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Diagnosis of paediatric tuberculosis by optically detecting two virulence factors on extracellular vesicles in blood samples

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Sensitive and specific blood-based assays for the detection of pulmonary and extrapulmonary tuberculosis would reduce mortality associated with missed diagnoses, particularly in children. Here we report a nanoparticle-enhanced immunoassay read by dark-field microscopy that detects two Mycobacterium tuberculosis virulence factors (the glycolipid lipoarabinomannan and its carrier protein) on the surface of circulating extracellular vesicles. In a cohort study of 147 hospitalized and severely immunosuppressed children living with HIV, the assay detected 58 of the 78 (74%) cases of paediatric tuberculosis, 48 of the 66 (73%) cases that were missed by microbiological assays, and 8 out of 10 (80%) cases undiagnosed during the study. It also distinguished tuberculosis from latent-tuberculosis infections in non-human primates. We adapted the assay to make it portable and operable by a smartphone. With further development, the assay may facilitate the detection of tuberculosis at the point of care, particularly in resource-limited settings.

Tuberculosis (TB) is the second leading cause of death from infectious disease and a top-ten cause of death globally¹–³, with an estimated 10 million new cases and 1.2 million deaths yearly. Despite sustained global efforts towards the control of TB, children aged less than 15 years account for ~1 million TB cases annually and for 230,000 TB-related deaths⁴. Most paediatric TB deaths (80%) occur in children less than 5 years old, and 96% of these deaths occur in children who are untreated owing to missed diagnoses⁵,⁶. The COVID-19 pandemic has hindered global TB-control efforts, and models from the World Health Organization (WHO) suggest that TB-notification decreases observed in TB-endemic countries in 2020 may result in hundreds of thousands of additional TB deaths⁷. Most microbiologic TB assays use culture or nucleic acid amplification to detect Mycobacterium tuberculosis (Mtb) bacilli in respiratory and extrapulmonary samples. However, young children frequently require invasive procedures to obtain respiratory samples that may contain few Mtb bacilli (paucibacillary TB), and often present with disseminated or extrapulmonary TB missed by respiratory sampling strategies. Mtb culture, the diagnostic gold standard, is slow (2–8 weeks) and detects only 30–62% of paedi- tric TB (ref. ⁸). PCR-based Xpert MTB/RIF (Xpert) and Xpert Ultra can provide diagnostic results in a few hours but detect only

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a fraction of the *Mtb*-positive sputum (65%) and gastric samples (73%) detected by *Mtb* culture. Xpert and Xpert Ultra may thus detect only 20–40% of all paediatric TB cases, and their sensitivity may be even lower in children living with HIV, who may have higher rates of culture-negative TB. Also, Xpert cannot distinguish DNA derived from viable and non-viable *Mtb* bacilli, reducing its utility for the evaluation of treatment responses.

Moreover, conventional immunoassays exhibit poor diagnostic performance because *Mtb*-derived factors may circulate at low concentration, exhibit poor specificity or are subject to masking effects. Also, they do not reliably distinguish latent TB infection from TB disease. We have reported that mass-spectrometry-coupled immunoassays can improve the diagnostic performance of such biomarkers, but these approaches may have restricted utility owing to equipment and technical-expertise requirements. Nanoparticle-enhanced IV immunonas (NEI) that are read by dark-field microscopes (DFMs) found in many research and clinical laboratories can be used for the ultrasensitive detection of cancer-associated extracellular vesicle (EV) biomarkers in serum, but these approaches require manual searches to identify fields of interest, making them prone to operator error and bias.

In this study, we show that automated NEI and machine learning can be used for the detection of EVs secreted by *Mtb*-infected cells (*Mtb* EVs), by leveraging the surface expression of factors abundantly expressed on the *Mtb* membrane. The NEI used in this study targeted the glycolipid lipoarabinomannan (LAM), a major component of the mycobacterial cell wall that accounts for up to 15% of *Mtb* biomass and that regulates *Mtb* virulence, as well as the membrane protein LprG, which is required for LAM distribution to the outer cell envelope. Multiple studies have evaluated the potential of urine LAM assays for the diagnosis of TB, but few have examined serum or plasma LAM assays, and none appear to have examined the TB diagnosis potential of serum or urine LprG assays. We hypothesized that EV LAM and LprG expression might serve as a serum biomarker of pulmonary and extrapulmonary TB since EVs accumulate in serum and can carry factors derived from *Mtb*-infected macrophages. We therefore confirmed the EV surface expression of LAM and LprG using EVs from *Mtb*-infected macrophages, investigated the ability of an integrated LAM and LprG marker to distinguish active TB disease from latent TB infection in a non-human primate model that permits accurate classification, and evaluated the ability of this integrated biomarker to diagnose TB in paediatric cohorts with and without HIV infection to address the effect of HIV on TB diagnosis. Our findings indicate that NEI had similar diagnostic performance for pulmonary and extrapulmonary TB and for culture-confirmed and clinically diagnosed TB cases in children with and without HIV.

**Results**

**EVs secreted by *Mtb*-infected macrophages express LAM and LprG.** To evaluate the diagnostic potential of EV LAM and LprG expression, we first examined EVs secreted by *Mtb*-infected macrophages. EVs isolated from macrophage cultures infected with or without *Mtb* had similar morphologies, but *Mtb*-infected macrophages secreted more and larger EVs (2.3 \times 10^6 to 1.1 \times 10^7 EVs per ml), as determined by nanoparticle tracking analysis (Supplementary Fig. 1a–c). LAM and LprG were significantly enriched in EV versus cytosal and membrane fractions of macrophages infected with *Mtb* strains exhibiting variable growth rates and immunogenicity, indicating their potential utility as strain-independent biomarkers of *Mtb* infection (Fig. 1b,c and Supplementary Fig. 1c). Western blot analysis of *Mtb*, *M. avium*, *M. intracellulare* and *M. kansasi*, and *M. smegmatis* culture filtrate protein (CFP) samples using our selected LAM and LprG antibodies detected species-specific signals. LAM signals were detected in *Mtb* and *M. smegmatis*, which rarely causes pulmonary infections, and strong and weak LprG signals were detected in *Mtb* and *M. intracellulare*. Thus, among the four mycobacteria that are major causes of pulmonary infection, our assay antibodies detected significant signals for both LAM and LprG signal only in *Mtb*, and thus dual detection of these factors is *Mtb*-specific (Fig. 1d). Neither of these factors was detected in EV fractions isolated from macrophages infected with bacteria responsible for common hospital infections (Supplementary Fig. 2). EV-ELISA, a gold standard for detection of EV surface factors, detected a dose-dependent increase in LAM and LprG signals (Fig. 1e and Supplementary Fig. 1e) in serial dilutions of EVs isolated from *Mtb*-infected macrophage cultures by ultracentrifugation. These ultracentrifugation samples could contain both EVs and bacteria-derived membrane vesicles (BMVs) due to their similar physical properties. However, the LAM and LprG EV-ELISAs used a host-specific EV protein for EV capture and detected no appreciable signal in BMV fractions from *Mtb* cultures (Fig. 1f and Supplementary Fig. 3), indicating the EV origin of the LAM and LprG signals detected in these samples.

The potential of EV-ELISA and LAM and LprG signals to distinguish serum from TB, latent TB infection (LTBI) and healthy control groups was next evaluated using serum from non-human primates that were *Mtb*-naive or that developed LTBI or pulmonary TB following *Mtb* exposure. Non-human primate (NHP) models were employed in this analysis to allow more confident TB and LTBI classification since in patients it is difficult to distinguish LTBI cases (*Mtb*-infected without TB disease) from infections likely to progress to TB, but which lack clinical symptoms in the absence (incipient TB) or presence (subclinical TB) of radiographic abnormalities or microbiologic findings. Serum EVs isolated from *Mtb*-naive NHPs had low LAM and LprG signals (Fig. 1g and Supplementary Table 1); EVs from the LTBI group had elevated LAM or LprG expression, with most revealing elevated LprG signal alone; and EVs from the pulmonary TB group had elevated expression of both LAM and LprG, suggesting that these factors might serve a composite biomarker for TB diagnosis. However, the mechanism(s) involved in this apparent differential expression in TB and LTBI, and its functional significance, is not known and should be replicated in larger, well-characterized longitudinal human LTBI and TB cohorts.

EV-ELISA analysis of EVs isolated from archived serum from a small, well-characterized case-control cohort of children (Supplementary Tables 2 and 3) enrolled at the Vietnam National Lung Hospital (NLH) as pilot of a large, population-based TB surveillance study found that mean LAM and integrated LAM and LprG signals, but not LprG signal, differed between the TB and non-TB groups when used to directly analyse their serum (Fig. 1h). EV-ELISAs were conducted to analyse additional factors that were reported to be abundantly secreted, membrane-associated and/or able to distinguish TB and LTBI cases, and which had available specific antibodies. Only LAM and LprG revealed differential expression on serum EVs from TB versus non-TB cases, potentially due to low or absent EV surface expression of the additional selected factors (Supplementary Fig. 4).

NEI sensitively detects LAM and LprG expression on *Mtb* EVs. LAM and LprG EV-ELISAs did not detect differences between the NLH TB and non-TB groups when used to directly analyse their serum (Supplementary Fig. 5), unlike results obtained with EVs enriched from these samples, demonstrating that EV-ELISA lacks the sensitivity required for a direct serum assay. We therefore investigated the feasibility of using NEIs to directly capture EVs from serum and sensitively detect gold nanorod (AuNR) probes bound to LAM or LprG on these EVs by high- or low-magnification dark-field microscope (DFM) image analysis, each of which has advantages and disadvantages. High-magnification analyses allow ultrasensitive detection but require manual focusing to detect plasmic signals from interacting nanoparticles, which can introduce selection bias due to sampling of limited areas of the assay wells.

980 NATURE BIOMEDICAL ENGINEERING | VOL 6 | AUGUST 2022 | 979–991 | www.nature.com/natbiomedeng
Fig. 1 | EV LAM and LprG expression as a biomarker for TB diagnosis. a, Rationale and assay schematic. Created with BioRender.com. b, c, Western blot densitometry of LAM (b) and LprG (c) expression in cytosolic (Cyto), EV and cell membrane (CM) fractions of macrophage cultures infected with the indicated Mtb strains. Data indicate mean ± s.d., n = 3 biologically independent repeats; P values obtained by one-way ANOVA with Dunnett’s post-test. a.u., arbitrary units. d, Western blot analysis of LAM and LprG from CFP samples of patients with sequence-confirmed M. smegmatis, M. tuberculosis, M. avium, M. intracellulare and M. kansasii infections. e, f, EV-ELISA for LAM on EVs from Mtb-infected macrophages (e) and BMVs isolated from Mtb culture medium (f) by ultracentrifugation when captured by host EV-specific antibody. g, EV-ELISA for LAM and LprG on serum EVs of non-human primates with pulmonary TB (PTB), LTBI or their healthy controls (Ctrl) (mean ± s.d. of three technical replicates). h, EV-ELISA for LAM, LprG and integrated LAM and LprG (LAM+LprG) expression on isolated EVs from serum of children with TB (N = 10) and without evidence of TB (Ctrl; N = 5). Mean ± s.e.m., P values obtained by two-sided Mann-Whitney U test.

Low-magnification DFM analyses can be automated and are thus more suitable for clinical applications, but are subject to artefacts that can increase background noise and reduce sensitivity.

We therefore established an NEI workflow (Fig. 2a) to permit automatic capture of low-magnification DFM images that were then processed with a custom noise-reduction algorithm to reduce artefacts from serum aggregates, particulates and surface scratches introduced during the assay. This algorithm detected and cropped the region of interest (ROI), converted each image into HSB (hue, saturation and brightness) colour space, and normalized and applied thresholds to each HSB channel to identify AuNR-derived signals (Supplementary Fig. 6). This markedly reduced signal artefacts, as indicated by the enhancement of AuNR signals in processed images and their pixel intensity maps (Fig. 2b,c). For signal detection, analysis of an AuNR dilution curve by quantifying positive pixels and mean pixel intensity found that the latter approach had greater linearity with less variation (Fig. 2d).

EVs isolated from Mtb-infected macrophages were hybridized with dilutions of anti-LAM antibody-conjugated AuNRs to determine the probe concentration required to maximize the signal-to-noise ratio at low target EV concentrations (Fig. 2e). Subsequent evaluation of capture and detection antibody pairs determined that anti-CD81 capture antibody and two anti-LAM (A194-01) and anti-LprG (Clone B) detection antibodies produced...
the best signal-to-noise ratios (10.21 and 7.25, respectively; Fig. 2f and Supplementary Fig. 7), and good linearity and variability over a broad EV concentration range (0–150 ng EV protein per ml; Fig. 2g). Target EVs spiked into pooled human serum were detected with high sensitivity (5 ng ml⁻¹), linearity (R² = 0.98) and variability (mean coefficient of variation (c.v.) = 4.6%, 2.1–5.3%) using the optimized NEI and analysis workflow, but with reduced sensitivity (40 ng ml⁻¹), linearity (R² = 0.81) and reproducibility (mean c.v. = 65.2%, 20.3–152.9%) when analysed without image processing (Fig. 2h). No LAM or LprG NEI signal was detected from BMVs isolated from Mtb culture medium (Supplementary Fig. 8). Major EV signal decreases were detected in spiked samples stored at 4°C or ambient temperature, presumably due to EV lysis or biomarker proteolysis, but not in cryopreserved samples (Supplementary Fig. 9).

NEI analysis of serum from a small discovery cohort found that serum EV LpG and LAM signals had similar ability to distinguish the TB and non-TB groups, but that EV LAM and LprG signals integrated via a logistic model (see Methods) had superior performance (Fig. 2i), as indicated by the ability of thresholds derived from each analysis to differentiate these groups (Fig. 2i–l). Substantial signal overlap occurred between these groups near the LAM or LpG signal thresholds, resulting in three false negatives and one false positive, while the integrated LAM and LpG threshold produced a single false negative. The NEI LAM and LpG signal was detected in EV-enriched but not in EV-depleted serum fractions, confirming the EV-specificity of the NEI signal (Supplementary Fig. 10). However, the NEI signal did not differ when paediatric TB cases were stratified by age, sex or TB manifestation (pulmonary or extrapulmonary) (Supplementary Fig. 11), although there was a tendency for higher NEI signal in patients with extrapulmonary TB, which might reflect reduced immune containment associated with this form of TB. The diagnostic performance observed in the NLH cohort was validated using serum from an ongoing paediatric TB study where patients had more detailed clinical information available for case classification (Supplementary Fig. 12 and Table 4). The NEI signal yielded diagnostic sensitivities of 88.2% (15/17) and 81.8% (9/11) for confirmed and unconfirmed TB cases, respectively, with most having positive findings for both symptoms and CXR findings (66.6% vs 71.2%; Fig. 3a), although a subgroup was reclassified from unlikely to unconfirmed TB on the basis of TBTx response or TB-related death as determined by an expert review panel (Supplementary Table 7 and Fig. 3a, subgroup A). Children classified as ‘unlikely TB’ frequently had symptoms or CXR findings consistent with TB (66.7% vs 30.2%), but a subset positive for both criteria was classified as unlikely TB due to symptom improvement following ART without TBTx initiation (Fig. 3a, subgroup B).

NEI analyses performed on PUSH cohort serum by operators blinded to clinical information and using positive signal thresholds determined in the NLH discovery cohort detected confirmed TB and unconfirmed TB with 83.3% and 76.1% diagnostic sensitivity (Table 2). NEI results had similar sensitivity for unconfirmed TB cases with and without clinical TB diagnoses (Table 2), and detected all but 2 confirmed TB cases (Fig. 3b). NEI results also detected the majority (54.4%) of children with unlikely TB who had at least one criterion required for unconfirmed TB diagnosis (Table 2). Since an age-matched low-risk cohort was not available, NEI diagnostic specificity was estimated in a subgroup of children with unlikely TB who lacked all NIH criteria for unconfirmed TB. Median Mtb EV signal in this group was significantly lower than in all other groups. Positive TBTx response rates were high in children with confirmed and unconfirmed TB (87.0% vs 70.0%; Table 1). Mortality after TBTx initiation was higher in children with confirmed vs unconfirmed TB (30.0% vs 8.7%; Table 2), but even higher (50.0%) in children with unconfirmed TB who were not diagnosed or treated during the study. Mortality was lower in children with unlikely TB who had no vs any TB criteria (0% vs 17.9%), although children with unlikely TB who died frequently had missing microbiologic or CXR results (Fig. 3a). Urine LAM tests performed during the study had poor diagnostic sensitivity for confirmed (37.5%; 3 of 8) and unconfirmed (5.8%; 3 of 52) TB cases with valid test results, and employed serum collected from children with HIV in the multi-site Paediatric Urgent vs post-Stabilization Highly active antiretroviral therapy (ART) initiation trial (PUSH) in Kenya who were hospitalized and not yet initiated on ART at enrolment (Supplementary Fig. 15 and Table 1). Most children in this study exhibited severe immunosuppression as well as some symptoms consistent with TB. Children were retrospectively classified on the basis of the 2015 NIH paediatric clinical TB case definitions, as confirmed TB using microbiologic evidence (positive Mtb culture or positive Xpert results for respiratory or stool samples); unconfirmed TB (if positive for ≥2 of the following criteria: TB-associated symptoms; abnormal TB-consistent chest radiograph (CXR); close TB exposure or evidence of Mtb infection (that is, positive tuberculin skin test (TST))); or positive response to TB treatment (TBTx); or unlikely TB (if they lacked two criteria for unconfirmed TB) (Supplementary Table 6).

Children with confirmed and unconfirmed TB had high rates of symptoms (75.0% vs 84.9%) and CXR findings (91.7% vs 84.9%) consistent with TB, with most having positive findings for both symptoms and CXR findings (66.6% vs 71.2%; Fig. 3a), although a subgroup was reclassified from unlikely to unconfirmed TB on the basis of TBTx response or TB-related death as determined by an expert review panel (Supplementary Table 7 and Fig. 3a, subgroup A). Children classified as ‘unlikely TB’ frequently had symptoms or CXR findings consistent with TB (66.7% vs 30.2%), but a subset positive for both criteria was classified as unlikely TB due to symptom improvement following ART without TBTx initiation (Fig. 3a, subgroup B).

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moderate specificity for unlikely TB cases (88.9%; 48 of 54), including children with no TB criteria (81.8%; 9 of 11).

Serum *Mtb* EV signal correspondence with TB reclassifications and treatment. Most children with unconfirmed TB were diagnosed using enrolment data, but a few were reclassified from unlikely to unconfirmed TB (15.1%; 10/66) on the basis of subsequent findings (Supplementary Table 8). Reclassification decisions were made without knowledge of EV results, but most unlikely to unconfirmed TB reclassifications (80%; 8/10) occurred in children who had detectable *Mtb* EV NEI signal at baseline, and this signal remained positive in children who had sera available after reclassification (Fig. 3c). Conversely, all unconfirmed to unlikely TB reclassifications occurred in children who had symptom improvement.
without TBTx initiation, most of whom had positive \( Mtb \) EV NEI signal at baseline (75%; 9/12) that decreased by the time of reclassification (88.9%; 8/9; Fig. 3d). Most of these children had HIV viral load and CD4+ T-cell count improvements following ART initiation (Fig. 3e,f), suggesting that improved immune function may have contained nascent TB cases. Median NEI signal also markedly decreased from pre- to post-TBTx initiation (median 5.5 months, <20%; age <12 months, <25%; age 12-35 months, <20%; age ≥36 months, <15%) or, in the absence of CD4-percentage data, CD4 count (age <12 months: <1,500 cells per μl; age 12-35 months, <750 cells per μl; age ≥36 months, <350 cells per μl). Among children 5 years and under: WHZ < −2 or MUAC <12.5 cm. Persistent cough (>14 d), fever (>7 d), failure to thrive, or lethargy (>7 d). Failure to thrive corresponds to wasted (WHZ < −2 or MUAC <12.5) or underweight (WAZ < −2) at enrolment (growth trajectories unavailable before enrolment). Sputum or gastric aspirate. †Includes one child with unconfirmed TB who had indeterminate Stool Xpert results. ‡Includes one child with unconfirmed TB and 2 children with unlikely TB who had invalid urine LAM results. Colour change corresponding to manufacturer reference card grade ≥1 was considered positive at the time of the study. *Two children with confirmed TB died before initiating TB treatment. †Within 6 months of enrolment.

### Discussion

We have evaluated the utility of adopting an NEI approach to rapidly detect \( Mtb \) EV biomarkers in small serum volumes for TB diagnosis, particularly in children with HIV—a population that is often missed by sputum-based diagnostics. Refining a standard NEI approach permitted automated image capture and the ultra-sensitive detection of the target signal. The assay captured EVs from serum, eliminating the common EV-immunoassay requirement for purified EV samples, which are normally isolated by methods that involve trade-offs between time, labour, expense and EV yield, purity and integrity that limit their clinical feasibility. The NEI workflow is similar to that employed by ELISAs and may be suitable for use in clinical-laboratory settings after further assay optimization and clinical-validation studies. Because there are currently no widely accepted EV biomarkers of TB, in this study we evaluated the potential diagnostic utility of measuring serum levels of EVs expressing two \( Mtb \)-derived factors associated with TB virulence—LAM and LprG. We found that the EV expression of these markers was enhanced in TB cases, and that multiplex detection of these two factors could differentiate LTBI and TB in an NHP model. LAM and LprG are both highly expressed in \( Mtb \) bacilli, which could

### Table 1 | Baseline characteristics of HIV-positive hospitalized ART-naïve children at high risk for TB evaluated by TB exosome assays for TB diagnosis (147 children)

| Characteristics | Number of children with results | Total, 147 children | With confirmed TB, 12 children | With unconfirmed TB, 66 children | With unlikely TB, 69 children |
|-----------------|---------------------------------|---------------------|-------------------------------|---------------------------------|-------------------------------|
| Age, years      | 147                             | 1.8 (0.8, 4.4)      | 2.9 (1.2, 6.7)               | 1.9 (1.1, 4.8)                 | 1.6 (0.7, 3.9)               |
| Female, number  | 147                             | 70 (47.6)           | 5 (41.7)                      | 30 (45.5)                      | 35 (50.7)                    |
| CD4, cell count, cells per μl | 146                             | 727.5 (317, 1,240)  | 487 (156, 800.5)             | 650 (202, 1,210)              | 767 (524, 1,445)            |
| CD4, %          | 146                             | 15 (9, 22)          | 13 (6, 21.3)                 | 12.9 (6, 19)                   | 17 (12, 24)                  |

| Number of children | With severe immunosuppression for HIV<sup>a</sup> | 146 | 102 (69.9) | 9 (75.0) | 50 (76.9) | 43 (62.3) |
|--------------------|---------------------------------------------------|-----|------------|----------|-----------|-----------|
| Wasted<sup>b</sup> | 114                                                | 72 (63.2) | 7 (87.5) | 34 (66.7) | 31 (56.4) |
| Underweight (WAZ < −2) | 147                                           | 95 (64.6) | 8 (66.7)  | 47 (71.2) | 40 (58.0) |
| With signs or symptoms suggestive of TB<sup>c</sup> | 147 | 111 (75.5) | 9 (75.0) | 56 (84.9) | 46 (66.7) |
| With TST ≥ 5 mm   | 134                                                | 7 (5.2)  | 1 (9.1)   | 5 (8.5)  | 1 (1.6)   |
| With TB exposure  | 147                                                | 25 (17.0) | 7 (58.3)  | 15 (22.7) | 3 (4.4)   |
| With CXR suggestive of TB | 141 | 86 (61.0) | 11 (91.7) | 56 (84.9) | 19 (30.2) |
| With positive respiratory \( Mtb \) culture or Xpert<sup>d</sup> | 147 | 11 (7.5)  | 0          | 0        |
| With Stool Xpert positive<sup>e</sup> | 130 | 7 (5.4)  | 0          | 0        |
| With urine LAM positive<sup>f</sup> | 116 | 12 (10.3) | 3 (37.5)  | 3 (5.8)  | 6 (10.7)  |
| With TBTx initiated | 147 | 57 (38.8) | 10 (83.3)<sup>g</sup> | 46 (69.7) | 1 (1.5)   |
| With TBTx response | 57                                                | 47 (82.5) | 7 (70.0)  | 40 (87.0) | 0         |
| Dead<sup>h</sup>   | 147                                                | 29 (19.7) | 5 (41.7)  | 14 (21.2) | 10 (14.5) |

<sup>a</sup>The numbers are provided as medians, with interquartile ranges or the percentage of participants with positive results (with respect to the number of children with results) given in brackets. WAZ, weight-for-age z-score; WHZ, weight-for-height z-score. †WHO age-specified percentage of CD4 cut-offs for severe immunosuppression (age <12 months, <25%; age 12-35 months, <20%; age ≥36 months, <15%) or, in the absence of CD4-percentage data, CD4 count (age <12 months: <1,500 cells per μl; age 12-35 months, <750 cells per μl; age ≥36 months, <350 cells per μl). †Among children 5 years and under: WHZ < −2 or mid-upper arm circumference (MUAC) <12.5 cm. Persistent cough (>14 d), fever (>7 d), failure to thrive, or lethargy (>7 d). Failure to thrive corresponds to wasted (WHZ < −2 or MUAC <12.5) or underweight (WAZ < −2) at enrolment (growth trajectories unavailable before enrolment). Sputum or gastric aspirate. †Includes one child with unconfirmed TB who had indeterminate Stool Xpert results. ‡Includes one child with unconfirmed TB and 2 children with unlikely TB who had invalid urine LAM results. Colour change corresponding to manufacturer reference card grade ≥1 was considered positive at the time of the study. *Two children with confirmed TB died before initiating TB treatment. †Within 6 months of enrolment.
NEI signal decreases (d) among children diagnosed as unconfirmed TB cases at baseline but reclassified as unlikely TB cases due to symptom improvement following ART initiation alone (Subgroup B in a) who were reclassified to unconfirmed TB by investigators blinded to EV results.

- **Fig. 3** | Mtbb EV NEI diagnostic performance in children living with HIV at high risk of TB. 

  a. NEI signal in children with confirmed, unconfirmed and unlikely TB as determined by positive respiratory culture/Xpert or stool Xpert results, TB-related symptoms meeting NIH criteria for the duration, chest X-ray (CXR) findings, close TB contact or positive TST, positive TBTx response, and/or TB-related death. Urine LAM results and serum Mtb EV results were not used for classification. Subgroup A: children reclassified from unlikely to unconfirmed TB on the basis of TBTx (TB treatment) response or TB-related death as determined by an expert review panel (see Supplementary Table 7 for criteria). Subgroup B: children reclassified from unconfirmed to unlikely TB on the basis of symptom improvement following ART initiation without TBTx initiation (alternate data for anti-TBTx response).

b. TB cases classified by NEI, Mtb culture and/or Xpert test results. c. Positive baseline NEI signal predicts subsequent TB reclassification in children with unlikely TB assignments.

c. **Table 2** | Diagnostic performance of Mtb EV for TB among HIV-positive hospitalized ART-naive children at high risk for TB (147 children)

| Confirmed TBa (12 children) | Unconfirmed TBb (66 children) | Unlikely TBc (69 children) | Any NIH criteriad (12 children) | No NIH criteriad (12 children) |
|----------------------------|-------------------------------|---------------------------|-------------------------------|-------------------------------|
| **Number of children positive according to Mtb EV** | 10 | 35 | 13 | 31 | 2 |
| **Viral load (copies per ml)** | 24.6 (6.9, 66.5) | 16.6 (6.9, 31.6) | 8.5 (3.9, 18.6) | 71 (1.9, 23.8) | 2.7 (0.6, 4.6) |
| **P value** | 0.001 | 0.0005 | 0.01 | 0.02 | Reference |

Confidence intervals (95%) for the sensitivities and specificities, and interquartile ranges for the Mtb EV levels are given in brackets. Includes 137 children with serum analysed at baseline; 7 at time of ‘unconfirmed TB diagnosis’ (6 at 2 weeks and 1 at 4 weeks post enrolment); and 3 ‘unlikely TB’ cases with missing baseline serum who had serum analysed at 2 weeks post enrolment. *Assessed post-hoc on the basis of international consensus clinical-case definitions: paediatric TB, children who were prospectively diagnosed during the study period by clinical staff and who received TBTx during the study period; no TB diagnosis or TBTx during study, children who were not clinically diagnosed and did not receive TBTx during the study period; NIH TB criteria, children who had at least one of the two diagnostic criteria required for TB diagnosis by the NIH algorithm. Assessed during the study. Clinical TB diagnosis: initiated TBTx. No clinical TB diagnosis: did not initiate TBTx. Positive CXR or TB exposure within the last two years; or TST ≥ 5 mm; or NIH TB symptoms: persistent cough (>14 d), fever (>7 d), failure to thrive, or lethargy (>7 d). Failure to thrive corresponds to wasted (WHZ < -2 or MUAC < 12.5) or underweight (WAZ < -2) at enrolment. Wilcoxon rank-sum test (two-sided), compared to ‘unlikely TB with no TB symptoms’ and ‘negative CXR’.
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Table 3 | Median Mtb EV levels at TB diagnosis and post initiation of TB treatment

| Number of children | Pre-TBTx initiation | Post-TBTx initiation | P value |
|--------------------|---------------------|----------------------|---------|
| All TB cases       | 50                  | 15.1 (7.5, 37.4)     | 4.9 (1.5, 13.4) | <0.0001 |
| Confirmed TB       | 9                   | 35.1 (9.1, 64.2)     | 5.3 (0.9, 13.5) | 0.0056  |
| Unconfirmed TB     | 41                  | 14.0 (6.9, 35.1)     | 4.7 (2.1, 13.1) | 0.0015  |

IQRs are given in brackets. *Median 5.5 months (IQR, 3.1, 5.7) between TBTx initiation and post-TBTx initiation. *Paired Wilcoxon signed-rank test (two-sided).

1 contribute to their diagnostic performance. However, other abundant Mtb-derived factors identified as potential targets by a literature search did not exhibit similar capacity to distinguish TB and non-TB cases, including the membrane protein Ag85b, a mycoloyl transferase required for efficient biosynthesis of the Mtb cell wall. In this analysis, it is not clear whether the secreted Mtb proteins analysed in this study (CFP10, ESAT6, MPT51 and MPT64) form stable membrane interactions that would permit their detection on serum EVs.

NEI analysis of an NHP model of LTBI and TB indicated that serum EV expression of both LAM and LprG was required to distinguish TB from LTBI. LAM is a virulence factor that is expressed on the Mtb cell wall, where it can bind to the macrophage mannose receptor to facilitate cellular entry of Mtb bacilli into host phagocytes, inhibit phagosome-lysosome fusion and modulate the immune response to promote continued intracellular survival of Mtb required for TB development. LprG plays an essential role in the localization of LAM to the outer cell envelope of Mtb bacilli, and LprG null Mtb mutants exhibit reduced LAM surface expression and virulence, decreased Mtb entry into host macrophages, reduced biogenesis and/or integrity of the Mtb cell envelope, failure to inhibit phagosome-lysosome fusion and reduced intracellular replication rates. LprG is thus essential for LAM activity, as LprG deficiency attenuates Mtb virulence without altering LAM expression. LprG expression might thus be expected to be downregulated in NHPs with LTBI cases; however, our results suggest the opposite case: downregulation of LAM and upregulation of LprG expression on serum EVs from NHPs diagnosed with LTBI, whereas both markers were elevated on serum EVs from NHPs diagnosed with TB. Several mechanisms could explain this finding, including the downregulation of LAM expression or the inhibition of LprG activity to limit LAM transport, both of which would be expected to limit LAM expression on the Mtb membrane and, presumably, LAM expression of EVs secreted by Mtb-infected cells during LTBI. However, the mechanisms responsible for this differential expression and its functional significance are unclear and merit further study, including replication in larger, well-defined LTBI and TB cohorts with less diagnostic uncertainty.

NEI analysis of Mtb EVs exhibited good diagnostic sensitivity for both confirmed and unconfirmed TB in a diagnostically challenging population of children living with HIV, including children not clinically diagnosed during the parent study despite extensive TB work-up. Notably, the NEI assay identified children with HIV who are often missed by sputum-based assays and diagnosed by physician judgement and non-specific findings. NEI diagnostic sensitivity in this group is of particular relevance because mortality was more than five times higher in untreated children who were not diagnosed with TB during clinical evaluation than those who were diagnosed and treated at evaluation, in keeping with reported high mortality rates in children who do not receive TBTx due to missed diagnoses. Notably, serum from several children exhibited positive Mtb EV signal before their TB diagnosis by clinical findings, suggesting that serum Mtb EV signal has potential as a means for early TB diagnosis, which is of particular importance in this population since a third of the children identified in NEI analysis died at or shortly after their diagnosis by conventional means. Most PUSH cohort children had valid urine LAM assay results, but these had poor diagnostic sensitivity for confirmed and unconfirmed TB (37.5% and 5.8%) compared with NEI (90.1% and 72.5%), although both assays showed similar diagnostic specificity (83.3% and 81.8% for NEI and urine LAM) for children with no TB criteria. There are several possible explanations for these differences. First, blood is homoeostatic unlike urine and may thus be less subject to dilution and degradation effects that could influence urine LAM detection. Specifically, serum EV LAM should achieve a steady state that balances secretion and lysis, phagocytosis and secretion rates in a constant volume, whereas urine LAM degradation and dilution effects can be influenced by variable urination intervals and dilution or concentration effects. Second, soluble LAM and EV LAM degradation rates may differ. Finally, urine LAM tests detect soluble LAM whereas EV LAM NEIs detect EV-bound LAM, hence the two methods are not directly comparable, although this question could be addressed in future studies.

NEI Mtb EV signal markedly decreased following TBTx initiation for both confirmed and unconfirmed TB cases, in agreement with TB symptom improvement, suggesting that Mtb EV level might be useful as a surrogate for TBTx response. Similar decreases were also observed in a subset of children who met the criteria for unconfirmed TB when evaluated by their baseline data, but who were reclassified as unlikely TB due to improvement of their TB suggestive symptoms following ART initiation without TBTx initiation. NEI Mtb EV signal decreases in this context suggest that the children may have had nascent TB which was at least partially contained by improved immune function following ART initiation. Quantifiable serum biomarker results may also serve as a surrogate when evaluating TBTx response, particularly for extrapolmonary TB cases which are not amenable to repeat sampling. This is a critical TB management need and the monitoring ability of NEI should thus be evaluated in studies designed and powered to address this question.

NEI Mtb EV signal was also detected in several children diagnosed with unlikely TB who had one of two algorithmic features required for unconfirmed TB classification. Median serum Mtb EV signal did not markedly differ between these groups, suggesting that a substantial fraction of the unlikely TB cases may have had nascent TB that was not diagnosed within the study evaluation period. NEI signal was markedly lower in the unlikely TB group that lacked a single positive result for any of the criteria required for TB diagnosis, with only two children having an EV signal above the established threshold for TB diagnosis (83% specificity). However, all these children were at high risk for TB and thus do not represent a clear non-TB control population. Specificity estimates for this study were thus compromised by the absence of a large non-symptomatic population that could more confidently be characterized as non-TB cases.

This study has limitations. First, we employed cryopreserved repository samples collected in different studies, although our results indicate that short-term sample-handling differences should not affect target EV signal. Second, although all children enrolled in the PUSH cohort underwent extensive TB evaluation and had at least two microbial test results, it is possible that missing data could have affected case classifications made using the modified NIH TB classification criteria. Furthermore, not all the PUSH cohort children who received TBTx had equivalent longitudinal serum samples available for analysis, preventing a detailed treatment-response
Fig. 4 | Design and performance of a smartphone-based NEI point-of-care TB diagnosis approach. a–c, Schematic of a portable smartphone-based DFM device for NEI assay readout (a) and its dark-field condenser mask (b), and the effect of this mask on NEI AuNR signal (red) intensity of the targeted Mtb EVs from images (c) collected without (top) and with (bottom) the condenser mask. d, Schematic of smartphone app menu workflow. e, EV NEI signal from serum samples of children with TB (N = 15) and with no evidence of TB (N = 15). Data indicate mean ± s.e.m.; dashed line indicates the TB detection threshold for TB positive (red) or TB negative (grey) sample assignment; P values were determined by two-sided Mann-Whitney U test. f, Comparison of integrated EV LAM and LprG NEI signals obtained for the samples in e using a desktop DFM and the portable smartphone DFM device, indicating the linear regression line and squared Pearson correlation coefficient.

Table 4 | Performance metrics for the diagnosis of children with TB, for the three cohorts and for the pooled datasets

| Cohort | Performance metric | Confirmed TB | Unconfirmed TB | Unlikely TB with any criteria | No NIH criteria and non-TB control |
|--------|-------------------|--------------|---------------|-------------------------------|----------------------------------|
| NLH (20 children) | Fraction of children | 14/15 | - | - | 0/5 |
| Sensitivity, % | 93.3 (68.1, 99.8) | - | - | - |
| Specificity, % | - | - | - | 100.0 (47.8, 100.0) |
| DR (43 children) | Fraction of children | 15/17 | 9/11 | 1/3 | 1/12 |
| Sensitivity, % | 88.2 (63.6, 98.5) | 81.8 (48.2, 97.7) | 33.3 (0.8, 90.6) | - |
| Specificity, % | - | - | 66.7 (9.4, 99.2) | 91.7 (61.5, 99.8) |
| PUSH (147 children) | Fraction of children | 10/12 | 48/66 | 31/57 | 2/12 |
| Sensitivity, % | 83.3 (51.6, 97.9) | 72.7 (60.4, 83.0) | 54.4 (40.7, 67.6) | - |
| Specificity, % | - | - | 45.6 (32.4, 59.3) | 83.3 (51.6, 97.9) |
| Pooled (210 children) | Fraction of children | 39/44 | 57/77 | 32/60 | 3/29 |
| Sensitivity, % | 88.6 (75.4, 96.2) | 74.0 (62.8, 83.4) | 53.3 (40.0, 66.3) | - |
| Specificity, % | - | - | 46.7 (33.7, 60.0) | 89.7 (72.6, 97.8) |

The fraction of children refers to the fractions that were positive according to Mtb EV. Confidence intervals (95%) for the sensitivities and specificities are given in brackets.
study following TBTx initiation. Larger prospective studies are warranted to confirm and extend the results in children and adults with and without HIV, with pulmonary and extrapulmonary TB disease, and with appropriate negative controls. However, despite the cohort-size limitations, TB diagnosis rates were similar in all three paediatric cohorts (Table 4), suggesting that HIV infection and severe immunosuppression did not degrade NEI diagnostic performance. NEI diagnosis rates and mean signal also did not markedly differ among children with and without HIV who had pulmonary or extrapulmonary TB. Thus, serum Mtb EV detection may be particularly valuable for TB diagnosis in young children, people with HIV and individuals with extrapulmonary TB, who can be difficult to diagnose using conventional methods and samples. Third, both LAM and its carrier protein LprG are required for normal cell-wall biogenesis in mycobacteria, but at least one antibody is reported to recognize a LAM epitope that is specific for Mtb complex species. No studies appear to have analysed the potential of LprG for TB diagnosis, but although several non-tuberculous mycobacteria (NTM) express LprG orthologues, those from the two NTM species responsible for most NTM respiratory infections—M. avium and M. intracellulare—have limited sequence identity with Mtb LprG.

Finally, we analysed LAM and LprG expression on EVs, but BMVs could also serve as potential biomarkers for TB diagnosis. A similar NEI assay to detect these BMVs directly from serum would require validation of a capture antibody that permits direct isolation of BMVs present in serum, and this needs to be addressed in future studies designed for this purpose.

Our current assay is suitable for use at sites with minimal laboratory equipment, but still requires blood collection and separation, and multiple pipetting and wash steps before being read via a microscope or a smartphone device. Efforts are ongoing to refine the assay for use in point-of-care settings that lack the resources to perform it, including adapting the assay to a lateral-flow format that could use fingerstick blood samples and be read by either a modified version of our existing smartphone device, or perhaps by direct inspection. This would also allow prospective real-time evaluation of an inexpensive portable device suitable for use in resource-limited settings, which could enhance testing options in regions with high endemic TB rates that account for 95% of new TB cases and mortality.

**Methods**

**EV isolation and characterization.** Mtb and cell culture and BMVs isolation are described in a previous study as a protocol for the isolation of mouse antibody specific for human CD81 EVs. Concentrated serum aliquots (1 ml) that were processed for EV isolation were rapidly thawed in a 37°C water bath, vortexed for 3 s, then 300 µl aliquots were transferred to 2 ml centrifugation tubes, mixed with 1.2 ml PBS, and then centrifuged at 1,200 r.p.m. for 15 min at 4°C to pellet large particulates and debris. Supernatants were centrifuged at 10,000 r.p.m. and 4°C for 45 min, and then at 110,000 g and 4°C for 90 min. Resulting EV pellets were suspended in 1 ml PBS, centrifuged at 110,000 g and 4°C for 3 h, and then suspended in 50 µl PBS and characterized by bicinchoninic acid assay and NanoSight nanoparticle tracking analysis (Malvern Panalytical; 5 µg ml⁻¹ with 5 replicates) to determine protein content and EV concentration and size distribution. Cell culture supernatants corresponding to the same number of H37Rv-infected macrophages and uninfected macrophages were concentrated for EV analysis by centrifugation at 4°C and 3,000 r.p.m. for 15 min using 30 ml 10 kDa Cospolymer Styrene ultratitration tubes (Millipore Sigma).

This procedure concentrated 200 ml of culture supernatant to 100 µl. Concentrated samples were processed for EV isolation and resulting EV suspensions were quantified by NanoSight nanoparticle tracking analysis at the same dilution factor to compare the number of EVs secreted by H37Rv-infected macrophages and uninfected macrophages. EV concentrations obtained represented the area under the curve of the number of particles detected over the entire analysed size range, as reported by the NanoSight NS 300 instrument. For transmission electron microscopy analyses, carbon-coated Cu grids (400 mesh) were incubated for 30 s at room temperature with 5 µL EV aliquots containing 1 mg ml⁻¹ protein, blotted with filter paper to remove excess liquid, incubated with 2 µl 1% osmium tetroxide for 2 min at room temperature to stain membranes, blotted to remove this solution, and imaged at the indicated magnification using a Tecnai F30 microscope (FEI Company) with an accelerating voltage of 200 kV. All EV samples isolated in this study were aliquoted and stored at −80°C until use, rapidly thawed in a 37°C water bath and immediately processed for EV assay or other analysis.

**EV-ELISA analyses.** Standard EV capture plates used for EV-ELISA were generated by adding 100 µl aliquots of mouse antibody specific for human CD81 (BioLegend) to each well of 96-well microtitre plates (5 µg antibody per ml in PBS) and incubating these plates for 16 h at room temperature. Wells were then washed 3x with 260 µl PBS (30 s per wash), then blocked by incubation with 250 µl blocking buffer (1% w/v BSA in PBS) for 2 h at 37°C, and again washed 3x with PBS. To test optimal EV capture conditions for NEI, wells were alternately incubated with 100 µl aliquots of mouse antibodies (5 µg ml⁻¹) specific for EV surface markers including CD9, CD63, CD81 and different clones of anti-LAM/LprG antibodies (1 µg ml⁻¹).

For EV-ELISAs, each wash was incubated overnight at 4°C with 100 µl dilutions of either EVs or serum, washed 3x with PBS and then incubated with 100 µl PBS containing 1 µg ml⁻¹ of the indicated detection antibodies, including monoclonal antibodies specific for Mtb LAM, LprG, LpH1, MPT64, CFP10, ESA16, MPT31, MPT32 and KatG and polyclonal antibodies specific for Mtb Ag85b and Ag85c, for 1 h at 37°C, washed 3x with PBS, then incubated with Mtb Ag85b containing 0.5 µg ml⁻¹ of the indicated detection antibody and incubated at room temperature with 5 µl TMB (Sigma) for 10 min at room temperature and then mixed with 50 µl of 2 M H₂SO₄ to stop the reaction, after which target EV for each well were read using a microplate reader (Tecan, Ultra) to measure optical density (OD)₅₄₀ values after subtracting OD₅₄₀ signal.

**NHP Mtb infection and sample collection.** No live Indian-origin rhesus macaques were used in this study. Cryopreserved NHP plasma analysed in this study was obtained from archived material from NHPs infected with Mtb in previously reported studies (approved by the respective Institutional Animal Care commission as well as the Institutional Biosafety Committee and the National Primate Research Center and Southwest National Primate Research Center). Briefly, specific-pathogen-free, retrovirus-free, mycobacteria-naive, adult rhesus macaques were assigned to three experimental groups that received different Mtb exposures (none, low and high). Samples for the negative control (Mtb naïve) cohort were obtained from four uninfected rhesus macaques that were not exposed to Mtb during the study period. Samples for the LTBI cohort were obtained from five rhesus macaques that were subjected to a high-dose Mtb aerosol exposure event (~200 c.f.u. of Mtb CDC1551), which developed active TB disease characterized by weight loss, pyrexia, elevated serum C-reactive protein levels, elevated chest radiograph scores consistent with TB, detectable Mtb c.f.u. levels in bronchoalveolar lavage fluid, higher lung bacterial burden and associated lung pathology at the study endpoint. Lung tissue collected at the study endpoint was randomly sampled by pathologists blinded to animal treatment using a grid, as described previously.

**NEI analyses.** EV capture slides used for NEI analyses were generated by adding 100 µl aliquots of mouse antibody specific for human CD81 (5 µg ml⁻¹) to each position of a 144-well mask affixed to a microscope slide and incubating these slides for 16 h at 4°C. All incubation steps in this analysis were performed in a humidified chamber to reduce evaporation effects. Following capture antibody binding, slides were washed 3x with PBS, blocked with 1 µl per well SuperBlock (PBS) blocking buffer (Cytiva) for 1 h at 4°C and then washed 3x with PBS before incubation with 1 µl of serum or isolated EV samples. Cryopreserved serum aliquots were stored at −80°C until use, rapidly thawed in a 37°C water bath and centrifuged at 10,000 g for 20 min to remove large debris. Serum or EV samples were incubated on EV capture slides for 16 h at 4°C, washed 3x with PBS, hybridized for 1 h at 37°C with 1 µl of the specified biotinylated detection antibody (100 µg ml⁻¹), washed 3x with PBS and then incubated with 1 µl of neutravidin-gold conjugate (1 µg ml⁻¹) at room temperature and then mixed with 1 µl of TMB reagent for 10 min at room temperature to generate a colour reaction, and subsequently stopped with 1 µl of 2 M H₂SO₄ to stop the reaction. Following the linearity of the NEI assay response and calculating its estimated limit of detection, an EV standard curve was generated by spiking healthy human serum with an EV fraction generated by ultracentrifugation of pooled supernatants of Mtb-infected macrophage cultures to produce a high concentration EV standard (60 ng ml⁻¹) that was then used to generate 40, 20, 10 and 5 ng ml⁻¹ EV dilutions using the same healthy human serum sample. A linear regression model with a single explanatory variable was used to calculate the slope and correlation coefficient (R²) of the NEI curves generated before and after image correction of the same samples. Coefficients of variation were calculated from triplicates of each concentration standard to determine the range of c.v. and averaged to determine the mean c.v. of the assay measurements. We determined the sensitivity of the assay to be the concentration of the lowest EV serial dilution.
Children were post-hoc categorized as confirmed TB (positive Mtb culture or Xpert result from a respiratory or stool sample), unconfirmed TB (≥2 of the following criteria: TB-associated symptoms, TB-consistent abnormal CXR, positive TST result (induration ≥ 5 mm) or known TB exposure, and/or TBTx response) or unlikely TB (not meeting other criteria) on the basis of NIH international consensus case-definition criteria for paediatric TB (Supplementary Table 6) 34. TB symptoms were defined as a cough lasting >2 weeks, weight loss/failure to thrive, fever lasting >1 week, and/or lethargy lasting >1 week. For children who died during the study, an expert panel reviewed cases and came to a consensus regarding whether death was considered to be likely, possibly, or unlikely related to TB. Death considered likely or possibly related to TB was employed as an additional criterion for unconfirmed TB classification (Supplementary Table 7). For the assay evaluation, children with unconfirmed TB were further stratified by whether or not they received a clinical TB diagnosis and started TBTx during the study. Children with TB were further stratified by the presence or absence of features employed for unconfirmed TB diagnosis. Children with cryopreserved serum available within 2 weeks of TB diagnosis, including within 2 weeks of TBTx initiation, were evaluated to estimate TB diagnostic performance of Mtb 68 NEI results. Children who initiated TBTx with samples within 2 weeks of TBTx initiation and at least one additional subsequent sample were eligible for the treatment-response analyses.

Participant characteristics were summarized by frequency and proportion for categorical variables, and by median and interquartile range (IQR) for continuous variables (Table 1). NEI diagnostic performance for TB diagnosis was estimated using a confidence interval (CI) and a binomial distribution assumption. Diagnostic performance was based on NIH paediatric TB clinical-case definitions (Table 2). Many children in the unlikely TB category either had CXR suggestive of TB or had TB symptoms (but not meeting unconfirmed TB criteria); therefore, we further stratified the unlikely TB category by the presence or absence of abnormal CXR and TB symptoms to identify a potentially more appropriate negative control group.

For the assay evaluation, children with unconfirmed TB were further stratified by whether or not they received a clinical TB diagnosis and started TBTx during the study. Children with TB were further stratified by the presence or absence of features employed for unconfirmed TB diagnosis. Children with cryopreserved serum available within 2 weeks of TB diagnosis, including within 2 weeks of TBTx initiation, were evaluated to estimate TB diagnostic performance of Mtb 68 NEI results. Children who initiated TBTx with samples within 2 weeks of TBTx initiation and at least one additional subsequent sample were eligible for the treatment-response analyses.

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Participant characteristics were summarized by frequency and proportion for categorical variables, and by median and interquartile range (IQR) for continuous variables (Table 1). NEI diagnostic performance for TB diagnosis was estimated using a confidence interval (CI) and a binomial distribution assumption. Diagnostic performance was based on NIH paediatric TB clinical-case definitions (Table 2). Many children in the unlikely TB category either had CXR suggestive of TB or had TB symptoms (but not meeting unconfirmed TB criteria); therefore, we further stratified the unlikely TB category by the presence or absence of abnormal CXR and TB symptoms to identify a potentially more appropriate negative control group.

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was performed using a two-sided $\alpha$ of 0.05 to evaluate significant differences. Both characteristics (LAM and LprG) were entered into this logistic regression model without other covariates. Data are presented as mean ± s.d. unless noted otherwise. The investigators were not blinded to the samples analysed in the training cohort, cell culture studies and the NHP cohort, but were blinded to the identification of the DR and PUSH cohort samples. No data points were excluded from the analyses.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data supporting the results in this study are available within the paper and its Supplementary Information. Source data are provided with this paper. The raw and analysed datasets generated during the study are too large to be publicly shared, yet they are available for research purposes from the corresponding author on request. De-identified patient data are available from the corresponding author, subject to IRB approval.

**Code availability**

Source code used in this work is available for non-commercial purposes from the corresponding author on request.

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Author contributions
W.Z. and T.Y.H. conceived the study, generated the hypotheses and designed the experiments. W.Z., F.Z., J.F., S.W., L.Z., D.B., L.Y., X.X., B.N. and C.L. performed all the experiments and analysed the data. W.Z., C.J.L. and T.Y.H. wrote the manuscript. D.K.K., D.K.S., M.K. and A.J.R performed the P3 infection study. D.K. and D.K.S. conceived and designed the non-human primate study. B.S., S.L. and R.L. designed the portable device and the software for image processing. W.Z., C.J.L., S.M.L., J.S., J.N.E., Z.H. and T.Y.H. performed clinical experiments, and analysed and interpreted the clinical data. G.J.-S. and D.C.W. designed the parent PUSH trial study and obtained grant funding. I.N., L.M.C., D.C.W. and G.J.-S. developed the PUSH protocol and study. I.N., E.M.-O., D.C.W., L.M.C. and G.J.-S. assessed initial clinical data. J.S., I.N.N., L.M.C. and S.M.L. and G.J.-S. analysed the primary trial data and/or subsequent TB diagnostic and clinical data. J.O., C.Y., C.D.M., E.P.-T., E.A.G., D.T.N., N.N., H.P. and C.M.B. contributed to the clinical sample protocol for this study. All authors contributed to the writing of the manuscript and to its revision.

Competing interests
T.Y.H. and W.Z. have a provisional patent (‘Method of detecting TB in blood’) submitted through Tulane University. The rest of the authors declare no competing interests.

Additional information
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- Data collection: NIS-Elements AR 5.0
- Data analysis: GraphPad Prism 9.0, Image J 1.53, Microsoft Office 365 Excel, Matlab R2020a, SAS OnDemand version 9.4, Stata 17.0. Source code used in this work is available for non-commercial purposes from the corresponding author on request.

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The data supporting the results in this study are available within the paper and its Supplementary Information. Source data is provided with this paper. The raw and analysed datasets generated during the study are too large to be publicly shared, yet they are available for research purposes from the corresponding author on reasonable request. De-identified patient data are available from the corresponding author, subject to IRB approval.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to predetermine sample sizes, which for each retrospective cohort were determined by the number of patients with sample available. |
| Data exclusions | No data were excluded from the analyses. |
| Replication | Information on experimental replication is provided in Methods and in figure legends. Replicate numbers were chosen on the basis of the minimum number required to detect differences between groups in cell-culture experiments, owing to constraints involved with the handling of increasing numbers of cells infected with Mycobacterium tuberculosis. Studies performed with archived samples from retrospective non-human-primate and patient cohorts employed all available samples, which were analysed using triplicate technical replicates. |
| Randomization | The cell samples and animals used in this study were randomly allocated to each experimental group prior to treatment. All individuals in the analysed study cohorts were categorized using predefined classification criteria, and all available samples from these individuals were analysed as described. |
| Blinding | The investigators were not blinded for the samples analysed in the training cohort, cell-culture studies and NHP cohort, but were blinded to the identification of the DR and PUSH cohort samples. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
| --- | --- |
| n/a | Involved in the study |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☒ | Palaeontology and archaeology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |
| ☑ | Clinical data |
| ☑ | Dual use research of concern |
| ☑ | Involved in the study |
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Antibodies

Mouse antibodies specific for human CD81 (Clone 5A6, Cat#: 349502) were purchased from BioLegend, mouse antibodies for human CD9 (Clone ALB 6, Cat#: sc-59140) and CD63 (Clone MX-49.129.5, Cat#: sc-5275) are from Santa Cruz Biotechnology, Inc., biotinylated mouse anti-human CD81 (Clone 5A6, Cat#: 349502), anti-CD9 (Clone H9a, Cat#: 312102) and anti-CD63 (Clone H5C6, Cat#: 353018) were purchased from BioLegend, USA.

Anti-LAM antibodies (Clone CS35, Cat#: NR-13811 and Clone CS40, Cat#: NR-13812) and anti-LprG antibodies (Clone A Cat#: NR-13806 and Clone B, Cat#: NR-51133) were obtained from BEI Resources. Anti-LAM antibody (A194-01, Cat#: HPAB-M0560-YC) was purchased from Creative Biolabs, USA.

Specific antibodies for Mtb LpqH (Clone IT54, Cat#: NR-13792), MPT64 (Clone B, Cat#: NR-50704), MPT51 (Clone B, Cat#: NR-50106), MPT32 (Rv1860, Cat#: NR-13817), Ag85c (Clone , Cat#: NR-13800), Ag85b (Clone , Cat#: NR-19362) and KatG (Clone B, Cat#: NR-50101) were obtained from BEI Resources.

HRP-labeled goat anti-mouse (Cat#: 115-035-166)/human (Cat#: 109-035-170)/rabbit (111-035-144) IgG are from Jackson ImmunoResearch Laboratories, USA.

Validation

All antibodies were obtained from commercial manufacturers, and employed without further validation. Validation statements are provided on the manufacturer’s websites.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) THP-1 monocytes were purchased from the American Type Culture Collection (ATCC; Manassas, VA).

Authentication Cell lines were obtained from the ATCC and used without further authentication.

Mycoplasma contamination All cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register) No misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals Specific-pathogen-free, retrovirus-free, and mycobacteria-naive adult rhesus macaques were assigned to three experimental groups that received different Mtb exposures.

Wild animals The study did not involve wild animals.

Field-collected samples The study did not involve samples collected from the field.

Ethics oversight No live rhesus macaques were used in this study. The cryopreserved NHP plasma was archived material obtained from NHPs infected with Mtb in previously reported studies (approved by the respective Institutional Animal Care and Use Committee as well as by the Institutional Biosafety Committee at Tulane National Primate Research Center and Southwest National Primate Research Center). Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics This study analysed archived samples and data collected in three independent studies.

The Pediatric Urgent vs. post-Stabilization Highly active anti-retroviral therapy initiation (PUSH) study was a randomized controlled trial (NCT02063880) performed in Kenya that evaluated whether urgent (<48 hours) vs. post-stabilization anti-retroviral therapy (7–14 days) improved survival in hospitalized HIV-infected children aged <12 years.

National Lung Hospital (NLH) cohort: Specimens and associated clinical data were collected 20 children aged ≤17 who consecutively visited the NLH in Ha Noi, Vietnam for clinical assessment and medical evaluation, using a protocol approved by the NLH IRB, which included an amendment approving the analyses performed in this study.

Dominican Republic (DR) cohort: Specimens and associated clinical data were collected from 48 children aged ≤18 years, who were enrolled and consecutively followed at the Robert Reid Children’s Hospital or the Hugo Mendoza Children’s Hospital.

Recruitment Recruitment details for these independent studies are briefly described in Methods, and fully described in publications describing these studies (cited in Methods).

Ethics oversight Permission to analyse the NLH cohort samples and clinical data at Tulane University was granted by the NLH Institutional Review Board.

Permission to analyse the PUSH cohort samples and clinical data at Tulane was granted by the IRB of Kenyatta National Hospital and the University of Nairobi.

Permission to analyse the DR cohort samples and clinical data at Tulane was granted by the IRB of the University Dominicana O&M.

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Clinical data

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All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration This study did not involve a clinical trial. It used archived samples and clinical data from a completed clinical trial (PUSH).

Study protocol —

Data collection —

Outcomes —