*. Indeed, the presence of B12 riboswitches has been documented in a plethora of prokaryotes, including intracellular pathogens as *Mycobacterium*, *Listeria*, or *Salmonella*²⁹.

These latter bacteria are known to reside within the macrophage phagolysosome sometime in their life cycles. Thus, it might not be casual that B12 is internalized within human cells through the lysosomal pathway^{30,31}, a cellular compartment expected to establish a close contact with intracellular bacteria. Indeed, molecular B12 sensing by *Listeria* to modulate virulence has been already demonstrated^{8,9}. Concerning *Salmonella*, two independent studies using either a pernicious anemia mice model, or a B12-depleted diet model, have demonstrated that B12 deficiency renders C57BL/6 mice more susceptible to *S. typhimurium* infection^{32,33}.

In *Mycobacterium*, a recent study identified virulence pathways in a panel of genetically diverse mice. Among these genes, it was found that *bacA*, *mutB*, and *PPE2* were associated with the ability of *M. tuberculosis* for adapting to diverse host environments³⁴. Of this pathway, *bacA* is a *M. tuberculosis* B12 transporter and its inactivation results in attenuated virulence^{35,36}, *mutB* encodes one of the constituents of the B12-dependent MutAB methylmalonyl-CoA-mutase³⁷, and *PPE2* is regulated by a B12 riboswitch²⁵ (Fig. 3A and Figure S8). Together, these genes integrate a B12-regulated network, and represent an independent finding supporting the role of B12 in *M. tuberculosis* pathogenesis.

Another line of evidence endorsing the implications of B12 in the host-pathogen cross-talk is the production of the antimicrobial itaconate by activated macrophages. Itaconyl-CoA is a molecule that structurally resembles methyl-malonyl-CoA, which is the substrate employed by the *M. tuberculosis* methylmalonyl-CoA-mutase MutAB. By molecular mimicry, Itaconyl-CoA strongly inhibits mycobacterial MutAB by undergoing an irreversible covalent attachment to the enzyme's B12 cofactor, and consequently reducing the intrabacterial B12 pool³⁸. Additionally, Itaconyl-CoA is also able to inhibit the own human methylmalonyl-CoA-mutase, resulting in inactivation of the human-derived B12 pool³⁹. Both Itaconyl-CoA-mediated mechanisms, might well serve to reduce the B12 intracellular availability in infected macrophages as a strategy to fight intracellular infections. Conversely, intracellular pathogens as *Y. pestis*, or *M. tuberculosis*, have counter evolved mechanisms to degrade itaconate and promote pathogenesis^{40,41}. Altogether, these evidences support the evolutionary arm-race of humans and pathogens to ensure access to B12.

The emergence of compensatory mutations is another genetic strategy of bacteria to efficiently survive in the presence of an external pressure. Here, this is illustrated by the compensatory polymorphisms arisen in the *metE* riboswitch of the *M. tuberculosis* Δ *metH* strain when B12 is present. Inspection of the literature indicates that the *M. tuberculosis* CDC1551 strain, which is naturally defective in the 3'-terminus of *metH*, and other isogenic *M. tuberculosis* *metH* mutants, also alleviate the B12-mediated repression through mutations in the *metE* riboswitch²⁵. These results are additional examples that reinforce the key role of the host B12 in the biology of *M. tuberculosis* through regulating the synthesis of an essential aminoacid.

In another context, our present-day society is composed by specific populations affected by deficits in B12⁴². In developing countries, deficiency is much more common, starting in early life and persisting

across the life span⁴³. In developed countries, B12 deficits affect predominantly to the elderly due to food-bound B12 malabsorption⁴⁴, to pregnant women⁴⁵, and to vegans and vegetarians improperly supplemented with B12⁴⁶. Our study establishes a direct connection between the optimal B12 serum levels and the virulence of *M. tuberculosis*, which in other words might be translated to lower bacterial virulence in B12 anemic conditions. Since our results come from an animal model, it might be debated that they are challenging to translate into humans. However, we endorse the applicability to humans based on two main arguments. On the one hand, unlike other phenotypes (i.e. immunology, pathogenesis) which are multifactorial, and/or depend on the specific genetic background of the host; the B12 deficiency in our model constitute a single factor (differential B12 serum levels), which closely mimic the human scenario. On the other hand, historic evidence after a *post-mortem* examination of more than 16.000 autopsies suggests that active TB is profoundly reduced in the context of pernicious anemia⁴⁷. Complementarily, more recent observations indicates a slight increase in the risk of developing TB during the first year of treatment for pernicious anemia⁴⁸. This antagonistic association between anemia and TB has been previously documented for other infectious diseases. A classic example is the protection afforded by sickle cell disease against malaria⁴⁹. At this point, we can theorize that adoption of agriculture 10.000 years ago might have alleviated the TB incidence by putatively decreasing B12 serum levels in Neolithic agrarian populations. It might be also possible that high burdens of TB in the past have selected human genetic variants associated with low B12 serum levels. Indeed, current genome wide association studies, has identified polymorphisms in European, Indian and Chinese populations associated with differential B12 serum concentrations⁵⁰⁻⁵², even if their associations with TB have not been yet established.

TB infection does not necessarily imply development of active disease, since *M. tuberculosis*-infected individuals can remain asymptomatic for some years⁵³, a period known as latent TB. This latent state might represent an evolutionary strategy of *M. tuberculosis*, and other pathogens, to ensure active infection only when the host environment is favorable. In this regard, it is plausible to propose that infection of B12 anemic hosts could restrain *M. tuberculosis* virulence until their B12 status improves. This argument somehow recapitulates the ecological theory of microorganisms, which predicts that when virulence is positively correlated with transmission, as is the case in TB, access to a larger number of susceptible hosts favours higher virulence and shorter latency periods⁵⁴. Thus, TB infection of people in developing countries with sub-optimal B12 serum levels, would result in lower virulence, and longer latency times, to guarantee the access to new hosts without decimating the susceptible population.

Our findings could be also translated into therapeutic approaches to treat TB. Even if reducing B12 serum concentrations under suboptimal levels in active TB patients is not an ethical possibility, maintaining B12 in a narrow physiological range could be an alternative strategy to accelerate recovery during the TB treatment. In this regard, it has been reported benefits in either prevention or treatment outcomes of TB as an heterologous side effect from the therapy with the type 2 diabetes drug metformin⁵⁵. This and other studies linking lower risk of active TB with metformin treatment have led to propose this drug as an adjunct anti-TB therapy⁵⁶. Despite the accurate mechanism of metformin to ameliorate TB is unknown, a

common undesirable effect of metformin is lowering B12 levels in treated patients⁵⁷, which according to our study, might establish a yet unknown association between metformin administration and improvement of TB disease.

In conclusion, our study reinterprets the natural evolution of *M. tuberculosis* shaped by its dependency on a host vitamin; and at the same time reconciles previous historical, clinical, therapeutical and experimental evidences^{34,47,48,56}.

Declarations

ACKNOWLEDGEMENTS

The authors would like to acknowledge Carlos Martín and Nacho Aguiló for the critical reading of the manuscript. We also acknowledge the use of the BSL3 facilities from “Servicio General de Apoyo a la Investigación-SAI” of the University of Zaragoza. The authors thank Irene Orera from the Proteomics Facility of the “Centro de Investigación Biomédica de Aragón” for the SRM analysis. Dr. Joaquín Surra Muñoz from “Escuela Politécnica Superior de Huesca” designed and provided a custom B12-deficient diet. Dr. Sofía Samper from the “Hospital Miguel Servet” provided clinical isolates from specific *M. tuberculosis* lineages. Drs. Luis Callén Sevilla and Luis Palomera Bernal, heads of the haematology section, from the “Hospital Clínico Universitario Lozano Blesa” kindly provided advice and facilities for cobalamin measurements.

AUTHOR CONTRIBUTIONS

Conceptualization, E. C.-P. and J.G-A.; Methodology, E. C.-P., S. U. and J.G-A.; Experimental research, E. C.-P., S. U. and J.G-A.; Writing–Original Draft, E. C.-P. and J.G-A.; Writing–Review & Editing, E. C.-P. and J.G-A.; Figure Design, E. C.-P. and J.G-A.; Funding Acquisition, J.G-A.; Supervision, J.G-A. All authors reviewed the final version of the manuscript.

COMPETING INTEREST DECLARATION

The authors have no conflict of interest to declare.

DATA AVAILABILITY

Data required to reproduce the content of this manuscript has been described elsewhere. In case of additional information, it will be provided upon request to the corresponding author.

FUNDING SOURCES

This work was supported by a grant PID2019-104690RB-I00 funded by MCIN/AEI/10.13039/501100011033 to J. G.-A. and by a grant FPU17/02909 funded by the Spanish Ministry of Universities to E. C.-P.

References

1. Minot, G. R. & Murphy, W. P. Treatment of pernicious anemia by a special diet. *Journal of the American Medical Association* **87**, 470-476, doi:10.1001/jama.1926.02680070016005 (1926).
2. Hodgkin, D. G. *et al.* The crystal structure of the hexacarboxylic acid derived from B12 and the molecular structure of the vitamin. *Nature* **176**, 325-328, doi:10.1038/176325a0 (1955).
3. Roth, J. R., Lawrence, J. G. & Bobik, T. A. Cobalamin (coenzyme B12): synthesis and biological significance. *Annu Rev Microbiol* **50**, 137-181, doi:10.1146/annurev.micro.50.1.137 (1996).
4. Allen, L. H. *et al.* Biomarkers of Nutrition for Development (BOND): Vitamin B-12 Review. *J Nutr* **148**, 1995S-2027S, doi:10.1093/jn/nxy201 (2018).
5. Nuccio, S. P. & Baumler, A. J. Comparative analysis of Salmonella genomes identifies a metabolic network for escalating growth in the inflamed gut. *mBio* **5**, e00929-00914, doi:10.1128/mBio.00929-14 (2014).
6. McNally, A., Thomson, N. R., Reuter, S. & Wren, B. W. 'Add, stir and reduce': Yersinia spp. as model bacteria for pathogen evolution. *Nat Rev Microbiol* **14**, 177-190, doi:10.1038/nrmicro.2015.29 (2016).
7. Thiennimitr, P. *et al.* Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. *Proc Natl Acad Sci U S A* **108**, 17480-17485, doi:10.1073/pnas.1107857108 (2011).
8. Mellin, J. R. *et al.* Riboswitches. Sequestration of a two-component response regulator by a riboswitch-regulated noncoding RNA. *Science* **345**, 940-943, doi:10.1126/science.1255083 (2014).
9. Mellin, J. R. *et al.* A riboswitch-regulated antisense RNA in *Listeria monocytogenes*. *Proc Natl Acad Sci U S A* **110**, 13132-13137, doi:10.1073/pnas.1304795110 (2013).
10. WHO. Global Tuberculosis Report 2022. (2022).
11. Riojas, M. A., McGough, K. J., Rider-Riojas, C. J., Rastogi, N. & Hazbon, M. H. Phylogenomic analysis of the species of the Mycobacterium tuberculosis complex demonstrates that Mycobacterium africanum, Mycobacterium bovis, Mycobacterium caprae, Mycobacterium microti and Mycobacterium pinnipedii are later heterotypic synonyms of Mycobacterium tuberculosis. *Int J Syst Evol Microbiol* **68**, 324-332, doi:10.1099/ijsem.0.002507 (2018).
12. Supply, P. *et al.* Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of Mycobacterium tuberculosis. *Nat Genet* **45**, 172-179, doi:10.1038/ng.2517 (2013).
13. Cole, S. T. *et al.* Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* **393**, 537-544, doi:10.1038/31159 (1998).
14. Minias, A., Minias, P., Czubat, B. & Dziadek, J. Purifying Selective Pressure Suggests the Functionality of a Vitamin B12 Biosynthesis Pathway in a Global Population of Mycobacterium tuberculosis. *Genome Biol Evol* **10**, 2326-2337, doi:10.1093/gbe/evy153 (2018).
15. Gopinath, K., Moosa, A., Mizrahi, V. & Warner, D. F. Vitamin B(12) metabolism in Mycobacterium tuberculosis. *Future Microbiol* **8**, 1405-1418, doi:10.2217/fmb.13.113 (2013).

16. Ngabonziza, J. C. S. *et al.* A sister lineage of the Mycobacterium tuberculosis complex discovered in the African Great Lakes region. *Nat Commun* **11**, 2917, doi:10.1038/s41467-020-16626-6 (2020).
17. Minias, A., Gasior, F., Brzostek, A., Jagielski, T. & Dziadek, J. Cobalamin is present in cells of non-tuberculous mycobacteria, but not in Mycobacterium tuberculosis. *Sci Rep* **11**, 12267, doi:10.1038/s41598-021-91430-w (2021).
18. Kawata, T. *et al.* Breeding severely vitamin B12-deficient mice as model animals. *Int J Vitam Nutr Res* **74**, 57-63, doi:10.1024/0300-9831.74.1.57 (2004).
19. Ghosh, S., Sinha, J. K., Putcha, U. K. & Raghunath, M. Severe but Not Moderate Vitamin B12 Deficiency Impairs Lipid Profile, Induces Adiposity, and Leads to Adverse Gestational Outcome in Female C57BL/6 Mice. *Front Nutr* **3**, 1, doi:10.3389/fnut.2016.00001 (2016).
20. Masiewicz, P., Brzostek, A., Wolański, M., Dziadek, J. & Zakrzewska-Czerwińska, J. A novel role of the PrpR as a transcription factor involved in the regulation of methylcitrate pathway in Mycobacterium tuberculosis. *PLoS One* **7**, e43651, doi:10.1371/journal.pone.0043651 (2012).
21. Munoz-Elias, E. J., Upton, A. M., Cherian, J. & McKinney, J. D. Role of the methylcitrate cycle in Mycobacterium tuberculosis metabolism, intracellular growth, and virulence. *Mol Microbiol* **60**, 1109-1122, doi:10.1111/j.1365-2958.2006.05155.x (2006).
22. Griffin, J. E. *et al.* Cholesterol catabolism by Mycobacterium tuberculosis requires transcriptional and metabolic adaptations. *Chem Biol* **19**, 218-227, doi:10.1016/j.chembiol.2011.12.016 (2012).
23. Savvi, S. *et al.* Functional characterization of a vitamin B12-dependent methylmalonyl pathway in Mycobacterium tuberculosis: implications for propionate metabolism during growth on fatty acids. *J Bacteriol* **190**, 3886-3895, doi:10.1128/JB.01767-07 (2008).
24. Eoh, H. & Rhee, K. Y. Methylcitrate cycle defines the bactericidal essentiality of isocitrate lyase for survival of Mycobacterium tuberculosis on fatty acids. *Proc Natl Acad Sci U S A* **111**, 4976-4981, doi:10.1073/pnas.1400390111 (2014).
25. Warner, D. F., Savvi, S., Mizrahi, V. & Dawes, S. S. A riboswitch regulates expression of the coenzyme B12-independent methionine synthase in Mycobacterium tuberculosis: implications for differential methionine synthase function in strains H37Rv and CDC1551. *J Bacteriol* **189**, 3655-3659, doi:10.1128/JB.00040-07 (2007).
26. Vitreschak, A. G., Rodionov, D. A., Mironov, A. A. & Gelfand, M. S. Regulation of the vitamin B12 metabolism and transport in bacteria by a conserved RNA structural element. *RNA* **9**, 1084-1097, doi:10.1261/rna.5710303 (2003).
27. Krohn, P. L., Oxnard, C. E. & Chalmers, J. N. Vitamin B12 in the serum of the rhesus monkey. *Nature* **197**, 186, doi:10.1038/197186a0 (1963).
28. Metz, J., Hart, D. & Harpending, H. C. Iron, folate, and vitamin B12 nutrition in a hunter-gatherer people: a study of the Kung Bushmen. *Am J Clin Nutr* **24**, 229-242, doi:10.1093/ajcn/24.2.229 (1971).
29. Nahvi, A., Barrick, J. E. & Breaker, R. R. Coenzyme B12 riboswitches are widespread genetic control elements in prokaryotes. *Nucleic Acids Res* **32**, 143-150, doi:10.1093/nar/gkh167 (2004).

30. Rutsch, F. *et al.* Identification of a putative lysosomal cobalamin exporter altered in the cblF defect of vitamin B12 metabolism. *Nat Genet* **41**, 234-239, doi:10.1038/ng.294 (2009).
31. Coelho, D. *et al.* Mutations in ABCD4 cause a new inborn error of vitamin B12 metabolism. *Nat Genet* **44**, 1152-1155, doi:10.1038/ng.2386 (2012).
32. Mottram, L. *et al.* Infection Susceptibility in Gastric Intrinsic Factor (Vitamin B12)-Defective Mice Is Subject to Maternal Influences. *mBio* **7**, doi:10.1128/mBio.00830-16 (2016).
33. Ge, Y., Zadeh, M. & Mohamadzadeh, M. Vitamin B12 coordinates ileal epithelial cell and microbiota functions to resist Salmonella infection in mice. *J Exp Med* **219**, doi:10.1084/jem.20220057 (2022).
34. Smith, C. M. *et al.* Host-pathogen genetic interactions underlie tuberculosis susceptibility in genetically diverse mice. *Elife* **11**, doi:10.7554/eLife.74419 (2022).
35. Gopinath, K. *et al.* A vitamin B(1)(2) transporter in Mycobacterium tuberculosis. *Open Biol* **3**, 120175, doi:10.1098/rsob.120175 (2013).
36. Domenech, P., Kobayashi, H., LeVier, K., Walker, G. C. & Barry, C. E., 3rd. BacA, an ABC transporter involved in maintenance of chronic murine infections with Mycobacterium tuberculosis. *J Bacteriol* **191**, 477-485, doi:10.1128/JB.01132-08 (2009).
37. Young, D. B., Comas, I. & de Carvalho, L. P. Phylogenetic analysis of vitamin B12-related metabolism in Mycobacterium tuberculosis. *Front Mol Biosci* **2**, 6, doi:10.3389/fmolb.2015.00006 (2015).
38. Ruetz, M. *et al.* Itaconyl-CoA forms a stable biradical in methylmalonyl-CoA mutase and derails its activity and repair. *Science* **366**, 589-593, doi:10.1126/science.aay0934 (2019).
39. Shen, H. *et al.* The Human Knockout Gene CLYBL Connects Itaconate to Vitamin B12. *Cell* **171**, 771-782 e711, doi:10.1016/j.cell.2017.09.051 (2017).
40. Sasikaran, J., Ziemski, M., Zadora, P. K., Fleig, A. & Berg, I. A. Bacterial itaconate degradation promotes pathogenicity. *Nat Chem Biol* **10**, 371-377, doi:10.1038/nchembio.1482 (2014).
41. Wang, H. *et al.* An essential bifunctional enzyme in Mycobacterium tuberculosis for itaconate dissimilation and leucine catabolism. *Proc Natl Acad Sci U S A* **116**, 15907-15913, doi:10.1073/pnas.1906606116 (2019).
42. Green, R. *et al.* Vitamin B(12) deficiency. *Nat Rev Dis Primers* **3**, 17040, doi:10.1038/nrdp.2017.40 (2017).
43. Allen, L. H. How common is vitamin B-12 deficiency? *Am J Clin Nutr* **89**, 693S-696S, doi:10.3945/ajcn.2008.26947A (2009).
44. Gaskell, H., Derry, S., Andrew Moore, R. & McQuay, H. J. Prevalence of anaemia in older persons: systematic review. *BMC Geriatr* **8**, 1, doi:10.1186/1471-2318-8-1 (2008).
45. Sukumar, N. *et al.* Prevalence of vitamin B-12 insufficiency during pregnancy and its effect on offspring birth weight: a systematic review and meta-analysis. *Am J Clin Nutr* **103**, 1232-1251, doi:10.3945/ajcn.115.123083 (2016).
46. Pawlak, R., Lester, S. E. & Babatunde, T. The prevalence of cobalamin deficiency among vegetarians assessed by serum vitamin B12: a review of literature. *Eur J Clin Nutr* **70**, 866,

doi:10.1038/ejcn.2016.81 (2016).

47. Barron, M. Pernicious anemia and tuberculosis: is there an antagonism?: A review of the occurrence of tuberculosis in ninety-three cases of pernicious anemia as found in 16,600 postmortem examinations, with a report of two clinical cases. *Journal of the American Medical Association* **100**, 1590-1592, doi:10.1001/jama.1933.02740200024007 (1933).
48. Ramagopalan, S. V., Goldacre, R., Skingsley, A., Conlon, C. & Goldacre, M. J. Associations between selected immune-mediated diseases and tuberculosis: record-linkage studies. *BMC Med* **11**, 97, doi:10.1186/1741-7015-11-97 (2013).
49. Allison, A. C. Protection afforded by sickle-cell trait against subtertian malarial infection. *Br Med J* **1**, 290-294, doi:10.1136/bmj.1.4857.290 (1954).
50. Laisk, T. *et al.* Genome-wide association study identifies five risk loci for pernicious anemia. *Nat Commun* **12**, 3761, doi:10.1038/s41467-021-24051-6 (2021).
51. Nongmaithem, S. S. *et al.* GWAS identifies population-specific new regulatory variants in FUT6 associated with plasma B12 concentrations in Indians. *Hum Mol Genet* **26**, 2551-2564, doi:10.1093/hmg/ddx071 (2017).
52. Lin, X. *et al.* Genome-wide association study identifies novel loci associated with serum level of vitamin B12 in Chinese men. *Hum Mol Genet* **21**, 2610-2617, doi:10.1093/hmg/dds062 (2012).
53. Behr, M. A., Edelstein, P. H. & Ramakrishnan, L. Revisiting the timetable of tuberculosis. *BMJ* **362**, k2738, doi:10.1136/bmj.k2738 (2018).
54. Gagneux, S. Host-pathogen coevolution in human tuberculosis. *Philos Trans R Soc Lond B Biol Sci* **367**, 850-859, doi:10.1098/rstb.2011.0316 (2012).
55. Zhang, M. & He, J. Q. Impacts of metformin on tuberculosis incidence and clinical outcomes in patients with diabetes: a systematic review and meta-analysis. *Eur J Clin Pharmacol* **76**, 149-159, doi:10.1007/s00228-019-02786-y (2020).
56. Singhal, A. *et al.* Metformin as adjunct antituberculosis therapy. *Sci Transl Med* **6**, 263ra159, doi:10.1126/scitranslmed.3009885 (2014).
57. Safety update: metformin and vitamin B(12). *Drug Ther Bull* **60**, 131, doi:10.1136/dtb.2022.000039 (2022).

Materials And Methods

Genomic analysis of the B12 biosynthetic pathway

Sequences of each of the 16 *cob* genes present in *M. tuberculosis* H37Rv¹ were aligned using the NCBI Nucleotide BLAST (BLASTn) tool, against the rest of the MTBC genomes available. Mutations were assigned to specific lineages based on inspection of literature and/or description of the sequenced strains. Experimental confirmations of specific mutations were performed in clinical isolates from our laboratory collection previously assigned to MTBC lineages. After DNA extraction from each isolate, the 16 *cob* genes were PCR amplified and sequenced by Sanger methodology to detect polymorphisms.

Bacterial strains and growth conditions

Mycobacteria were cultured at 37°C in Middlebrook 7H9 liquid medium (Difco) containing 0.05% (vol/vol) Tween-80 (Sigma) and supplemented with 10% (vol/vol) of ADC (0,5% bovine serum albumin, 0.2% dextrose, 0.085% NaCl and 0.0003% beef catalase) growth supplement (Middlebrook). For solid media, Middlebrook 7H10 broth containing 10% (vol/vol) ADC was used. For B12 uptake *in vitro* experiments AdoCbl (Sigma-Aldrich) or CNCbl (Sigma-Aldrich) were added to the liquid medium at a concentration of 10 mg/mL. In *in vitro* growth assays, both liquid and solid were supplemented with AdoCbl and L-Methionine (Sigma-Aldrich) when required at concentrations of 10 mg/mL and 25 mg/mL, respectively. When necessary, 50 mg/mL hygromycin (Hyg) or 20 mg/mL kanamycin (Kan) were added to the media to select mutant strains. Bacterial suspensions of *M. tuberculosis* H37Rv, *M. canettii* C59, and their derivatives used for intranasal infections of mice were properly diluted in PBS from previously quantitated glycerol stock solutions. *Escherichia coli* was cultured in liquid media Luria-Bertani (LB) broth or in solid media LB agar at 37°C, or at 30°C for the recombineering event in the case of pKD46 temperature-sensitive plasmid containing strains. When required, media were supplemented with 100 mg/mL ampicillin (Amp), 12.5 mg/mL chloramphenicol (Cm) or 20 mg/mL Kan. A detailed description of each strain used in the study is provided in (Table 1).

Measures of endogenous B12 production and exogenous B12 uptake by bacteria

For B12 production assays, strains from *M. canettii*, MTBC, environmental, and opportunistic mycobacteria were cultured at 37°C until log phase ($OD_{600}=0,6$) in 10 ml 7H9-0.05% Tween-80-ADC. Cells were harvested by centrifugation at 4000 x *g* for 10 min and extensively washed three times with sterile PBS 1x. Pellets were resuspended in PBS containing 1% Triton X-100 and transferred into tubes containing glass beads (MP Biomedicals). Suspensions were disrupted by Fast-Prep (6.5 m/s, 45 s) twice and samples were cooled on ice between the cycles. Supernatants containing soluble fractions were filtered through a 0.22 µm-pore-size filter after cold centrifugation at 14000 x *g* for 5 min. Protein content in the whole-cell extracts were quantified using QuantiPro BCA assay (Sigma Aldrich) for subsequent normalization of B12 measurements. Bacterial whole-cell extracts were used for the quantification of B12 levels in a Cobas analyser from the haematology section of the University Clinical Hospital “Lozano Blesa” (Zaragoza) based on a competitive electrochemiluminescent binding assay. The values of total B12 obtained in pg/mL were normalized against the total protein values in mg/mL. For B12 uptake assays *M. canettii* and MTBC strains were cultured at 37°C in 30 ml 7H9-0.05% Tween-80-ADC supplemented when required with AdoCbl (10 mg/mL) and CNCbl (10 mg/mL). Ten mL of culture were taken at day 2, day 7 and 2 months, and bacteria were processed as reported for B12 production studies.

RNA extraction and quantitative Reverse Transcription-PCR (qRT-PCR)

These protocols were previously described in ². Sequences of primers used are specified in (Table 2). Melting curves were performed for each amplicon to verify specificity. Four replicates of each gene C_T value were obtained and normalized to the C_T of the *sigA* housekeeping gene (amplified from the same

samples), obtaining a $\Delta CT = C_{T,j} - C_{T,sigA}$, where j is a gene different from *sigA*. We calculated a $\Delta\Delta C_T$ value specific for the B21-supplementation condition ($\Delta\Delta C_T(+B12)$), subtracting the ΔCT mean value for each gene obtained from non-B12-supplemented condition from the ΔCT obtained for each gene from + B12 condition. Finally, change of expression, denoted as Relative quantity, was calculated with the equation $2^{-\Delta\Delta C_T(+B12)}$.

RNA-seq analysis

RNA-seq analysis were performed by STAB-vida (Portugal). First, the library construction of cDNA molecules was carried out using a Ribosomal Depletion Library Preparation Kit. The generated DNA fragments (DNA library) were sequenced in the Illumina Novaseq platform, using 150bp paired-end sequencing reads, and the analysis of the generated raw sequence data was carried out using CLC Genomics Workbench 12.0.3. The bioinformatics analysis started with trimming of raw sequences to generate high quality data only. The high-quality sequencing reads were mapped against the reference genomes, *M. tuberculosis* H37Rv (NC_000962.3) or *M. bovis* AF2122/97 (NC_002945.3) when appropriate, using the following parameters: length fraction = 0.8 and similarity fraction = 0.8. The result of mapping served to determine the gene expression levels based on the TPM (Transcripts per Million). To analyse variation and bring out strong patterns in the dataset Principal Component Analysis was performed. Differential expression analysis among samples from each strain and replicate was carried out. Fold changes were calculated from the Generalized Linear Model GLM, which corrects for differences in library size between the samples and the effects of confounding factors. The differentially expressed genes were filtered using the condition "Fold change ≥ 2 or ≤ -2 " and the genes that fulfilled that condition were listed (Tables S1 to S3).

Targeted proteomics by SRM/MS

The SRM/MS approach was used for the quantification of specific PrpC, PrpD and MetE peptides in whole-cell extracts of *M. tuberculosis* H37Rv and GC1237. Cultures were grown until logarithmic growth in 35 ml 7H9-0.05% Tween-80-ADC supplemented with or without AdoCbl. Cells were washed three times with sterile PBS 1x and pelleted. Pellets were resuspended in a volume equivalent to 10% of the initial culture volume with PBS containing 1% Triton X-100 and transferred into tubes containing glass beads (MP Biomedicals). Suspensions were disrupted by Fast-Prep (6.5 m/s, 45 s) twice and samples were cooled on ice between the cycles. Supernatants containing soluble proteins were filtered through a 0.22 μm -pore-size filter after cold centrifugation at 14000 x g for 5 min. 1.5 mL of each bacterial extract was taken and a volume equivalent to 10% of the initial volume of TCA. After overnight cold precipitation of the samples and a long cold centrifugation at 14000 x g, the supernatants were discarded, and the pellets washed with acetone. A second centrifugation was performed for 10 min and the supernatants were discarded again. The pellets were left at room temperature until completely dry. Finally, they were resuspended in 1 mL of 100 mM Tris solution and the total proteins present in the final samples were quantified using QuantiPro BCA assay (Sigma Aldrich). The SRM/MS was performed as previously described³. The identified and quantified peptides were as follows: for PrpC, GLAGVVVDTTAISK,

GELPTDAELALFSQR and VVPQTNSLTyr; for PrpD, IIDNAAVSAASMVR, FTELADGVVEPVEQQR and VPLPAPGEPK; and for MetE, SWLAFGAEK, VPSAEEMADSLR, IEAIVASGAHR and NVDEVTASLHNMVAAAR.

Construction of mutant strains in *M. canettii* and *M. tuberculosis*

Deletion of *cobM* and *cobK* genes in *M. canettii* C59 was achieved using the BAC-recombineering (BAC-rec) strategy⁴. Briefly, the thermosensitive plasmid pKD46 containing the red recombinase from lambda phage⁵ was co-transformed into the *E. coli* DH10B clone carrying BAC Rv412 (containing *cobMK* genes)⁶. DH10B Rv412 pKD46 transformants incubated with arabinose 0.15% were subsequently transformed with a PCR product obtained using “KO BAC *cobMK* Mcan-P1 Fw” and “KO BAC *cobMK* Mcan P1-Rv” primers. This PCR product consists on a Kan resistance cassette (Kan^R), with FRT sites from pKD4, flanked by 40 bp identity arms to the genes of interest. Recombinants were selected on LB agar containing Kan incubated at 30°C overnight, and genes deletion in the BAC was confirmed by PCR amplification using “Conf-KO BAC *cobMK* Mcan-Fw” and “P1 inv” primers for the 5' end and “P2 inv long” and “Conf-KO BAC *cobMK* Mcan-Rv” for the 3' end. Allelic exchange substrates (AES) containing the Kan^R flanked by approximately 1 kb identity arms for site-specific recombination were obtained by high fidelity PCR using BAC Rv412- Δ *cobM,K::Kan*^R as template and “Conf-KO BAC *cobMK*-Fw”/“Conf-KO BAC *cobMK*-Rv” primers. AESs sequences are shown in (Supplementary File 1). AES were transformed by electroporation in *M. canettii* C59 carrying pJV53H recombineering plasmid⁷ and cultured in the presence of 0.2% acetamide. The BAC-rec strategy was also used in *M. tuberculosis* H37Rv for construction of a Δ *metH::Kan*^R mutant. We followed the procedure described above but starting from BAC Rv73 containing the gene Rv2124c (*metH*). The AES (Supplementary File 1) were obtained using the BAC-knockouts as templates for a PCR using primers described in (Table 2). Then, *M. tuberculosis* H37Rv carrying the pJV53H was electroporated with the AES and recombinant colonies were selected and confirmed. The *M. tuberculosis* H37Rv Δ *metE::Kan*^R mutant was constructed using a synthetic AES (GenScript) (Supplementary File 1) introduced in H37Rv-pJV53H by electroporation. To favour the recombination event and recovery of recombinants, the transformation mixtures were incubated overnight in liquid medium without antibiotic and in the presence of AdoCbl, since MetH requires B12 as cofactor. The AESs used for the construction of H37Rv Δ *metE::Kan*^R and Δ *metH::Kan*^R mutants were also electroporated in the *M. canettii* C59 strain containing pJV53H in order to obtain *metE* and *metH* mutants in this strain following the same procedure explained above for *M. tuberculosis*. Finally, to construct the double recombinant *M. tuberculosis* H37Rv Δ *metH::Kan*^R Pr_{Ag85a}-*metE* (Hyg^R), a pMV361H integrative plasmid containing the *metE* gene regulated by the promoter of *fbpA* (Ag85a) (Supplementary File 1) was electroporated in H37Rv Δ *metH::Kan*^R.

For the final steps of mutant construction and confirmation, recombinant colonies were selected by plating on 7H10-ADC with the appropriate antibiotic/supplement and confirmed by PCR using specific primers (Table 2 and Figure S12). All plasmids used in this work are listed in (Table 3).

Construction of mutant strains in *M. smegmatis*

To construct a *metE* knockout in *M. smegmatis* mc²155 and to mimic the B12 phenotype of *M. tuberculosis*, B12 synthesis in mc²155 was first abrogated by deletion of *cobLMK* genes. *M. smegmatis* mc²155 carrying pJV53H⁷ were electroporated with AES containing the Kan^R flanked by approximately 50 bp identity arms for site-specific recombination. AES (Supplementary File 1) were synthesized by PCR amplification of the Kan^R cassette of pKD4 using primers containing 50 bp identity arms (Table 2). Transformants were plated on 7H10-ADC-Kan plates and recombinants were confirmed by PCR using specific primers (Table 2 and Figure S12)

Once constructed the *M. smegmatis* mc²155 Δ *cobLMK* strain, and after confirmation loss of pJV53H, this strain was electroporated with the pRES-FLP-*Mtb* plasmid to resolve the Kan^R resistance cassette. The resolved strain was transformed again with the pJV53H to subsequently generate double mutants. For the construction of the *metE* mutant, the AES -which contained a Kan^R cassette and the GFP gene optimized for its expression in mycobacteria (eGFP) (Supplementary File 1)- was synthesized by GenScript. Construction was confirmed by PCR (Figure S12) of transformant colonies grown on 7H10-ADC-Kan-L-Met plates.

Growth of methionine synthesis mutants in laboratory cultures

The L-Met synthesis mutants were first grown in 7H9-Tween-ADC liquid medium, both with and without B12 (AdoCbl, 10 μ g/mL), to assess their growth in the presence and absence of the vitamin and thus validate their phenotypes. To evaluate growth on solid media, in all cases -with the exception of the Δ *metE* mutant, which only grew in the presence of B12 and, therefore, plating with and without B12 was performed from liquid cultures supplemented with B12- the liquid cultures grown without B12 were plated in solid 7H10-ADC plates supplemented with or without AdoCbl (10 μ g/mL) and/or L-Met (25 μ g/mL).

Riboswitch structure prediction

Prediction of the secondary structure of the *metE* B12-riboswitch was based on previous studies^{8,9}. Using these studies, we identified invariant residues across approximately 200 B12-riboswitches, and *M. tuberculosis*-specific residues. We also identified a conserved B12-box.

Mouse model of B12 deficiency

Female C57BL/6 and SCID mice were fed with a diet similar to control group normal diet (Teklad 2014S, ENVIGO), but with no added B12 and with 5% Pectin (Tekland custom diet TD.170206, ENVIGO). Note that pectin has been shown to bind vitamin B-12 in the intestine making it less bioavailable¹⁰. Moreover, cages were renewed more frequent than usual to avoid coprophagia, as it could be an additional source of B12. After 8 weeks of feeding, concentrations of vitamin B12 were measured in plasma. For this, mice were euthanized, and blood was extracted by cardiac puncture. The blood extractions were incubated at 4°C for 24h and after centrifugation for 5 min at 4000 x g, the upper phase containing the serum was extracted. Serum samples were analyzed by electrochemiluminescence at the University Clinical Hospital

“Lozano Blesa” to quantify the B12-levels. For B12 supplementation experiments, we used the previously mentioned B12-deficient diet (Diet #1 in the text; Tekland custom diet TD.170206, ENVIGO), and a second independent diet reproducing the composition of the standard 2014S diet which was supplemented with a vitamin mix without B12 (Vitamin A, Vitamin D₃, Vitamin E, Vitamin K₃, Vitamin B₁, Vitamin B₂, Niacin, Vitamin B₆, Pantothenic Acid, Biotin, Folate and Choline) to ensure nutritional adequacy after autoclaving (Diet #2 in the text). Subcutaneous B12 supplementation of SCID mice subsequent to *M. tuberculosis* infection, in the corresponding group (Diet #1), consisted on 2.5 µg CNCbl (Optovite B12, Normon laboratories) per week and per mouse, which translates the treatment prescribed for patients with B12 deficits into the animal model.

Mouse infection experiments

All mice were kept under controlled conditions and observed for any sign of disease. Immunocompromised SCID and immunocompetent C57BL/6 female mice were infected with a low dose (\approx 200 CFUs) of the different *M. tuberculosis* wild type and mutant strains and with a high dose (\approx 50000 CFUs) of the *M. canettii* C59 wild type and the $\Delta cobMK$ mutant strain by the intranasal route. The animals were anesthetized by inhalation route with Isoflurane (Isboa Vet) using a vaporizer and intranasal administration was performed with two instillations of 20 µl of the bacterial suspension prepared in PBS. Bacterial suspensions for infection were plated in solid agar medium to confirm the CFUs used for *in vivo* challenges. For survival experiments, SCID mice were monitored daily for the development of clinical signs of disease and examined in case any abnormality of behavior was observed. Weight of mice was followed during the experiment. The euthanasia endpoint was defined at the point that the loss of weight was more than 20%. Four weeks postinoculation, the bacterial burden was evaluated in the lungs and spleen of C57BL/6 mice. For this, the organs were aseptically removed and homogenized in 1 ml of H₂O using a GentleMacs dissociator (Miltenyi Biotec). CFUs were determined by plating serial dilutions onto 7H10-ADC plates supplemented with AdoCbl when required.

Ethics statement

Experimental animal studies were performed in agreement with European and national directives for the protection of animals for experimental purposes. All procedures were carried out under Project License PI 31/21, approved by the Ethics Committee for Animal Experiments from the University of Zaragoza.

Statistics

Mice were randomly distributed in groups of 6 animals per cage prior to start experimental procedures. Results were not blinded for analysis. No statistical method was used to calculate sample size in animal experiments. GraphPrism software was used for statistical analysis. Statistical tests used for each experiment are indicated in the figure legends. All statistical tests were two-tailed and differences were considered significant at $p < 0.05$.

METHODS AND TABLES REFERENCES

- 1 Cole, S. T. *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537-544, doi:10.1038/31159 (1998).
- 2 Perez, I. *et al.* Live attenuated TB vaccines representing the three modern *Mycobacterium tuberculosis* lineages reveal that the Euro-American genetic background confers optimal vaccine potential. *EBioMedicine* **55**, 102761, doi:10.1016/j.ebiom.2020.102761 (2020).
- 3 Perez, I. *et al.* The *Mycobacterium tuberculosis* PhoPR virulence system regulates expression of the universal second messenger c-di-AMP and impacts vaccine safety and efficacy. *Mol Ther Nucleic Acids* **27**, 1235-1248, doi:10.1016/j.omtn.2022.02.011 (2022).
- 4 Aguilo, N. *et al.* Reactogenicity to major tuberculosis antigens absent in BCG is linked to improved protection against *Mycobacterium tuberculosis*. *Nat Commun* **8**, 16085, doi:10.1038/ncomms16085 (2017).
- 5 Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640-6645, doi:10.1073/pnas.120163297 (2000).
- 6 Brosch, R. *et al.* Use of a *Mycobacterium tuberculosis* H37Rv bacterial artificial chromosome library for genome mapping, sequencing, and comparative genomics. *Infect Immun* **66**, 2221-2229, doi:10.1128/iai.66.5.2221-2229.1998 (1998).
- 7 van Kessel, J. C. & Hatfull, G. F. Recombineering in *Mycobacterium tuberculosis*. *Nat Methods* **4**, 147-152, doi:10.1038/nmeth996 (2007).
- 8 Vitreschak, A. G., Rodionov, D. A., Mironov, A. A. & Gelfand, M. S. Regulation of the vitamin B12 metabolism and transport in bacteria by a conserved RNA structural element. *RNA* **9**, 1084-1097, doi:10.1261/rna.5710303 (2003).
- 9 Warner, D. F., Savvi, S., Mizrahi, V. & Dawes, S. S. A riboswitch regulates expression of the coenzyme B12-independent methionine synthase in *Mycobacterium tuberculosis*: implications for differential methionine synthase function in strains H37Rv and CDC1551. *J Bacteriol* **189**, 3655-3659, doi:10.1128/JB.00040-07 (2007).
- 10 Cullen, R. W. & Oace, S. M. Dietary pectin shortens the biologic half-life of vitamin B-12 in rats by increasing fecal and urinary losses. *J Nutr* **119**, 1121-1127, doi:10.1093/jn/119.8.1121 (1989).
- 11 Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T. & Jacobs, W. R., Jr. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* **4**, 1911-1919, doi:10.1111/j.1365-2958.1990.tb02040.x (1990).
- 12 Supply, P. *et al.* Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*. *Nat Genet* **45**, 172-179, doi:10.1038/ng.2517 (2013).

- 13 Garnier, T. *et al.* The complete genome sequence of *Mycobacterium bovis*. *Proc Natl Acad Sci U S A* **100**, 7877-7882, doi:10.1073/pnas.1130426100 (2003).
- 14 Keating, L. A. *et al.* The pyruvate requirement of some members of the *Mycobacterium tuberculosis* complex is due to an inactive pyruvate kinase: implications for in vivo growth. *Mol Microbiol* **56**, 163-174, doi:10.1111/j.1365-2958.2005.04524.x (2005).
- 15 Caminero, J. A. *et al.* Epidemiological evidence of the spread of a *Mycobacterium tuberculosis* strain of the Beijing genotype on Gran Canaria Island. *Am J Respir Crit Care Med* **164**, 1165-1170, doi:10.1164/ajrccm.164.7.2101031 (2001).
- 16 Jackson, M. *et al.* Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Mol Microbiol* **31**, 1573-1587, doi:10.1046/j.1365-2958.1999.01310.x (1999).
- 17 Gonzalo-Asensio, J. *et al.* PhoP: a missing piece in the intricate puzzle of *Mycobacterium tuberculosis* virulence. *PLoS One* **3**, e3496, doi:10.1371/journal.pone.0003496 (2008).
- 18 Broset, E. *et al.* MTBVAC-Based TB-HIV Vaccine Is Safe, Elicits HIV-T Cell Responses, and Protects against *Mycobacterium tuberculosis* in Mice. *Mol Ther Methods Clin Dev* **13**, 253-264, doi:10.1016/j.omtm.2019.01.014 (2019).
- 19 Broset, E. *et al.* Engineering a new vaccine platform for heterologous antigen delivery in live-attenuated *Mycobacterium tuberculosis*. *Comput Struct Biotechnol J* **19**, 4273-4283, doi:10.1016/j.csbj.2021.07.035 (2021).
- 20 Song, H. & Niederweis, M. Functional expression of the Flp recombinase in *Mycobacterium bovis* BCG. *Gene* **399**, 112-119, doi:10.1016/j.gene.2007.05.005 (2007).

Tables

Table 1
Bacterial strains used and constructed in this study

Mycobacterial strains used			
Species & strain designation	Description	Use in this study	Reference
M. smegmatis mc²155	Reference laboratory strain of <i>M. smegmatis</i>	<i>In vitro</i> B12 production experiments	11
M. mucogenicum	Strain provided by Hospital Miguel Servet (Zaragoza)	<i>In vitro</i> B12 production experiments	This study
M. fortuitum	Strain provided by Hospital Miguel Servet (Zaragoza)	<i>In vitro</i> B12 production experiments	This study
M. xenopii	Strain provided by Hospital Miguel Servet (Zaragoza)	<i>In vitro</i> B12 production experiments	This study
M. abscesus	Strain provided by Hospital Miguel Servet (Zaragoza)	<i>In vitro</i> B12 production experiments	This study
M. avium	Strain provided by Hospital Miguel Servet (Zaragoza)	<i>In vitro</i> B12 production experiments	This study
M. gordonae	Strain provided by Hospital Miguel Servet (Zaragoza)	<i>In vitro</i> B12 production experiments	This study
M. canettii C59 (STB-A) (CIPT 140010059)	Clinical isolate of <i>M. canettii</i>	<i>In vitro</i> B12 production and uptake experiments. <i>In vivo</i> experiments in mice	12
M. africanum HCU2744	Clinical isolate of <i>M. africanum</i> lineage 5 provided by Hospital Clínico Universitario Lozano Blesa (Zaragoza)	<i>In vitro</i> B12 production experiments	This study
M. africanum HCU2828	Clinical isolate of <i>M. africanum</i> lineage 6 provided by Hospital Clínico Universitario Lozano Blesa (Zaragoza)	<i>In vitro</i> B12 production and uptake experiments	This study
M. bovis AF2122/97	Reference laboratory strain of <i>M. bovis</i> clade A4	<i>In vitro</i> B12 production and uptake experiments	13

Mycobacterial strains used			
M. bovis AN5	Reference laboratory strain of <i>M. bovis</i> clade A4	<i>In vitro</i> B12 production experiments	14
M. tuberculosis GC1237	Clinical isolate of <i>M. tuberculosis</i> lineage 2	<i>In vitro</i> B12 production experiments	15
M. tuberculosis Mt103	Clinical isolate of <i>M. tuberculosis</i> lineage 4	<i>In vitro</i> B12 production experiments	16
M. tuberculosis H37Rv	Reference laboratory strain of <i>M. tuberculosis</i> lineage 4	<i>In vitro</i> B12 production and uptake experiments. <i>In vitro</i> growth characterization experiments. <i>In vivo</i> experiments in mice	1
<i>E. coli</i> strains used			
E. coli DH10B	<i>E. coli</i> strain carrying a <i>M. tuberculosis</i> H37Rv BAC library	BAC-rec for KO construction	6
E. coli DH10B BAC Rv412 pKD46	Clone carrying recombineering plasmid pKD46 and BAC Rv412 containing <i>cobMK</i> genes	BAC-rec for KO construction in <i>M. canettiii</i>	This study
E. coli DH10B BAC Rv73 pKD46	Clone carrying recombineering plasmid pKD46 and BAC Rv73 containing Rv2124c (<i>methH</i>) gene	BAC-rec for KO construction in <i>M. tuberculosis</i> & <i>M. canettii</i>	This study
Mycobacterial mutant strains constructed in this study			
Species & strain designation	Description	Use in this study	Construction method
M. canettii C59 ΔcobMK::Kan^R	C59 mutant of <i>cobMK</i> genes with a FRT-Kan ^R -FRT cassette	<i>In vivo</i> experiments in mice	BAC-recombineering
M. tuberculosis H37Rv ΔmetE::Kan^R	H37Rv mutant of <i>metE</i> (Rv1133c) gene with a FRT-Kan ^R -FRT cassette	<i>In vitro</i> growth characterization experiments. <i>In vivo</i> experiments in mice	Recombineering of synthetic AES

Mycobacterial strains used			
M. tuberculosis H37Rv ΔmetH::Kan^R	H37Rv mutant of <i>metH</i> gene (Rv2124c) with a FRT-Kan ^R -FRT cassette	<i>In vitro</i> growth characterization experiments. <i>In vivo</i> experiments in mice	BAC-recombineering
M. tuberculosis H37Rv ΔmetH::Kan^RPr_{Ag85a}-metE (Hyg ^R)	Complemented copy of the <i>metE</i> gene controlled by <i>fbpA</i> (Ag85a) promoter in H37Rv ΔmetH::Kan^R	<i>In vitro</i> growth characterization experiments. <i>In vivo</i> experiments in mice	Integration of pMV361H-Pr _{Ag85a} - <i>metE</i> plasmid
M. canettii C59 ΔmetE::Kan^R	C59 mutant of <i>metE</i> gene with a FRT-Kan ^R -FRT cassette	<i>In vitro</i> growth characterization experiments.	Recombineering of synthetic AES
M. canettii C59 ΔmetH::Kan^R	C59 mutant of <i>metH</i> gene with a FRT-Kan ^R -FRT cassette	<i>In vitro</i> growth characterization experiments	BAC-recombineering
M. smegmatis mc²155 ΔcobLMKΔmetE::eGFP_Kan^R	mc ² 155 ΔcobLMK mutant of <i>metE</i> gene with a FRT-Kan ^R -FRT cassette and eGFP gene	<i>In vitro</i> growth characterization experiments	Recombineering of PCR-constructed AES

Table 2
Primers used in this study

qRT-PCR analysis			
Primer	Sequence 5' -> 3'	Use in this study	Reference
qRT-Rv1129c-FW	ATGTCAACCAGTTGGAGAATGA	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-Rv1129c-RV	CGGAATCCGAGGAGAAATACTG	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-prpD-FW	CTGCGTATGGTGCGGATTAT	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-prpD-RV	GCGATCTTG TAGGCCATGT	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-PPE2-FW	ATCCCGCAACTATCGCTTT	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-PPE2-RV	GCGATGGTATCGGTGATGAT	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-cobQ1-FW	GCAGAACATGTCCAACAACCTC	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-cobQ1-RV	AGCAAGACCGGGTTCATC	qRT-PCR verification of "Core-B12 regulon" expression	This study
qRT-metE-FW	GTTGACACCAACTACCACTAC	qRT-PCR verification of "Core-B12 regulon" expression and qRT-PCR of <i>metE</i> expression in L-Met synthesis mutants	This study
qRT-metE-RV	CTTGCCCTAACGCCTCTTT	qRT-PCR verification of "Core-B12 regulon" expression and qRT-PCR of <i>metE</i> expression in L-Met synthesis mutants	This study
RT-sigA-FW	CCGATGACGACGAGGAGATC	Normalization of gene expression in qRT-PCR analysis	17
RT-sigA-RV	CGGAGGCCTTGTCCTTTTC	Normalization of gene expression in qRT-PCR analysis	17
Knockouts construction and PCRs of verification			
Primer	Sequence 5' -> 3'	Use in this study	Reference
KO BAC cobMK Mcan P1-Fw	ACGCTCACCCGGGTGGCGACGCTG TCACCCCATACCGCCGGTGAGGA GTGTAGGCTGGAGCTGCTTC	AES synthesis PCR for KO of <i>cobMK</i> in <i>E. coli</i> DH10B BAC Rv412 pKD46	This study
KO BAC	GGCGAGCCAGTACCAGGTACGGGA	AES synthesis PCR for KO of	This

cobMK Mcan P1- Rv	GCCCGAGCTCACCGCACACCTGCGC GCATATGAATATCCTCCTTAGT	<i>cobMK</i> in <i>E. coli</i> DH10B BAC Rv412 pKD46	study
P1-inv	GAAGCAGCTCCAGCCTACAC	PCR verification of deleted genes, inside Kan ^R cassette	2
P2-inv long	CTTCGGAATAGGAACTAAGGAGG ATATTCATATG	PCR verification of deleted genes, inside Kan ^R cassette	2
Conf-KO BAC cobMK Mcan-Fw	GCACGACGGGCAGATCACAAA	PCR verification of <i>cobMK</i> deletion in <i>E. coli</i> and AES amplification PCR for KO of <i>cobMK</i> in <i>M. canettii</i>	This study
Conf-KO BAC cobMK Mcan-Rv	TTACCGCACTCGCCTTGTCG	PCR verification of <i>cobMK</i> deletion in <i>E. coli</i> and AES amplification PCR for KO of <i>cobMK</i> in <i>M. canettii</i>	This study
Confirm-KO cobMK C59-Fw	G TTCAGCGTGCTCGAACAGCTT	PCR verification flanking <i>cobMK</i> deletion in <i>M. canettii</i>	This study
Confirm-KO cobMK C59-Rv	TCTACGACGACCGGTCGTAGAC	PCR verification flanking <i>cobMK</i> deletion in <i>M. canettii</i>	This study
KO BAC Rv2124c FRT Kan- FW	GAGCGCTGTCAACGACTGAGGAA ATTTTCATAGGCCGACTATCCTTGC CATGTGTAGGCTGGAGCTGCTTC	AES synthesis PCR for KO of Rv2124c (<i>metH</i>) in <i>E. coli</i> DH10B BAC Rv73 pKD46	This study
KO BAC Rv2124c FRT Kan-RV	GCCGACGTCCTGTGCGCAGCCGATG CTCCGCACACGTGGGACGGGTCAG A CATATGAATATCCTCCTTAGT	AES synthesis PCR for KO of Rv2124c (<i>metH</i>) in <i>E. coli</i> DH10B BAC Rv73 pKD46	This study
Conf-KO BAC meth- FW	TTCGGTGGGTGCGACACATAGT	PCR verification of <i>metH</i> deletion in <i>E. coli</i> and AES amplification PCR for KO of <i>metH</i> in <i>M.</i> <i>tuberculosis</i>	This study
Conf-KO BAC meth- RV	TCATCGCCCAGGTGTTGGACTG	PCR verification of <i>metH</i> deletion in <i>E. coli</i> and AES amplification PCR for KO of <i>metH</i> in <i>M.</i> <i>tuberculosis</i>	This study
Conf-KO Mtb meth- FW	TTCGGCGATCGTCTCGGTGATC	PCR verification flanking <i>metH</i> deletion in <i>M. tuberculosis</i>	This study
Conf-KO Mtb meth- RV	TGCGCTATCTGGCTGTTGAGCT	PCR verification flanking <i>metH</i> deletion in <i>M. tuberculosis</i> and in <i>M. canettii</i>	This study
PCR-Frag KO metE Mtb-FW	GCGAACTCCGGCTTCCAGAACA	AES amplification PCR for KO of <i>metE</i> in <i>M. tuberculosis</i>	This study

PCR-Frag KO metE Mtb-RV	CGCCTGGCAGATCGTGCTG	AES amplification PCR for KO of <i>metE</i> in <i>M. tuberculosis</i>	This study
Conf-KO Mtb metE- FW	ACTCCGGAGACCTGAACACCG	PCR verification flanking <i>metE</i> deletion in <i>M. tuberculosis</i> and in <i>M. canettii</i>	This study
Conf-KO Mtb metE- RV	TTGCCCTCTACGCACTGCTG	PCR verification flanking <i>metE</i> deletion in <i>M. tuberculosis</i> and in <i>M. canettii</i>	This study
Km pKD4 out1	CCACGATAGCCGCGCTGCCTCG	PCR verification of deleted genes, inside Kan ^R cassette	18
Km pKD4 out2	GGGCTGACCGCTTCCTCGTGCT	PCR verification of deleted genes, inside Kan ^R cassette	18
pMV361C	GATCCGGAGGAATCACTTC	PCR verification of integration of pMV361H-Pr _{Ag85a} - <i>metE</i> plasmid in <i>M. tuberculosis</i> Δ <i>metH</i> : Kan ^R	19
pMV361B	CCTCGAGCAAGACGTTTCC	PCR verification of integration of pMV361H-Pr _{Ag85a} - <i>metE</i> plasmid in <i>M. tuberculosis</i> Δ <i>metH</i> : Kan ^R	19
Conf-KO Mcan metH-Fw	ACCTCGACGTTCGAGCACG	PCR verification flanking <i>metH</i> deletion in <i>M. canettii</i>	This study
KO cobLMK Msmeg FRT Kan-FW	CTGGCGCAGGATGCCGCGATGAGC GCTCATGCGAAGAGCCGAGGACAC CGGTGTAGGCTGGAGCTGCTTC	AES synthesis PCR for KO of <i>cobLMK</i> in <i>M. smegmatis</i>	This study
KO cobLMK Msmeg FRT Kan-RV	GAGCGCCCCGTGGTCAGGAACACG CGCGAAAACCCGCGCTGCGCCACC ACCATATGAATATCCTCCTTAGT	AES synthesis PCR for KO of <i>cobLMK</i> in <i>M. smegmatis</i>	This study
Conf-KO cobLMK Msmeg- FW2	TCCAGTTTGGGCTGTGTCATGTC	PCR verification flanking <i>cobLMK</i> deletion in <i>M.</i> <i>smegmatis</i>	This study
Conf-KO cobLMK Msmeg- RV2	TTATGTCTACGGTTCGGCCAAGG	PCR verification flanking <i>cobLMK</i> deletion in <i>M.</i> <i>smegmatis</i>	This study
PCR-Frag GFP + Kan KO metE Msmeg-FW	GAAGACCTGCCAGCCGTACC	AES synthesis PCR for KO of <i>metE</i> in <i>M. smegmatis</i>	This study

PCR-Frag GFP + Kan KO metE Msmeg-RV	CTCCAGACCATCGCGATGACG	AES synthesis PCR for KO of <i>metE</i> in <i>M. smegmatis</i>	This study
Conf-KO 5'metE Msmeg-FW	AGTAGGTGTGCGTCAGCGCA	PCR verification flanking <i>metE</i> deletion in <i>M. smegmatis</i>	This study
Conf-KO 3'metE Msmeg-RV	TCGGGCTTGTCAACTTGTCAGG	PCR verification flanking <i>metE</i> deletion in <i>M. smegmatis</i>	This study
eGFP-RV	CGTCGCCGTCCAGCTCGACCAG	PCR verification flanking <i>metE</i> deletion in <i>M. smegmatis</i> , inside eGFP gene	19

Table 3. Plasmids used in this study

Plasmid	Description	Reference
pKD46	Thermosensitive and replicative plasmid for <i>E. coli</i> containing the λ -red recombinase system from lambda phage inducible by arabinose, and an Amp-resistance marker	5
pKD4	Replicative plasmid for <i>E. coli</i> containing the FRT-Kan-FRT resistance marker	5
pJV53H	Replicative plasmid for <i>E. coli</i> and <i>Mycobacterium</i> containing Che9c-gp60-61 recombinase system inducible by acetamide and a Hyg-resistance marker	7
pRES-FLP-Mtb	Replicative plasmid for <i>E. coli</i> and <i>Mycobacterium</i> containing the <i>flp</i> recombinase from <i>Saccharomyces cerevisiae</i> with codon usage adapted to <i>Mycobacterium</i> and a Hyg-resistance marker	20 2
pMV361H-PrAg85a_metE	Integrative mycobacterial vector with attP site and <i>int</i> gene from mycobacteriophage L5, an origin of replication for <i>E. coli</i> , a Kan-resistance marker and a genetic construction cloned in the <i>NdeI</i> site containing the CDS of H37Rv <i>metE</i> gene controlled by the H37Rv <i>fbpA</i> promoter	This study

Figures

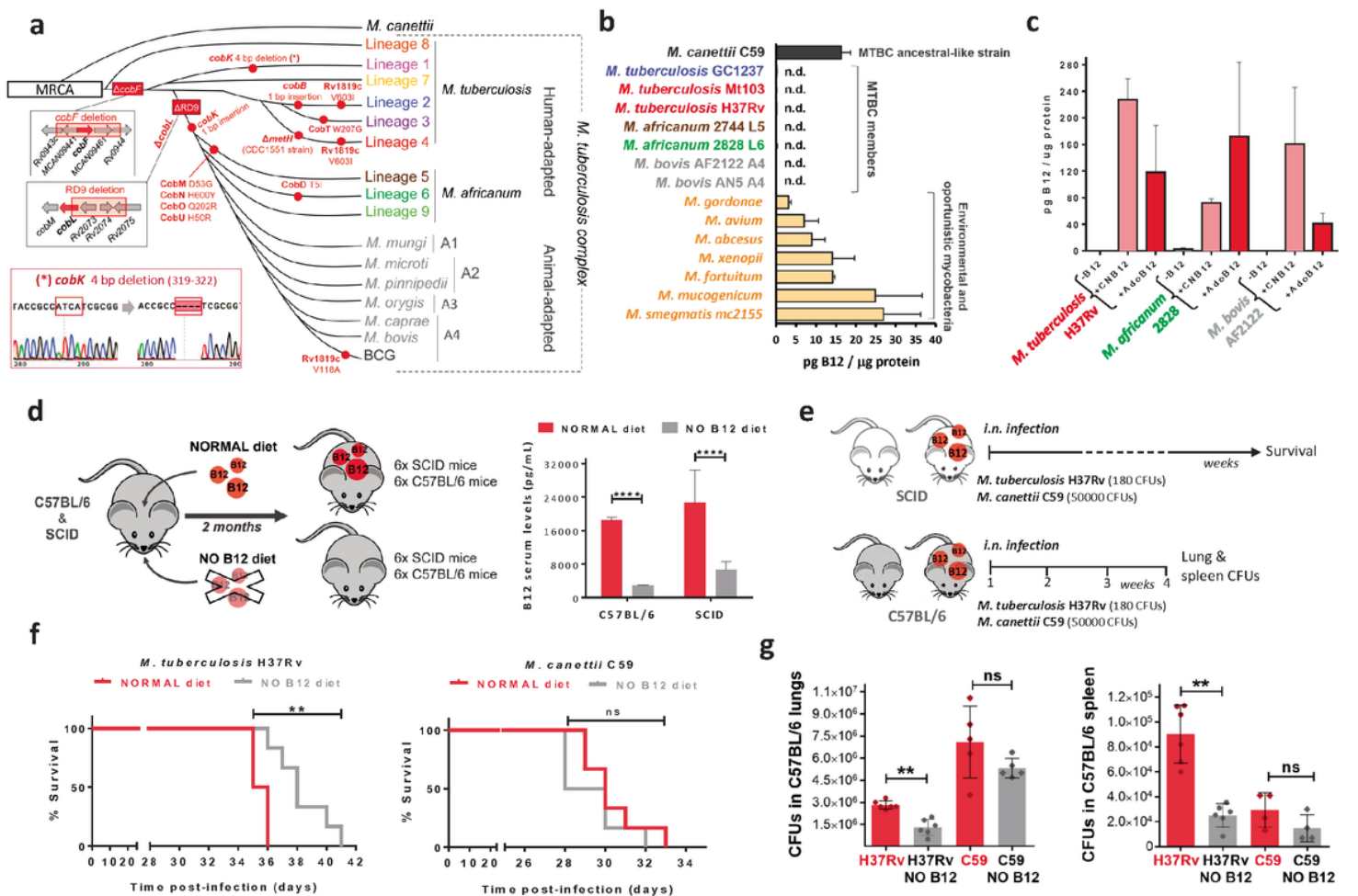


Figure 1

M. tuberculosis, but not its ancestor *M. canettii*, exhibits reduced virulence in B12 deficient mice models due to the inability of MTBC bacteria to synthesize endogenous B12. (A) Identification of mutations (red dots) and large genomic deletions (red boxes) affecting the *de novo* B12 synthesis pathway in MTBC strains and in its last known common ancestor (*M. canettii*). The bottom left box shows the validation by Sanger sequencing of one of these mutations. Additional confirmations by Sanger sequencing are provided in Figure S1. (B) B12-levels synthesized *in vitro* by MTBC species (represented with the same colour code as in (A)), in *M. canettii* (dark grey) and in environmental and opportunistic mycobacteria (yellow). "n.d." denotes non detected B12-levels. Bars and error bars are the average and SD from at least three biological replicates. (C) Cyanocobalamin (CNB12) and adenosylcobalamin (AdoB12) scavenged by MTBC members in *in vitro* cultures containing B12 grown until exponential growth-phase. Data represented are the mean and standard deviation (SD) from at least three biological replicates. (D) Development of mouse B12 anemic models and confirmation of decreased serum B12-levels in the treated mice. Graph data are mean \pm SD of at least three biological replicates. Statistical analysis was performed using two-way ANOVA followed by Sidak's post-test. Asterisks indicate **** 0.0001 > p. (E) Experimental set-up of the survival and virulence experiments in SCID and C57BL/6 anemic mice infected

with the wild type strains of *M. tuberculosis* H37Rv and *M. canettii* C59. **(F)** Survival rates from groups of 6 SCID mice for each type of diet inoculated by the intranasal route with *M. tuberculosis* H37Rv or *M. canettii* C59. P values are indicated as follows: ** 0.01 > p > 0.001; ns: not significant, p ≥ 0.05 [Log-rank (Mantel-Cox) test]. **(G)** Bacterial loads in the lungs and spleens of control and anemic C57BL/6 mice after 4 weeks infected with *M. tuberculosis* H37Rv or *M. canettii* C59. Data represent mean ± SD of each mice group, and statistical analysis was performed using Mann-Whitney test for each strain. P values are indicated as follows: ** 0.01 > p > 0.001; ns: not significant, p ≥ 0.05.

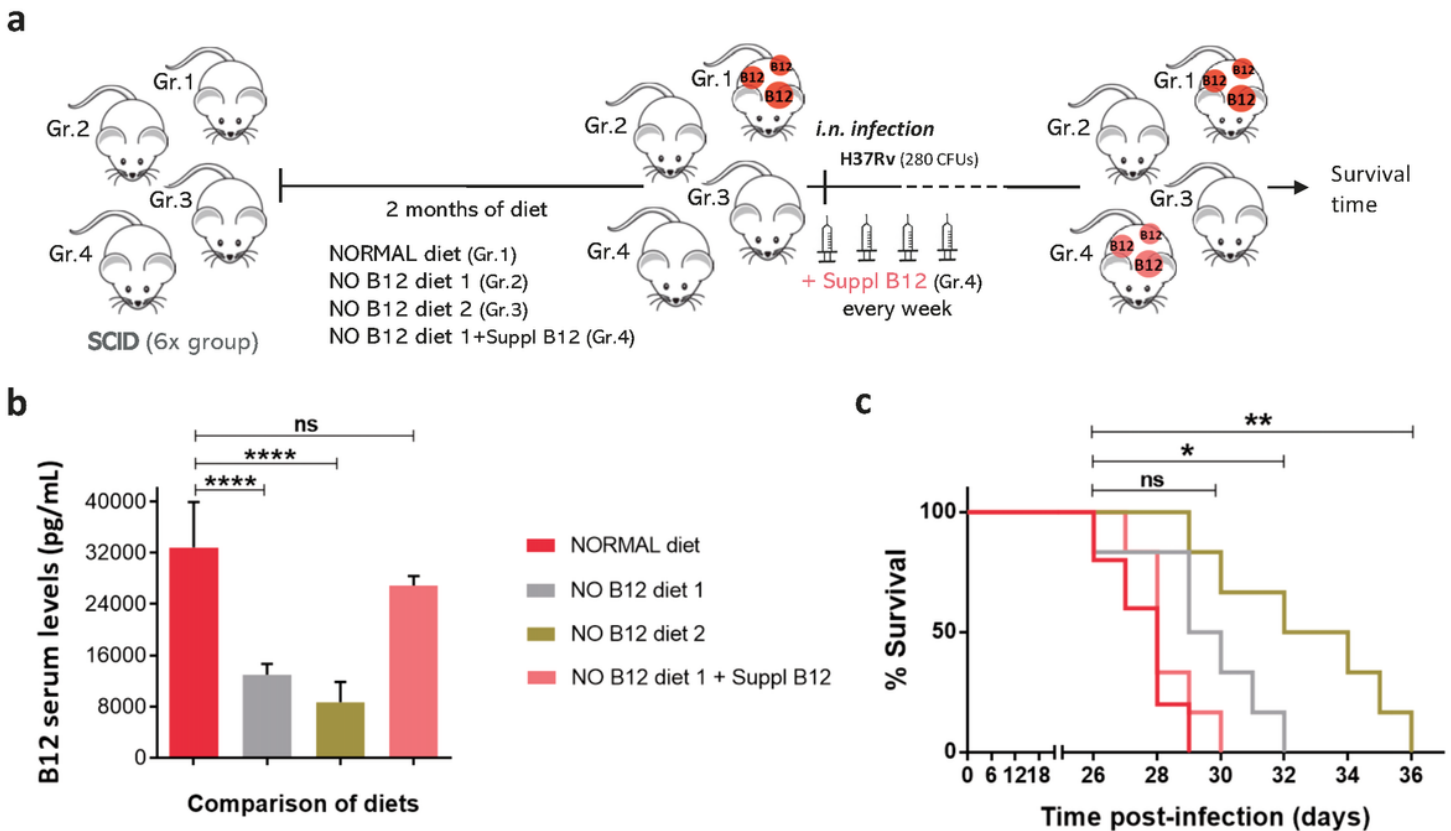


Figure 2

Subcutaneous B12 supplementation efficiently restores B12 serum levels in anemic mice and results in enhanced *M. tuberculosis* virulence. **(A)** Experimental design and timelines of the different B12 treatments, B12 subcutaneous supplementation, and *M. tuberculosis* infection, in each SCID mice group. **(B)** B12 serum levels in animal groups described in panel A after sacrifice at the humane endpoint. B12 levels remained significantly decreased in animals treated with B12 restricted diets relative to the control group throughout the course of the experiment. Note that weekly B12 supplementation restored standard B12 serum levels in mice. Graph data are mean ± SD of six biological replicates. Statistical analysis was performed using One-Way ANOVA followed by Tukey post-test. Asterisks indicate the following p values:

**** 0.0001 > p; ns: not significant, $p \geq 0.05$. **(C)** Survival rates from groups of 6 SCID mice for each type of treatment after infection by the intranasal route with *M. tuberculosis*. P values are indicated as follows: * 0.05 > p > 0.01; ** 0.01 > p > 0.001; ns: not significant, $p \geq 0.05$ [Log-rank (Mantel-Cox) test].

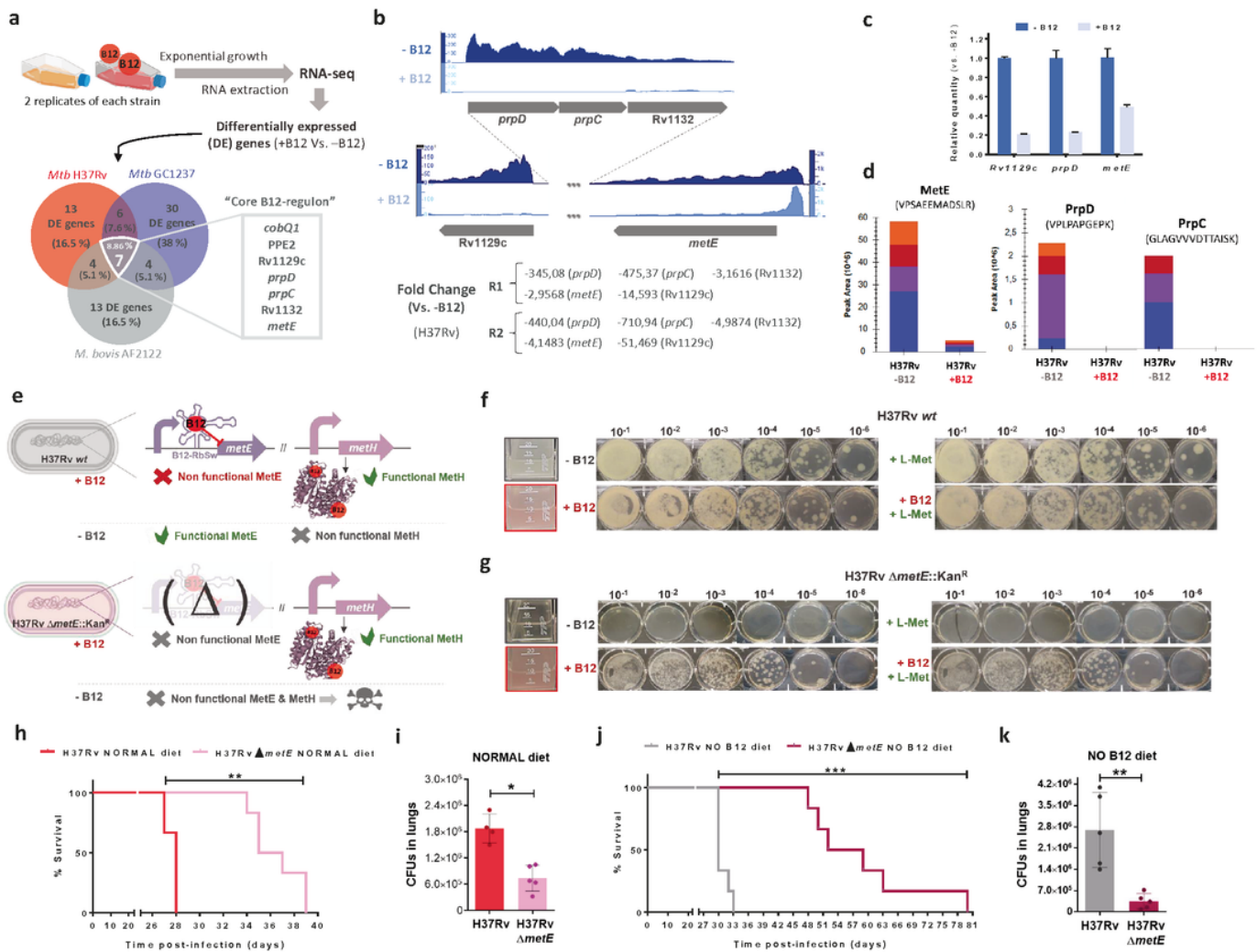


Figure 3

The core B12-dependent transcriptome identifies a gene cluster regulated by B12 in the MTBC and establishes a link with methionine metabolism. **(A)** Experimental procedure for RNA-seq analysis of differentially expressed (DE) genes in response to B12 supplementation of MTBC cultures. Venn diagrams show the resulting DE genes from two independent replicates of *M. tuberculosis* H37Rv (L4), *M. tuberculosis* GC1237 (L2), and the animal-adapted *M. bovis* AF2122. Those DE genes common to the three strains are indicated as the "core B12-regulon", and consist on 7 downregulated genes in the presence of B12. **(B)** RNA-seq profiles showing downregulated gene expression of the gene cluster Rv1129c (*prpR*)-*prpD*-*prpC*-Rv1132-*metE* in *M. tuberculosis* H37Rv in response to exogenous B12 supplementation (light blue) relative to the experimental control without B12 (dark blue). Note that in order to visualize expression differences, each genetic section has been represented with its corresponding scale. Expression profiles of the remaining genes from the core B12 regulon are indicated in Figure S8. **(C)** qRT-PCR measures of Rv1129c (*prpR*), *prpD* and *metE* in *M. tuberculosis* H37Rv cultures

grown with or without B12. Relative quantity refers to the differential expression of the selected genes in the presence of B12 in comparison with its expression in the absence of B12. Each gene was normalized against *sigA* expression in each sample. Graphs represents mean \pm SD from three biological replicates. Expression of additional genes of the regulon is shown in Figure S8. **(D)** Quantification of MetE, PrpD and PrpC protein levels of *M. tuberculosis* H37Rv both in presence and absence of B12 by MRM-MS. Bars represent the area under the curve for every transition from a specific peptide of each protein in both experimental conditions. Equivalent results were obtained for the rest of the analyzed peptides (Figure S9) in two biological replicates of H37Rv and GC1237 strains of *M. tuberculosis*. **(E)** Expected phenotypes of the *M. tuberculosis* H37Rv *DmetE* knockout compared to its wild type strain in presence and absence of B12. **(F) and (G)** *in vitro* growth in liquid (boxed images) and in solid media of the *M. tuberculosis* H37Rv wild type strain (F) and its *DmetE* mutant (G) in the absence or presence of exogenous B12 and/or L-methionine. **(H) and (J)** Survival curves from groups of 6 SCID mice inoculated by the intranasal route with *M. tuberculosis* H37Rv and H37Rv *DmetE* fed with normal diet (H) and a B12-deficient diet (J). P values are indicated as follows: ** 0.01 > p > 0.001; *** 0.001 > p > 0.0001 [Log-rank (Mantel-Cox) test]. **(I) and (K)** Bacterial loads in the lungs of normal (I) and anemic (K) C57BL/6 mice after 4 weeks infected with *M. tuberculosis* H37Rv and its *DmetE* mutant. Data are mean \pm SD of at least four replicates. Statistical analysis was performed using Mann-Whitney test for each strain. P values are indicated as follows: * 0.05 > p > 0.01; ** 0.01 > p > 0.001. Results show that inactivation of the methionine synthase MetE attenuates the *M. tuberculosis* virulence more markedly in B12 anemic mice relative to controls.

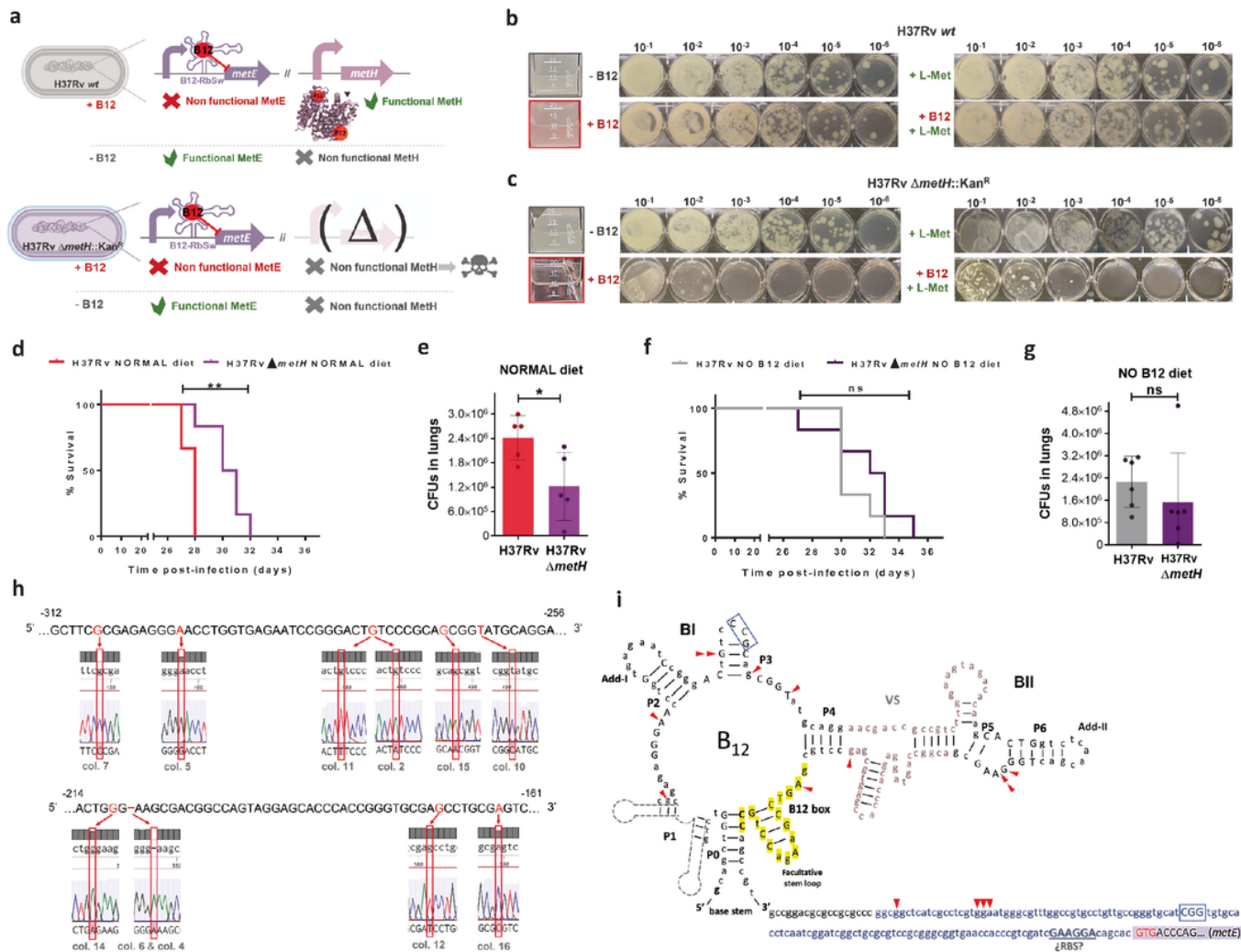


Figure 4

Inactivation of the methionine synthase MethH causes B12 toxicity in *M. tuberculosis* resulting in alleviating compensatory mutations in the *metEB12*-Riboswitch. (A) Expected phenotypes of the *M. tuberculosis* H37Rv *DmethH* knockout compared to the wild type strain in presence and absence of B12. **(B) and (C)** *in vitro* growth in liquid (boxed images) and in solid media of the *M. tuberculosis* H37Rv wild type strain (B) and its *DmethH* mutant (C) in absence or presence of exogenous B12 and/or L-methionine. **(D) and (F)** Survival curves from groups of 6 SCID mice fed with normal diet (D) and B12-deficient diet (F), and inoculated by the intranasal route with *M. tuberculosis* H37Rv and its *DmethH* mutant. P values are indicated as follows: ** 0.01 > p > 0.001; ns: not significant, p ≥ 0.05 [Log-rank (Mantel-Cox) test]. **(E) and (G)** Bacterial loads in the lungs of control (E) and B12 anemic (G) C57BL/6 mice after 4 weeks infected with *M. tuberculosis* H37Rv and its *DmethH* mutant. Data are mean ± SD of at least five replicates. Statistical analysis was performed using Mann-Whitney test for each strain. P values are indicated as follows: * 0.05 > p > 0.01; ns: not significant, p ≥ 0.05. Results show that inactivation of the MethH isoform attenuates the mutant exclusively in animals with normal serum B12-levels **(H)** Sequence of the *M. tuberculosis* H37Rv genomic region comprising the *metEB12*-Riboswitch. Negative numbers refer to nucleotides immediately upstream of the *metE* initiation codon. Those bases affected by

mutations identified in *M. tuberculosis* H37Rv *DmetH* colonies grown with B12 are highlighted in red in the Sanger chromatograms located below the riboswitch sequence. Additional mutations identified in the 5'UTR region immediately upstream of the *metE* initiation codon are shown in Figure S11. **(I)** Prediction of the secondary structure of the *metE* B12-riboswitch. Location of the identified mutations in the B12-resistant *M. tuberculosis* H37Rv *DmetH* colonies are indicated by red arrows. Invariant residues across approximately 200 B12-riboswitches are indicated in capital letters. Lowercase letters indicate *M. tuberculosis* specific residues. The conserved B12-box is highlighted in yellow. The *metE* 5'UTR region is shown in blue letters with the possible RBS underlined. The start of the *metE* CDS is shown with a purple arrow and the initiation codon is highlighted in red.

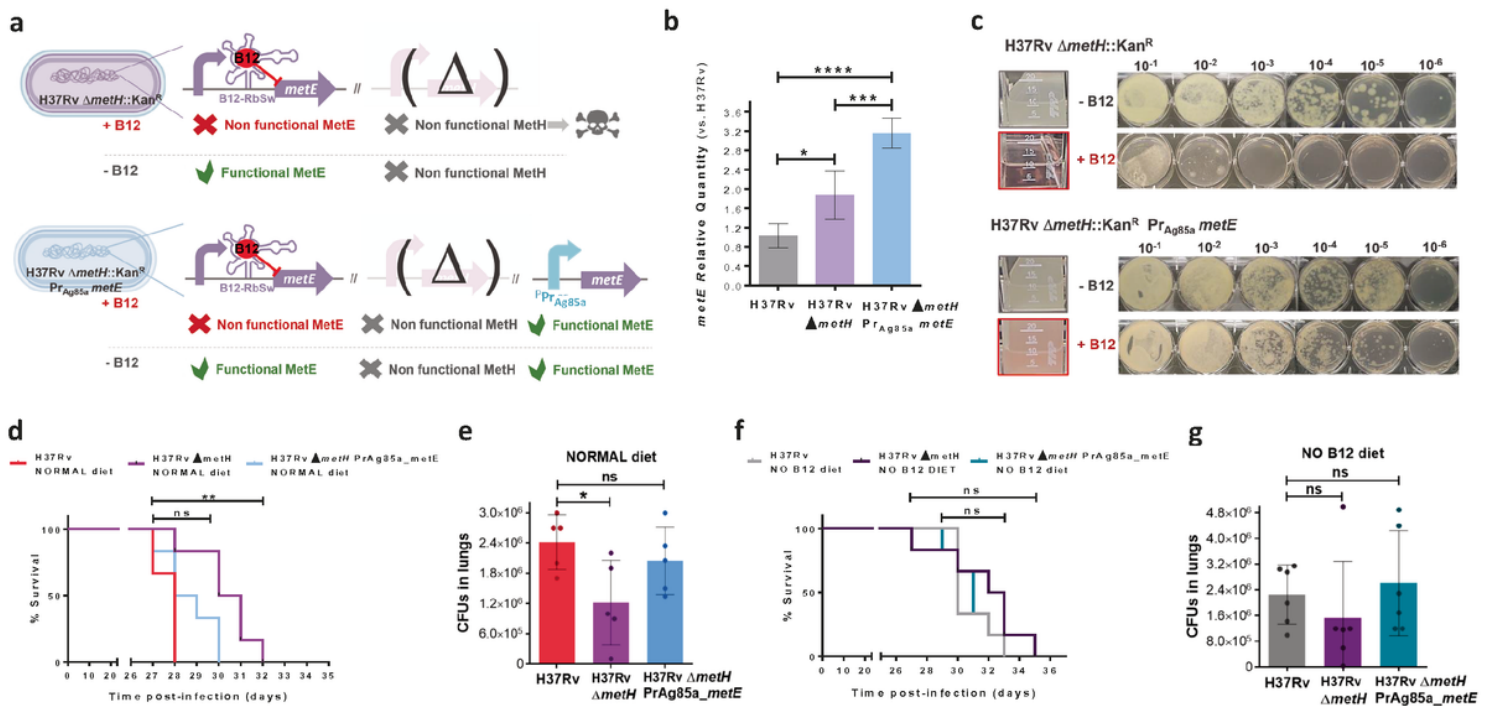


Figure 5

Complementation of the *M. tuberculosis* *DmetH* knockout with a B12-independent *metE* gene restores virulence to wild type levels. (A) Expected phenotypes of the *M. tuberculosis* H37Rv *DmetH* Pr_{Ag85a} *metE*::Kan complemented strain compared to the parental *DmetH* strain in presence and absence of B12. **(B)** Experimental growth in liquid (boxed images) and in solid media of the *M. tuberculosis* H37Rv *DmetH* and the *DmetH* Pr_{Ag85a} *metE*::Kan strains in presence and absence of B12. **(C)** Validation by qRT-

PCR of the *metE* complementation and its stable expression in the complemented mutant *M. tuberculosis* H37Rv *DmetH*Pr_{Ag85a}*metE*::Kan compared to the parental *DmetH* and the wild type strains. Relative quantity refers to the differential *metE* gene expression in the two mutant strains in comparison with its expression in the wild type H37Rv. Each gene was normalized against *sigA* expression in each sample. Graphs represents mean \pm SD from one experiment with three replicates. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons post-test. **(D) and (F)** Survival curves from groups of 6 SCID mice fed with normal diet (D) or B12-deficient diet (F), and inoculated by intranasal route with *M. tuberculosis* H37Rv, its *DmetH* mutant or the *DmetH* Pr_{Ag85a}*metE*::Kan complemented strain. P values are indicated as follows: * 0.05 > p > 0.01; ns: not significant, p \geq 0.05 [Log-rank (Mantel-Cox) test]. **(E) and (G)** Bacterial loads in the lungs of normal (E) and B12 anemic (G) C57BL/6 mice after 4 weeks infected with *M. tuberculosis* H37Rv, the *DmetH* knockout, or the *DmetH* Pr_{Ag85a}*metE*::Kan Data are mean \pm SD of at least five replicates. Statistical analysis was performed using Mann-Whitney test for each strain. P values indicated as ns means not significant. Results show that complementation with a B12-independent *metE* restores virulence in the *M. tuberculosis* *DmetH* mutant independently of the B12 status of the host.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalTable1Mbovis.xlsx](#)
- [SupplementalTable2MtbGC1237.xlsx](#)
- [SupplementalTable3MtbH37Rv.xlsx](#)
- [SupplementaryFiguresB12intuberculosis.pdf](#)