Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*

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Global spread and limited genetic variation are hallmarks of *M. tuberculosis*, the agent of human tuberculosis. In contrast, *Mycobacterium canettii* and related tubercle bacilli that also cause human tuberculosis and exhibit unusual smooth colony morphology are restricted to East Africa. Here, we sequenced and analyzed the whole genomes of five representative strains of smooth tubercle bacilli (STB) using Sanger (4–5× coverage), 454/Roche (13–18× coverage) and/or Illumina DNA sequencing (45–105× coverage). We show that STB isolates are highly recombinogenic and evolutionarily early branching, with larger genome sizes, higher rates of genetic variation, fewer molecular scars and distinct CRISPR-Cas systems relative to *M. tuberculosis*. Despite the differences, all tuberculosis-causing mycobacteria share a highly conserved core genome. Mouse infection experiments showed that STB strains are less persistent and virulent than *M. tuberculosis*. We conclude that *M. tuberculosis* emerged from an ancestral STB-like pool of mycobacteria by gain of persistence and virulence mechanisms, and we provide insights into the molecular events involved.

*M. tuberculosis* is a pervasive human pathogen currently estimated to infect 2 billion people throughout the world1. The bacterial population resulting from this massive spread is very large, yet the genetic diversity within the classical members of the *M. tuberculosis* complex (MTBC), comprising *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium pinnipedi* and *M. tuberculosis* is very limited. Tuberculosis is therefore assumed to be a recent human disease2,3 linked to clonal expansion of its causative organism4–6.

In contrast to MTBC strains, STB clinical isolates, which have a distinctive smooth colony phenotype on culture media, named *M. canettii* and/or *Mycobacterium prototuberculosis*7–10, are less genetically restricted. Initial genotyping analysis suggested that these isolates possess higher diversity with traces of intraspecies horizontal gene transfer and might therefore represent early-branching lineages of tuberculosis-causing mycobacteria. Since their first isolation by Georges Canetti in 1969, less than 100 STB strains have been identified. All have been obtained from humans with tuberculosis, mostly from (or with connection to) East Africa8,11. Thus, a collection of a few tens of STB strains from a geographically restricted region seems to contain greater genetic diversity than the worldwide population of MTBC strains. This observation raises intriguing questions about

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the origin of tuberculosis and provided an opportunity to examine the molecular and evolutionary events involved in the emergence of *M. tuberculosis*. Here, we report the whole-genome sequence analysis of five diverse STB isolates and compare the physiopathological properties of these mycobacteria to those of *M. tuberculosis*, as well as the whole-genome shotgun sequences of four additional STB strains for secondary screening and confirmation purposes.

**RESULTS**

**Ancestral features of STB genomes**

We applied multilocus sequence typing (MLST) based on 12 housekeeping genes to a panel of 55 available STB isolates and identified a total of 13 sequence types (Fig. 1 and Supplementary Tables 1 and 2). From analyses of the concatenated sequences, we inferred a highly reticulated phylogeny, suggestive of conflicting phylogenetic signals and possible horizontal gene transfer between the MLST target genes. We then selected five representative isolates for comprehensive genomic analysis. This selection included the original strain isolated by George Canetti of sequence type A (STB-A) and an isolate from the most prevalent group of sequence type D (STB-D)\(^9\), as well as strains from the most distant sequence types STB-L, STB-J and STB-K (Fig. 1 and Supplementary Fig. 1).

Comparison of these five STB genomes with those of *M. tuberculosis* H37Rv\(^12\) and other MTBC members\(^13\) showed very similar overall organization between STB and MTBC strains, with a high percentage of syntenic genes, ranging from 93% for STB-K to 96% for STB-A, compared to only 77% between *Mycobacterium marinum*, one of the phylogenetically closest non-tuberculous mycobacterial species\(^14,15\), and *M. tuberculosis* H37Rv. No major chromosomal rearrangements or plasmids were detected (Fig. 1). Pairwise analyses between the conserved STB and MTBC genome sequences showed that all combinations had average nucleotide identities of at least 97.3%, above the 95% threshold proposed for classification into the same species\(^15\). However, the genomes of the STB strains were 10–115 kb larger than those of the MTBC members and thus represent the largest genomes known for tubercle bacilli, although they are still much smaller than those of *M. marinum* (6.6 Mb)\(^16\) and the other most closely related nontuberculous species *Mycobacterium kansasi* (6.4 Mb)\(^17\). Excluding repetitive sequences such as PE_PGRS- and PPE_MPTR-encoding regions, which account for ~8% of the coding capacity of *M. tuberculosis*\(^12\),
STB and MTBC strains shared >89.3% of their genomes, representing a core genome for tubercle bacilli of >3.398 Mb. This core comprises 96.3% of the 774 \textit{M. tuberculosis} H37Rv genes predicted to be essential for \textit{in vitro} growth and all 194 genes required for mycobacterial survival during mouse infection\(^{18-20}\), further reflecting the close affiliations of STB strains and \textit{M. tuberculosis}. The accessory genomes of individual STB strains harbor from 124 (STB-A) to 366 (STB-J and STB-K) genes not present in MTBC members that enlarge the known pan-genome of tubercle bacilli by 890 predicted coding sequences, representing a supplement of more than 20% relative to the gene pool of \textit{M. tuberculosis} (Supplementary Fig. 2a,b and Supplementary Table 3). Notably, only nine of these predicted coding sequences were common to all five STB genomes analyzed (Supplementary Fig. 2c and Supplementary Table 3). Conversely, 51 genes partially overlapping with genomic islands\(^{21}\) present in MTBC members were not found in any of the STB strains (Supplementary Table 4). These genes encode derivatives of mobile elements, such as the phiRv1 and phiRv2 prophage-like regions (24 coding sequences), 3 transposases, 5 unique members of a glycine-rich protein family (for example, \textit{PE_PGRS33}; Supplementary Fig. 3a) and 19 other hypothetical proteins (Supplementary Fig. 3b). It is noteworthy that Rv1989c-Rv1990c from one such MTBC-specific region showed around 90% identity with proteins encoded on a plasmid from \textit{Mycobacterium gilvum} and \textit{Mycobacterium sp.} KMS, raising intriguing questions about possible transmission routes by which the corresponding genes were introduced into the MTBC genomes. Several other MTBC-specific hypothetical proteins had no or only weak amino-acid similarity with other mycobacterial proteins (Supplementary Table 4), suggesting horizontal gene transfer into the MTBC lineage from distant donors after its separation from the STB lineages.

We also identified prominent horizontal gene transfer–related differences in clustered, regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR-Cas) systems between STB and MTBC strains. These systems may confer adaptive immunity against phages and plasmids in bacteria and archaea via repeat-spacer–derived RNAs\(^{22}\). The genomes of STB-A and STB-D contain a single CRISPR-Cas locus encoding a system of major type III-A that is similar to that of MTBC genomes but with a few \textit{crispr} spacers in common\(^{7,10}\) and substantially lower sequence similarity for their Cas proteins (down to 75%) than is seen for the core proteins (98–100%) (Fig. 2). The same genomic region in the more distant STB-J, STB-K and STB-L strains is occupied by a completely different CRISPR-Cas system of a rare type I-C variant (Fig. 2), most closely related to those of environmental actinobacteria, such as \textit{Gordonia amarae}, or purple sulfur bacteria from the \textit{Thioalkalivibrio} genus. Furthermore, in STB-K, the presence of a second CRISPR-Cas module of a different type I-C was identified 260 kb upstream of the other locus (Fig. 2), encoding Cas proteins that were most similar to those of \textit{Moorella} or \textit{Thiorhodovibrio} species. Finally, screening of whole-genome shotgun–derived sequences from strains STB-E, STB-G, STB-H and STB-I, located at well-distributed intermediate positions on the STB MLST-based network (Fig. 1), showed the existence of yet another type I-E module in STB-G and STB-I that was most closely related to those of environmental actinobacteria, such as members of the \textit{Saccharomonospora} genus, whereas, in the two remaining STB-E and STB-H strains, a type I-C variant similar to those in strains STB-J, STB-K and STB-L was found (Fig. 2a). As CRISPR-Cas systems have not been identified in non-tuberculous mycobacterial species, these different systems were most likely acquired by independent horizontal gene transfer events that occurred after the divergence of the STB and MTBC lineages. Although it is not known whether the CRISPR systems in tubercle bacilli are functional, their disparate origins suggest that the distinct respective \textit{crispr} spacer sets might not necessarily reflect genetic records of recent encounters of tubercle bacilli with distinct phage transgressors but could also represent older traces of interaction of donor organisms encoding the respective CRISPR-Cas systems with non-mycobacterial phages. The identification of a 55-kb prophage region in the whole-genome shotgun–derived sequence of STB-I that is large enough to encode a potentially complete virion\(^{23}\) (Fig. 2), which to our knowledge represents the first such finding in tubercle bacilli, provides a promising future model for testing the functionality of mycobacterial CRISPR-Cas systems on adaptive immunity against phages.

Progressive genome downsizing is a hallmark of mycobacterial pathogen evolution\(^{17,24}\). Therefore, the larger genome sizes of STB compared with MTBC strains argue for the ancestral status of the STB lineages. Further evidence for the ancestral nature of STB genome structures comes from inspection of interrupted coding sequences (ICDSs), thought to reflect molecular scars inherited during the pseudogenization of the MTBC genomes\(^{25,26}\). Among the 81 reported ICDSs in MTBC strains, most were found to be also interrupted in STB strains and more distantly related mycobacteria, suggesting that they are evolutionary ancient mycobacterial scars (Supplementary Table 5). However, we identified four ICDS orthologs—for example, \textit{pks8} belonging to a multigene family encoding polyketide synthases that are involved in the biosynthesis of important cell envelope lipids\(^{16,27}\)—which were intact in the genomes of STB strains (in one case, \textit{rv3741c-rv3742c}, the region was absent from STB-J) and in the \textit{M. marinum} and/or \textit{M. kansasi}i outgroup genomes (Supplementary Fig. 4). Thus, these scars occurred in the most recent common ancestor of the MTBC lineage after divergence from STB-like progenitors. The opposite situation, that is, ICDSs shared by the STB genomes corresponded to intact coding sequences in MTBC strains, was not observed, further supporting the ancestral status of the STB genome structure. In addition, we detected four independent loci (\textit{narX}, \textit{pks5}, \textit{pknH} and \textit{lppV}) where a likely ancestral gene organization, present both in the mycobacterial outgroups \textit{M. marinum} and/or \textit{M. kansasi}i and in STB strains, was rearranged to result in a single hybrid gene and loss of intervening gene(s) in MTBC genomes (Supplementary Fig. 5), similar to what has been observed for \textit{pknH} in \textit{M. africanum}\(^{28}\).

Ancient branching of STB lineages is also consistent with the much higher number of SNPs detected in STB genomes compared to those of MTBC strains. Pairwise comparisons of STB-A, STB-D, STB-J, STB-K and STB-L genome sequences with the \textit{M. tuberculosis} H37Rv reference uncovered 16,168–61,228 SNPs (Fig. 1). This number is within the range of 9,525–65,744 SNPs observed in the group of STB strains alone and up to 25-fold higher than the 741–2,437 SNPs previously observed in members of the MTBC lineage\(^{1,29,30}\). Consistent with MLST data (here and in ref. \(^{9}\), a NeighborNet analysis based on pairwise comparisons of the genome-wide SNP data showed that MTBC strains form a single compact group within a much larger reticulated network of STB genotypes (Fig. 1). Consistently, this reticulation was even increased when whole-genome shotgun–derived sequence data from four additional STB strains were included (Supplementary Fig. 6), further confirming the MLST-derived phylogeny at the genome level. The Phi test for recombination was highly significant (\(P = 1 \times 10^{-6}\)). Notably, the relative compactness of the MTBC branch was additionally confirmed by the structure of the phylogenetic tree, obtained after exclusion of the genome portions affected by recombination and/or horizontal gene transfer (Fig. 3a). These results thus show that the worldwide MTBC population represents a
genetically homogeneous subset branching from the larger diversity of recombinogenic STB isolates. Taken together with independent lines of evidence pointing to earlier branching, these findings suggest that the STB lineages diverged from the common ancestor of all tubercle bacilli well before the successful clonal radiation of MTBC strains began.

**Impact of selection and recombination**

To compare the effect of selection on the evolution of the STB and MTBC genomes, we calculated global ratios of nonsynonymous versus synonymous SNPs (dN/dS). The genome-wide dN/dS ratio is unusually high in MTBC strains, which has been suggested to reflect relaxed purifying selection against nonsynonymous changes that are in general slightly deleterious. The dN/dS ratios in different gene categories among the STB strains were only about a third of those found in the MTBC strains (Table 1) and are thus compatible with a much longer time during which STB strains were exposed to purifying selection, given the time dependence of dN/dS ratios for closely related bacteria and assuming that purifying selection pressures were the same for STB strains as for MTBC ones.

As an important exception, the sequences encoding the protective human CD4+ and CD8+ T-cell antigens and the epitopes of *M. tuberculosis* have been described to be under purifying selection, suggesting that MTBC members do not use T-cell antigen variation to escape human immune responses but might instead benefit from recognition by T cells. Similarly, we found that the dN/dS ratios based on pairwise concatenated codon alignments of the 65 T-cell antigen–encoding STB genes conserved across all STB genomes were on average lower than those of the 2,300 genes classified as nonessential and similar or slightly lower than those of the 710 essential
Figure 3 Interstrain recombination segments between the STB and MTBC genomes. (a) Phylogenetic tree inferred using the Neighbor-Joining algorithm on nucleotide $p$ distances, after concatenation of the sequence alignments of 2,047 genes of the predicted clonal portion of the STB-MTBC core genome (after exclusion of the genes affected by recombination and gapped regions). (b) SNP distribution among STB and MTBC aligned genome segments, showing probable recombination regions involving genes $rv1936-rv1937$ between STB-J and $M. tuberculosis$. Each of the three panels shows a comparison of two STB or $M. bovis$ strains (top and bottom) relative to $M. tuberculosis$ $H37Rv$ (middle). Red lines indicate individual SNPs identified between pairwise-compared genomes. Thicker or uneven red lines result from multiple SNPs in close proximity or shifts due to small indels. Note the SNP-free identical genome segments in close proximity or shifts due to small indels. The extensive impact of recombination among STB genomes, as identified by our comparative genome analysis, might be partly responsible for the discrepancy in dN/dS ratios between the MTBC and STB groups, as it could more efficiently oppose fixation of slightly deleterious mutations than in the more clonal MTBC population$^{39}$. Consistent with this contention, strong variations in the local distribution of SNPs were observed throughout the aligned STB and MTBC genomes, suggestive of numerous recombination events. Approximately one-third of the core genome alignment consists of zones with significantly lower or higher nucleotide differences between each pair of genomes. Stringent selection of informative regions in the predicted recombination-free blocks led to the identification of a minimal clonal backbone of 1,794,643 characters (~33% of the core genome), which was used to infer a phylogenetic tree (Fig. 3a). Inspection of the genomic regions with unexpected SNP densities allowed us to identify >110 blocks of up to 14 kb that each included from 1 to 12 complete genes (Supplementary Table 6), with homoplasic SNP distributions (relative to the tree), indicative of likely interstrain recombination events among STB strains and/or between STB and MTBC strains (Fig. 3b and Supplementary Fig. 7a,b). The extensive impact of recombination...
was independently confirmed by the finding that ~8–15% of the protein-coding sequence alignments from the core genome had mosaic structures indicative of interstrain intragenic recombination events. In contrast, the influence of exogenous importation from more distant mycobacterial species on core genome sequence diversity seemed to be minimal, as inferred by the detection of only a few regions with unexpectedly high SNP densities in STB strains, yielding BLAST best hits closer to non-tuberculous mycobacteria than to STB and MTBC strains (Supplementary Fig. 7c).

Notably, the gene blocks in *M. tuberculosis*, whose sequences perfectly match those of one or more STB strain, showed SNPs in the orthologous region in *M. bovis* and/or other MTBC strains (Fig. 3b), suggesting that gene flux between *M. tuberculosis* and the pool of STB strains existed even well after the divergence of the MTBC lineage and perhaps still exists. We also found intermediate situations where the SNP distribution clearly suggested recombination events that were more ancient and likely followed by accumulation of a few mutations in the recipient or the donor strains (Supplementary Fig. 7a). These data provide new solid evidence for the discussion on potential interstrain gene flux in *M. tuberculosis*40,41. Our findings also raise puzzling questions about the (micro)environments and mechanisms that favor or have favored such extensive DNA exchanges. The high number of apparently recent recombination episodes, as suggested by numerous perfect large sequence matches detected among sequences from different STB lineages together with the almost exclusive isolation of STB strains from affected individuals around the Horn of Africa strongly suggests a common local source. Aquatic environments rich in mycobacteria, potentially residing in protozoan hosts24,42, are one possible opportunity for genetic exchange to occur, as suggested by a recent report on the detection of MTBC DNA in rural water sources in Ethiopia (E. Wellington, personal communication). The presence of a 55-kb genomic segment corresponding to a putative complete phage-encoding region inserted into the *lysT* gene (Lys tRNA) of the STB-I strain (Fig. 2) suggests a possible mediation by phages, although alternative mechanisms, such as DNA transfer by conjugation, reported for *Mycobacterium smegmatis* under biofilm conditions43, could also be involved.

**STB strains show reduced persistence**

To determine whether these genetic differences between the STB and MTBC strains affect host-pathogen interactions, we first measured the growth of these strains in *in vitro* cultures. Most STB strains grew 2 to 3 times faster than *M. tuberculosis* strains, both in liquid media (Supplementary Fig. 8) and on solid media (data not shown) at 30 °C and 37 °C, in line with previous observations10,11. Following infection of BALB/c mice (Fig. 4) and C57BL/6 mice (Supplementary Fig. 9) by aerosol, the STB strains effectively multiplied in the lungs and disseminated to the spleens during the acute infection phase but consistently persisted less well during the chronic infection phase compared to *M. tuberculosis*. Whereas the latter was able to persist in the lungs for up to 30 weeks at levels close to those seen in the acute phase (peaking at 3 weeks with around 1 × 10^7 to 1 × 10^8 colony-forming units (CFUs)), the infection levels of all STB strains dropped by at least 1 log at all (and by 2 to 3 logs at most) later time points in these organs (P = 0.05 by Mann-Whitney test, except for day 130 for STB-D, STB-K and STB-L). The strongest difference with *M. tuberculosis* was observed for STB-K, the strain phylogenomically most distant from MTBC strains, for which bacterial counts were undetectable after 30 weeks in BALB/c mice (Fig. 4c,d). Similar trends were observed in spleens, with STB-K also almost completely cleared at day 210. In parallel, histopathological analyses showed less intense lung lesions and inflammation 128 d after infection with the STB strains compared to *M. tuberculosis* infection, with STB-K showing the least damage (Fig. 4 and Supplementary Table 7). Furthermore, C57BL/6 mice intravenously infected with high doses of STB survived in contrast to controls infected with *M. tuberculosis* strains of different lineages (data not shown), confirming the decreased virulence of STB strains.

Finally, we determined whether these variations could be correlated to differences in the innate or adaptive immune responses elicited by infection. The STB and *M. tuberculosis* strains were similarly able to induce maturation of innate immunity cells *in vitro*, such as dendritic cells derived from wild-type, *Tlr2*-null, *Tlr4*-null or double-knockout C57BL/6 mice (data not shown), suggesting shared major pathogen-associated molecular patterns (PAMPs)44. Consistently, substantial recruitment of activated innate immune cells, for example, CD11b+BST-2+ and CD11c+MHC-II^hi^ cells, was observed *in vivo* in the lung parenchyma of severe combined immunodeficient (SCID) mice after 3 weeks of infection by STB strains but to a lesser extent than with *M. tuberculosis* infection (data not shown). With regard to adaptive responses, massive recruitment of activated CD4+ or CD8+...
T cells, showing CD44 modulation and CD45RB, CD27 and CD62L downregulation, was detected in the lungs of C57BL/6 mice after 13 weeks of infection by strains with smooth colony morphology. Again, the responses were overall quantitatively lower for STB strains compared to M. tuberculosis strains, especially for STB-K (Supplementary Fig. 10), in line with the lower virulence and persistence of the STB strains.

DISCUSSION

With the larger pan-genome reflecting the ancestral, wider gene pool of tubercle bacilli, their lower virulence and faster growth, especially at temperatures below 37 °C, plausibly reflecting broader environmental adaptability, STB strains might thus come nearer to the as-yet-unknown missing link between the obligate pathogen M. tuberculosis and environmental mycobacteria. We propose that M. tuberculosis has evolved its widespread, pathogenic lifestyle starting from a pool of STB-like mycobacteria by gaining additional virulence and persistence mechanisms through a potential combination of (i) loss of gene function, (ii) acquisition of new genes via horizontal gene transfer, (iii) intraspecies recombination of gene clusters and (iv) fixation of SNPs. The data presented here suggest further experiments to examine which of these genetic events were involved. Primary candidates are MTBC-specific genes (Supplementary Table 4), including the prophage-like phiRv1- and phiRv2-encoding regions reported to be important for late infection 45, the genes encoding Supplementary Table 4.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The complete genome sequence for the STB-a strain (CIPT 140010059) was deposited under accession HE872590. Genome sequences of strains STB-D (CIPT 140060008), STB-I (CIPT 140070017), STB-K (CIPT 140070010) and STB-L (CIPT 140070008) were deposited in the European Molecular Biology Laboratory (EMBL) database under project/accession codes PRJEB94/FO203507, PRJEB93/FO203508, PRJEB92/FO203509 and PRJEB95/FO203510, respectively. Illumina-derived whole-genome shotgun sequences for strains STB-E (CIPT 140070002), STB-G (CIPT 140070005), STB-H (CIPT 140070013) and STB-I (CIPT 140070007) were deposited in the EMBL whole-genome sequence repository project/accession codes PRJEB584/CAOLO0000000, PRJEB585/CAOM00000000, PRJEB586/CAOON00000000 and PRJEB587/CAOO00000000, respectively.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.S.-M., C.-G. and R.B. designed the study. P.S. and R.B. analyzed data and wrote the manuscript, with comments from all authors. M.M., D.W., J.P., C.M. and S.D.B. annotated the genomes. S.M., E.C.B., M.A.Q., L. Ma, C.B. and V.B. performed and/or verified the finishing and assembly of sequences. D.R. and S.C. performed SNP analyses and database management, with the support of G.S. C.R., A.P., W.F., R.S., A.-S.D., E.W. A. Casciroferro and M.-G. performed mouse infection experiments, in vivo data analyses and/or mycobacterial growth assays. L. Majlessi, F.S., C. Locht and C. Leclerc conducted and/or analyzed immune assays. J.T., A. Criscuolo and S.B. conducted MLST, recombination and/or phylogenetic analyses. I.F. conducted histopathological analyses. V.K., M.O. and C.P. created bioinformatics tools and analyzed data. M.F. isolated STB strains. T.S. and T.P.S. conducted core genome and NeighborNet analyses.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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Illumina HiSeq technology and single lanes. Resulting reads that covered up to data from strains STB-E, STB-G, STB-H and STB-I were generated using were mapped at a resulting genome coverage of >40×. SNPs with base coverage reads of 36 bp (STB-A, STB-D, STB-J and STB-L) or trimmed at 50 bp (STB-K) Recombination.

Annotation and genome compari -

Bootstrap consensus trees were inferred with RAxML (2002) with the pairwise nucleotide p distances. All regions of ≥500 characters in length with a significantly high or low number of SNPs were inspected visually for the pairwise homoplasy index62, the maximum X² test63 and the neighbor similarity score64.

Bacterial growth assays. The growth rates of STB and reference MTBC strains in liquid media were measured using a BACTEC 460 system (Beckton-Dickinson) as recommended by the manufacturer.

Mouse infection experiments and histopathological and cell analyses. Mice were maintained according to the Institut Pasteur de Lille and Paris guidelines for laboratory animal husbandry. Animal experiments were approved by the Nord-Pas-de-Calais ethical committee (CEEA 15/2009) and the Institut Pasteur Hygiene Committee (authorization number 75-1469), in accordance with European and French guidelines (Directive 86/609/CEE and Decreed 87–848). Eight-week-old female BALB/c mice were infected with the intranasal route with 1,000 CFUs of either STB or M. tuberculosis H37Rv strains. At the indicated times, four mice per group were sacrificed, and colony counting was performed from homogenized individual lungs and spleens as described65. For histopathological evaluation, whole lungs were harvested from three BALB/c mice for each group 128 d after infection, fixed in 4% formalin and embedded in paraffin. Four-mm-thick sections were stained with hematoxylin and eosin. Virulence and cell analysis–based immunological assays using C57BL/6 and/or SCID mice were performed as described66,67. Adaptive immune cells from infected mice were prepared, incubated with conjugated monoclonal antibodies (Beckton-Dickinson), fixed and analyzed using a CyaN system and Summit (Beckman Coulter) and FlowJo (TreeStar) software.

ONLINE METHODS

Bacterial strains and MLST. The 55 STB and 10 reference MTBC isolates are described in Supplementary Table 1. Twelve housekeeping genes were selected for MLST37 (Supplementary Table 2). Phylogenetic groupings were identified by split decomposition analysis26 on the concatenated target sequences.

Recombination. The genomes of M. tuberculosis H37Rv, M. bovis AF2122/97 and the five STB strains were aligned using progressiveMauve60. Given a pair of aligned genomes i and j, the number of SNPs \( x_{ij} \) observed between i and j within a region of length l follows a binomial distribution \( B(l, p_{ij}) \), where \( p_{ij} \) is the expected proportion of recombination-free nucleotide differences between taxa i and j. Regions containing at least one pair of sequences i and j with an unexpectedly high or low number of \( x_{ij} \) SNPs, that is, \( \min(P(X \geq x_{ij}), P(X \leq x_{ij}) < 0.05, \) where \( P(X = B(l, p_{ij})) \) were identified using a 200-character-long sliding window along the conserved (core) portions of the multiple-genome alignment. The value \( p_{ij} \) inside each window was estimated as the proportion of SNPs between i and j within the 10,000 aligned characters flanking the sliding window on both sides. To obtain a reference phylogeny, all regions of ≥2500 characters in length (excluding gaps) that did not contain an unexpected number of SNPs were concatenated. The derived supermatrix was used to infer a phylogenetic tree with the Neighbor-Joining algorithm61 on the pairwise nucleotide p distances. All regions of ≥500 characters in length with a significantly high or low number of SNPs were inspected visually for detection of the concentration of homoplastic characters using ACT53, leading to the identification of similarities between strains incongruent with the phylogenetic tree. The proportion of protein-coding sequences within the core genome probably affected by interstrain recombination was assessed with the pairwise homoplasy index62, the maximum X² test63 and the neighbor similarity score64.

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