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Estudio de rutas de vacunación en
modelo murino para el uso
profiláctico o terapéutico de
vacunas atenuadas de
tuberculosis

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Tesis Doctoral

ESTUDIO DE RUTAS DE VACUNACIÓN EN MODELO MURINO PARA EL USO PROFILÁCTICO O TERAPÉUTICO DE VACUNAS ATENUADAS DE TUBERCULOSIS

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EXPONEN:

Que la Tesis Doctoral se ha realizado bajo su dirección y se corresponde con el proyecto de tesis aprobado en su momento por la Comisión de Doctorado de la Universidad de Zaragoza, no habiéndose producido ninguna variación.

Que dicha Tesis Doctoral reúne los requisitos necesarios para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizan su presentación para que pueda ser juzgado por el Tribunal correspondiente.

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TESIS DOCTORAL COMO COMPENDIO DE PUBLICACIONES

Esta tesis es un compendio de trabajos previamente publicados. A continuación se relacionan las referencias completas de los 4 artículos que constituyen el cuerpo de la tesis.

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A mis padres (aita siempre en la mente)
A mis hijos
A Lourdes

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RESUMEN

La tuberculosis (TB) es una de las enfermedades infecciosas que produce mayor mortalidad en el mundo. El agente causante es *Mycobacterium tuberculosis* que convive estrechamente con el hombre desde sus inicios como *Homo sapiens*. De momento, la única vacuna disponible es BCG (derivada de *Mycobacterium bovis*) pero esta no es efectiva contra la TB pulmonar en adultos, que es además la forma responsable de la transmisión de la enfermedad. Actualmente muchos equipos de investigación están trabajando en el desarrollo de nuevas vacunas y tratamientos eficaces, siendo MTBVAC la única vacuna candidata basada en la atenuación del agente patógeno causante de la TB humana. La eficacia de esta vacuna está siendo actualmente estudiada en ensayos clínicos en Fase III de eficacia. Previo a la fase clínica todos los estudios conllevan una fase de estudios preclínicos *in vitro* y después en animales de experimentación. El modelo murino es el modelo de elección en animales de experimentación, principalmente por su facilidad de manejo, economía de estabulado y disponibilidad de reactivos específicos de ratón para estudios inmunológicos. En esta tesis relacionamos distintos modelos de experimentación en ratones para lo cual hemos seleccionado 4 estudios de investigación basados en las vacunas vivas atenuadas MTBVAC y BCG.

En el primer estudio exploramos la eficacia de MTBVAC en el tratamiento de cáncer vesical, obteniendo resultados significativamente mejores que con el actual tratamiento con BCG.

En el segundo estudio utilizamos la vía canónica de vacunación subcutánea, y la vía sistémica intraperitoneal, para comparar la protección y atenuación de 3 variantes de MTBVAC obtenidas de los 3 principales “linajes modernos” de transmisión en humanos de *M. tuberculosis*. Los resultados mostraron que los tres linajes conferían una protección similar y una atenuación superior para MTBVAC procedente del linaje 4, el más común en Europa, África y América.

En el tercer estudio valoramos una pauta de vacunación por vía pulmonar (intranasal) con MTBVAC inactivada, analizando su potencial de refuerzo inmunitario frente a la vacunación previa con BCG. Los resultados confirman que esta pauta de

refuerzo confiere una mayor producción de anticuerpos protectores en la mucosa pulmonar contra *M. tuberculosis*.

Finalmente, en el cuarto estudio exploramos la utilidad de BCG y MTBVAC como terapia contra el asma, de nuevo en modelo murino. Los resultados confirman la reversión de la hiperreactividad en las vías altas respiratorias de ratón por la instilación de micobacterias vivas atenuadas BCG o MTBVAC.

ABSTRACT

Tuberculosis is one of the infectious diseases that produces the highest mortality in the world. The causative agent is the bacterium *Mycobacterium tuberculosis*, which has lived closely with man since his beginnings as *Homo sapiens*. At the moment, the only vaccine available for its control is BCG (derived from *Mycobacterium bovis*), but this is not effective against pulmonary tuberculosis in adults, which is also the form responsible for dissemination. Currently many research teams are working on the development of new vaccines and effective treatments against tuberculosis. Among the candidate vaccines we have MTBVAC, which is the only attenuated vaccine that is being tested in Clinical Phase III. Prior to the clinical phase, all studies involve a phase of preclinical studies *in vitro* and in experimental animals. Mice are the experimental animals of choice, primarily because of their ease of handling, economy of housing, and availability of mouse-specific reagents. In this thesis, the experimental models in mice applied in 4 different research studies based on the tuberculosis vaccine candidate MTBVAC compared to BCG.

In the first study, the efficacy of the MTBVAC in the treatment of a type of bladder cancer was explored, obtaining significantly better results than with the current treatment with BCG.

In the second study, we compared the attenuation and protection of 3 MTBVAC variants obtained from the 3 main *M. tuberculosis* pathogenic lineages and the result gives us a similar protection in the three lineages and an improved attenuation for the MTBVAC vaccine from the most common lineage 4.

In the third study, we evaluated a pulmonary (intranasal) vaccination schedule with inactivated MTBVAC vaccine, analyzing its potential for immune reinforcement compared to simple BCG vaccination. The results confirm that this pattern confers a higher presence of protective antibodies in the pulmonary mucosa.

Finally, in the fourth study, the usefulness of BCG and MTBVAC vaccines as therapy against asthma was explored, again in a murine model. The results confirm the reversal of hyperresponsiveness in mouse upper airways by instillation of both live attenuated mycobacteria.

ABREVIATURAS

ADC	Albúmina, dextrosa y catalasa
ADN	Ácido desoxirribonucleico
BAL	Lavado bronco alveolar
BCG	Bacilo de Calmette-Guérin
COVID-19	Enfermedad producida por el coronavirus Sars-Cov-2
DEX	Dexametasona
ESAT-6	Antígeno de secreción temprana de 6KDa
<i>fadD26</i>	Gen de virulencia de micobacterias <i>fadD26</i>
HDM	Antígeno del ácaro del polvo doméstico
HK	Inactivada por calor
MTBC	Complejo <i>Mycobacterium tuberculosis</i>
MTBVAC	Candidata a vacuna de <i>Mycobacterium tuberculosis</i>
OMS	Organización Mundial de la Salud
OVA	Ovoalbúmina
PAS	Periodic Acid-Schiff
PBS	Solución tampón fosfato
PDIM	Dimicoserosato tiocerol
PET/CT	Tomografía por emisión de positrones
<i>phoP</i>	Gen de virulencia de micobacterias <i>phoP</i>
PNH	Primates no humanos
RD	Regiones diferenciales del genoma
RD1	Región diferencial 1
SARS-CoV-2	Coronavirus 2 del Síndrome Agudo Respiratorio
SCID	Inmuno deficiencia severa combinada
SIV	Virus de la inmunodeficiencia del simio
SNPs	Polimorfismo de un nuceótido simple
SPF	Libre de patógenos específicos
TB	Tuberculosis
UFC	Unidades formadoras de colonias
VIH	Virus de la inmunodeficiencia humana

INTRODUCCIÓN Y OBJETIVOS

INTRODUCCIÓN

TUBERCULOSIS

La tuberculosis (TB) acompaña al hombre desde la más remota antigüedad, esta enfermedad está producida por la bacteria *Mycobacterium tuberculosis*, también conocido como el bacilo de Koch. Esta bacteria es el patógeno microbiano responsable de la muerte de más personas a lo largo de la historia. Todavía hoy, la TB sigue siendo una lacra sanitaria a nivel mundial, y está clasificada como pandemia por la Organización Mundial de la Salud desde el año 1993.

A pesar del uso generalizado de BCG, única vacuna aceptada contra la TB, cepa atenuada de *Mycobacterium bovis*, la TB sigue siendo una de las principales enfermedades infecciosas, transmisible por vía aérea, que aqueja a la población mundial. BCG tiene el mérito de mantenerse en uso desde hace 100 años, pero a pesar de ello tenemos una necesidad urgente de mejorar la eficacia de esta vacuna ya que no es efectiva contra la TB respiratoria, la forma transmisible de la enfermedad. Las tasas de incidencia y mortalidad han ido disminuyendo lentamente desde principios del siglo XXI, a pesar de ello en 2020 la OMS notificó 10 millones de casos nuevos y más de 1,4 millones de muertes a nivel mundial [1]. Entre los agentes infecciosos la mortalidad por TB solo ha sido superada por la pandemia de COVID-19 causada por SARS-CoV-2 que causó 1,8 millones de muertes en 2020 [2]. Tanto la TB como la COVID-19 son enfermedades de transmisión respiratoria y se estima que, como resultado de la COVID-19, las muertes por TB podrían aumentar hasta en un 16% en los próximos cinco años [3]. En países desarrollados la TB afecta principalmente a personas de edad avanzada e inmunodeprimidos, mientras que en los entornos de bajos recursos cada año la TB se cobra principalmente la vida de los adultos jóvenes y de mediana edad, el intervalo de edad más productivo de la sociedad. Hay que destacar también que entre los 10 millones de nuevos infectados anuales aproximadamente medio millón de casos son resistentes al tratamiento de uno o varios antibióticos.

La TB humana está causada principalmente por la infección de *M. tuberculosis*, aunque también puede ser causada en menor medida por otras especies de lo que se denomina *Complejo Mycobacterium tuberculosis* (MTBC) que son un grupo de distintas especies de micobacterias, como *M. bovis* o *Mycobacterium caprae*, con un alto grado de

identidad genética (>99%) con *M. tuberculosis*, que producen patología en distintos tipos de animales. Cuando algunas de estas distintas especies animales conviven en estrecho contacto con el hombre, como ganado bovino y caprino, también son capaces de causar TB en humanos, aunque la transmisión en estos casos no ocurre normalmente por vía respiratoria.

La TB es una enfermedad que tiene consecuencias igualmente graves para los seres humanos y una amplia gama de especies animales, se debería considerar el problema desde el enfoque de "Una sola salud" (One Health). Sin embargo, todavía, los casos de TB en humanos y animales se tratan comúnmente como problemas separados.

La infección por *M. tuberculosis* puede producir diversas formas de enfermedad con cursos y sintomatología variables. Aunque principalmente es una enfermedad que afecta al aparato respiratorio, la bacteria puede colonizar y causar patología en casi todos los órganos y tejidos, incluidos el sistema nervioso central y los huesos. La tuberculosis puede cursar de forma aguda o primaria; de forma crónica con una lenta progresión; o la infección puede estar latente, en este último caso la infección inicial está controlada pero no se termina de eliminar totalmente el agente patógeno por el huésped y permanece inactivo o silente.

El problema de la infección latente tiene su importancia ya que la eliminación total del bacilo tuberculoso a través de mecanismos de inmunidad del huésped es extremadamente difícil, el bacilo suele replicar en el interior de los macrófagos y se puede mantener durante años inactivo o latente en una estructura anatómica característica de la enfermedad que se denomina granuloma tuberculoso. Se estima que aproximadamente en el 10% de las personas con infección latente, la enfermedad se reactiva debida normalmente a algún periodo de inmunosupresión, aunque a menudo se desconoce el desencadenante de la reactivación.

La forma más frecuente de la enfermedad es la TB pulmonar y normalmente es de curso crónico, se prolonga durante meses y los síntomas que presenta el paciente con TB activa son: tos persistente, fiebre alterna, fatiga, pérdida de peso, sudores nocturnos y tos con sangre. Los enfermos con TB activa son los responsables de la transmisión de la enfermedad ya que expulsan las bacterias al exterior a través de la tos y estas permanecen suspendidas en el aire y son a su vez respiradas por otros

individuos que serán contagiados. Normalmente el individuo que entra en contacto con la bacteria por su respiración controla la infección en un estadio inicial y este permanece asintomático. Se estima que un 20% de la población mundial ha estado en contacto con el bacilo tuberculoso, pero sólo entre 5-10% de las personas infectadas llegarán a padecer la enfermedad a lo largo de su vida.

La TB humana es una enfermedad social y está muy relacionada con la pobreza, sobre todo con el hacinamiento. Otras enfermedades con alteraciones del sistema inmunitario predisponen también a contraer la TB, como pueden ser la infección por VIH o la diabetes mellitus.

Tuberculosis a lo largo de la historia.

Aunque hay autores que consideran que el ancestro genético de *M. tuberculosis* estaba ya presente en África Oriental y coevolucionó con los primeros homínidos hace unos 3 millones de años [3]. Otros autores estiman que la historia de adaptación del bacilo con la humanidad es más reciente, surgiendo hace unos 70.000 años a la vez que *Homo sapiens* [4], y que desde entonces la bacteria se ha ido adaptando a su hospedador, compartiendo sus migraciones fuera de África y extendiéndose por los distintos continentes del planeta desde el Neolítico hasta la actualidad.

Para estudiar el origen, la coevolución, la dispersión y la diversificación genética del patógeno se han utilizado técnicas de secuenciación genómica masiva de distintos aislados clínicos procedentes de todos los continentes, que dan como resultado la identificación actual de seis linajes genéticos principales para la TB humana (Océano Índico, Asia Oriental, Asia Central, Europa, y dos linajes en África Occidental) siendo los linajes denominados “modernos” (L2, L3 y L4) los que con más frecuencia causan la enfermedad en humanos (Figura 1).

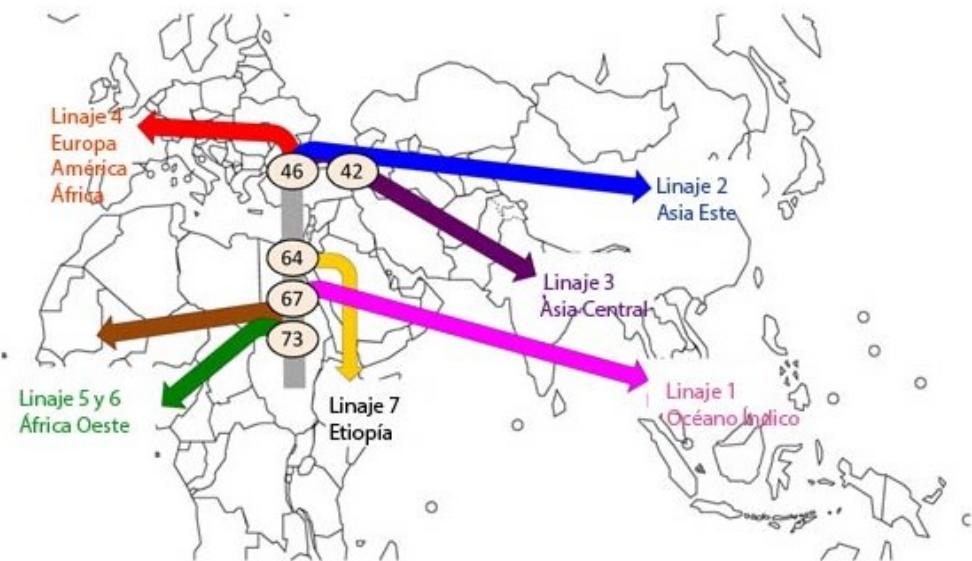


Figura 1. Linajes genéticos identificados en la tuberculosis humana, además de un séptimo linaje exclusivo del Cuerno de África. Adaptado de: Comas *et al.*-Microbe World [4].

En cualquier caso, la TB está documentada en el antiguo Egipto y en momias peruanas de 5.000 y 1.000 años, respectivamente. Las momias egipcias revelan deformidades esqueléticas típicas de esta patología, estos hallazgos han sido respaldados además por la detección de ADN micobacteriano en algunas de estas lesiones, así como en esqueletos de momias precolombinas [5][6].

En cuanto a documentación escrita, Hipócrates (460-370 a. C.) usó ya la palabra "tisis" (que significa descomposición) para describir la enfermedad [7]. En el siglo XVII, la TB se convirtió en epidemia en Europa, con una tasa de mortalidad del 20%, y persistió durante los siguientes 200 años. En 1679 Franciscus Sylvius de la Böe describió en su "Ópera Médica" los nódulos tumorales presentes en los pulmones de los muertos por TB, denominándolos "tubérculos" por su aspecto. A partir de la segunda mitad del siglo XVIII durante el proceso de la industrialización, las transformaciones socioeconómicas, tecnológicas y culturales desencadenan el desplazamiento de la población agraria hacia las ciudades, se deterioran las condiciones sanitarias y sociales, entramos en superpoblación con viviendas mal

ventiladas y desnutrición, todo ello contribuye a la propagación de la enfermedad en Europa y América del Norte [8].

Entre los múltiples tratamientos que a través de los siglos se han recomendado para luchar contra la TB, destacan medidas como dietas restringidas, purgas y sangrías, que además de no ser efectivas contra el bacilo, incluso perjudicaban la salud general de los pacientes, mermándoles de respuesta inmunitaria para luchar contra la infección.

La enfermedad será denominada definitivamente como “tuberculosis” en 1834 por el médico alemán Johann Lukas Schönlein quien la describe ya con ese nombre y como una entidad clínica específica.

El impulso definitivo en el avance sobre el conocimiento de la TB se da a partir de 1882 cuando Robert Koch presenta su libro "Die Aetiologie der Tuberkulose", donde describe ya al bacilo tuberculoso o “bacilo de Koch” como el agente etiológico en animales y humanos. Utiliza para sus estudios técnicas innovadoras de tinción y cultivo y describe además los famosos “postulados de Koch” demostrando la relación entre el agente causal y la enfermedad [9], y sentando así las bases de la Microbiología Médica moderna. Todo ello le hace merecedor del Premio Nobel de Medicina y Fisiología en 1905.

A partir de mediados del siglo XIX se va extendiendo una tendencia o corriente médica para tratar y curar la TB, su fundamento está basado en tratar al enfermo de TB en “sanatorios para tuberculosos” donde estos están aislados de su entorno familiar y social, expuestos al aire puro de montaña, con buena exposición solar, alimentación abundante, ejercicio moderado y control higiénico sanitario periódico. Los sanatorios antituberculosos proliferan y se extienden por Europa y Estados Unidos, funcionan hasta mediados del siglo XX. A partir de este momento y con el descubrimiento de los antibióticos los sanatorios son sustituidos por los tratamientos en el domicilio de cada paciente, la efectividad de estos fármacos antituberculosos y su ventaja económica respecto al internamiento produce el declive de los sanatorios antituberculosos [10].

Una vez Robert Koch identifica y aísla el bacilo responsable de la TB, se implementan investigaciones sobre la enfermedad más importante de la época y otro de los avances decisivos para combatir la enfermedad se produce con el desarrollo de una vacuna por parte del médico Albert Calmette y el veterinario Camille Guérin. La

vacuna se obtuvo tras atenuar el bacilo causante de la TB bovina, *M. bovis*, por pases sucesivos en medio de cultivo. Esta cepa atenuada se llamó *Bacille Calmette-Guérin* o BCG, se demostró que era efectiva contra la TB en diferentes modelos animales, y se administró por primera vez en humanos en 1921. Hoy en día BCG sigue siendo, cien años después, la única vacuna aceptada en la actualidad para prevenir la TB demostrando su eficacia contra las formas graves de TB como la TB meníngea y diseminada pero no contra la TB pulmonar [11].

Un hito destacable en la historia de la enfermedad, como vimos anteriormente, fue el descubrimiento de fármacos antibióticos, con ellos se dio un vuelco decisivo en el pronóstico de la TB. La enfermedad por fin tiene un tratamiento farmacológico efectivo. En 1944, la estreptomicina, aislada por Selman A. Waksman, fue el primer antibiótico efectivo contra *M. tuberculosis* [12]. Desde el primer momento se observó que la pauta de tratamiento debería ser prolongada para conseguir la curación, a pesar de que en algunos pacientes no mostraba eficacia. En los años siguientes, se descubrieron nuevos fármacos antituberculosos como el ácido p-aminosalicílico (1948), la isoniazida (1952), la pirazinamida (1954), la cicloserina (1955), el etambutol (1962) y la rifampicina (1963). Todos estos compuestos fueron progresivamente aplicados con más o menos éxito, ya sea como tratamientos simples o preferiblemente como tratamientos combinados ya que las monoterapias producen la aparición de cepas resistentes [13]

Desde mediados del siglo XIX la mortalidad de la TB va disminuyendo a pesar de mantener su alta prevalencia. Esta caída de letalidad es debida a distintos motivos: primero tenemos una mejora de las condiciones socioeconómicas, paralelamente mejora la aplicación de medidas de Salud Pública con en el aislamiento y seguimiento de los enfermos, se produce el desarrollo de las terapias por cirugía de resección pulmonar, y la utilización de la vacuna BCG, y finalmente los tratamientos antibióticos. Todas estas medidas dan como resultado una disminución pronunciada de la incidencia de TB. Se consiguió en gran medida disminuir la enfermedad, aunque nunca se logró erradicar (Figura 2).

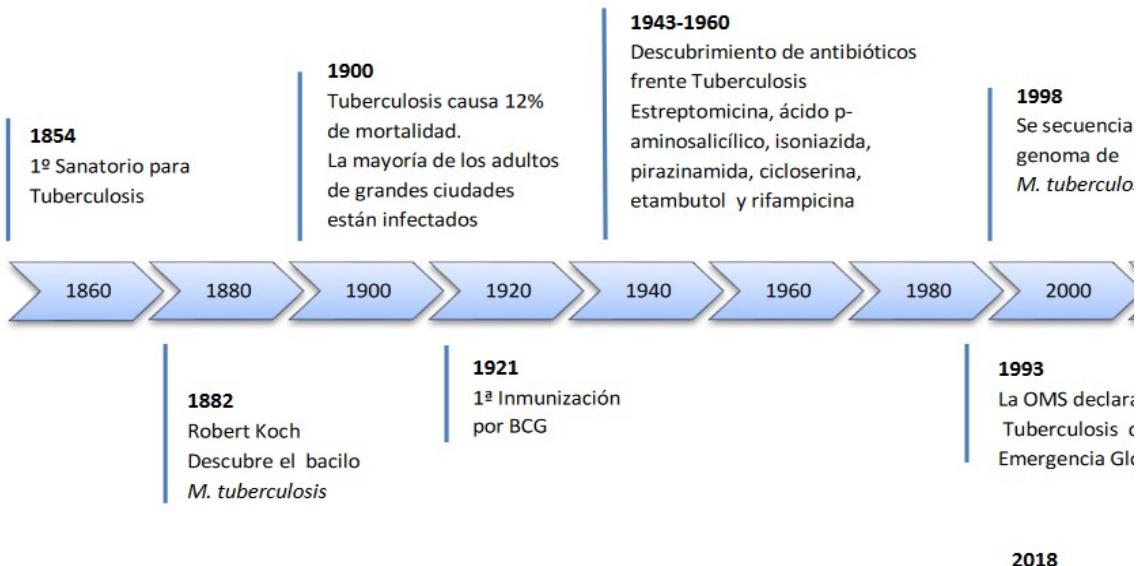


Figura 2. Cronología de la historia de la enfermedad de TB. Los eventos más relevantes en la historia reciente de la TB. Adaptado de [8].

A partir de los años 80, y coincidiendo con el comienzo de la pandemia del virus de la inmunodeficiencia humana (VIH), y con otros factores como el fin de los programas de control de la TB, la aparición de cepas resistentes a los medicamentos, y la protección variable de BCG contra la TB pulmonar, se produjo un importante rebrote de la enfermedad a nivel mundial, aumentando la incidencia global. La OMS declaró en 1993 a la TB como una “emergencia global” con el fin de llamar la atención sobre el alarmante estado en que se encontraba la enfermedad y para controlarla de forma efectiva [14]. Se puso en marcha un programa global para el desarrollo de nuevas estrategias con el objetivo de erradicar la TB para el horizonte del año 2050. Estas estrategias incluyen la investigación de nuevas vacunas y terapias más eficaces, así como nuevos métodos de diagnóstico más precisos y fiables [14]. La pandemia causada por SARS-CoV2 sufrida recientemente puede suponer un retraso en los esfuerzos por erradicar la TB ya que se produjo un desvío de la financiación en investigación, pero además también se relajaron las medidas de Salud Pública en relación a enfermedades que no son COVID-19.

DISEÑO DE UNA VACUNA CONTRA LA TUBERCULOSIS

BCG, la vacuna centenaria.

Actualmente la única vacuna autorizada en uso para prevenir la TB es BCG. Como hemos indicado, se trata de una vacuna viva atenuada que procede de una cepa de *M. bovis*, que fue aislada originalmente por Edmond Nocard en 1902 de una vaca que padecía mastitis tuberculosa. El bacilo bovino se fue atenuando (por Calmette y Guérain) mediante pases sucesivos en medio de cultivo. En concreto fueron necesarios 230 pases que se realizaron entre 1908 y 1919 en medio de patata saturado en bilis de ternera y con 5% de glicerina. La cepa atenuada resultante se seleccionó porque no reprodujo enfermedad al ser inoculada en cobayas, conejos, vacas o caballos. Una vez confirmada la seguridad de BCG, la vacuna se administró a humanos por primera vez en 1921 por vía oral [15] [16]. Desde entonces, BCG ha sido ampliamente utilizada, ya que confiere una fuerte protección contra las formas graves de la enfermedad en los niños (meningitis tuberculosa y tuberculosis miliar). Sin embargo, la eficacia de BCG contra la tuberculosis pulmonar en adultos, la forma habitual de transmisión de la enfermedad, es variable y limitada [17].

Hoy en día, BCG sigue siendo una de las vacunas más utilizadas, con más de la mitad de la población mundial vacunada y más de 100 millones de nuevos vacunados cada año. Se recomienda como parte de los programas de inmunización infantil en función de la tasa de incidencia de TB de cada país, entre los países de alta incidencia de todo el mundo la cobertura al nacimiento está cercana al 90% [18] (Figura 3). BCG se administra en una dosis única después del nacimiento por vía intradérmica, con una concentración de partículas vivas que varía de 50.000 a 3 millones por dosis, dependiendo de la formulación y de la cepa de BCG administrada [19].

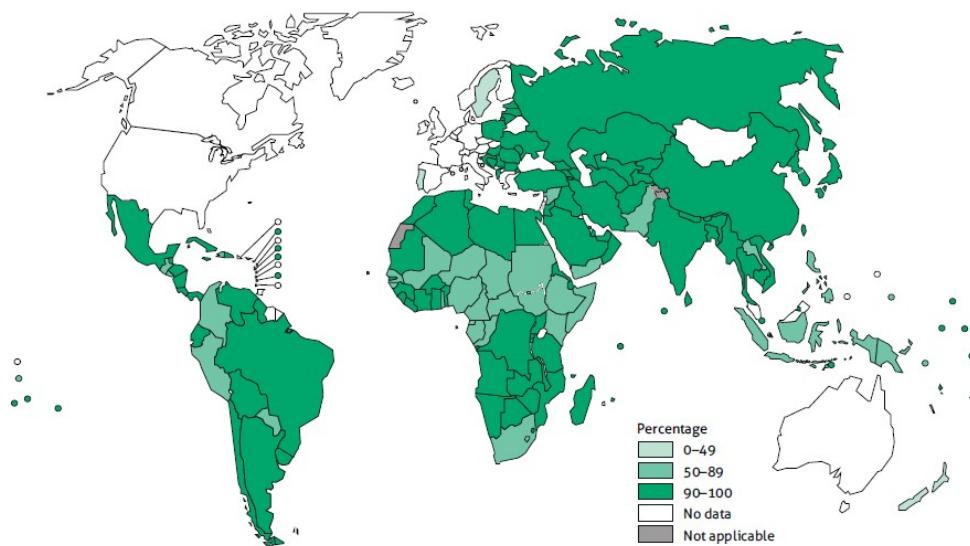


Figura 3. Distribución de la vacuna BCG en 2018. Adaptado de [1].

Después de la primera inmunización con BCG, la cepa original se distribuyó a diferentes laboratorios en todo el mundo, donde se mantuvo por subcultivo en condiciones no estandarizadas hasta la llegada del método de conservación por liofilización en la década de 1960. A consecuencia de esta replicación dispersa, aparecieron diferentes líneas de BCG, que al ser comparadas mostraron diferencias genotípicas e inmunogénicas. Las diferentes estirpes o cepas de BCG fueron denominadas con el nombre del laboratorio o país con el que estaban asociados [20].

Las cepas BCG más utilizadas actualmente en los programas internacionales de inmunización en todo el mundo son: BCG Pasteur 1173 P2; BCG Danish 1331; BCG Glaxo 1077; BCG Tokyo 172-1; BCG Russia-I; y BCG Moreau RDJ [21].

La comparación genética de las distintas estirpes reveló diferencias en las Regiones Diferenciales (RD), en mutaciones puntuales (SNPs), en secuencias de inserción y delecciones, así como en la presencia de duplicaciones en tandem. El estudio de estas diferencias ha permitido la construcción de una genealogía entre las variantes vacunales, que se dividen en cuatro grupos principales (según las duplicaciones en tandem DU) junto con otras variaciones en marcadores genéticos específicos y regiones RD) [15][22]. Por otro lado hay también variaciones en los principales factores de

virulencia micobacteriana entre las distintas estirpes geográficas, como los polimorfismos en el sistema de dos componentes PhoP/PhoR y la producción del lípido implicado en virulencia PDIM, que se ha perdido independientemente en las cepas de BCG Japón, BCG Moreau y BCG Glaxo [23][24] (Figura 4).

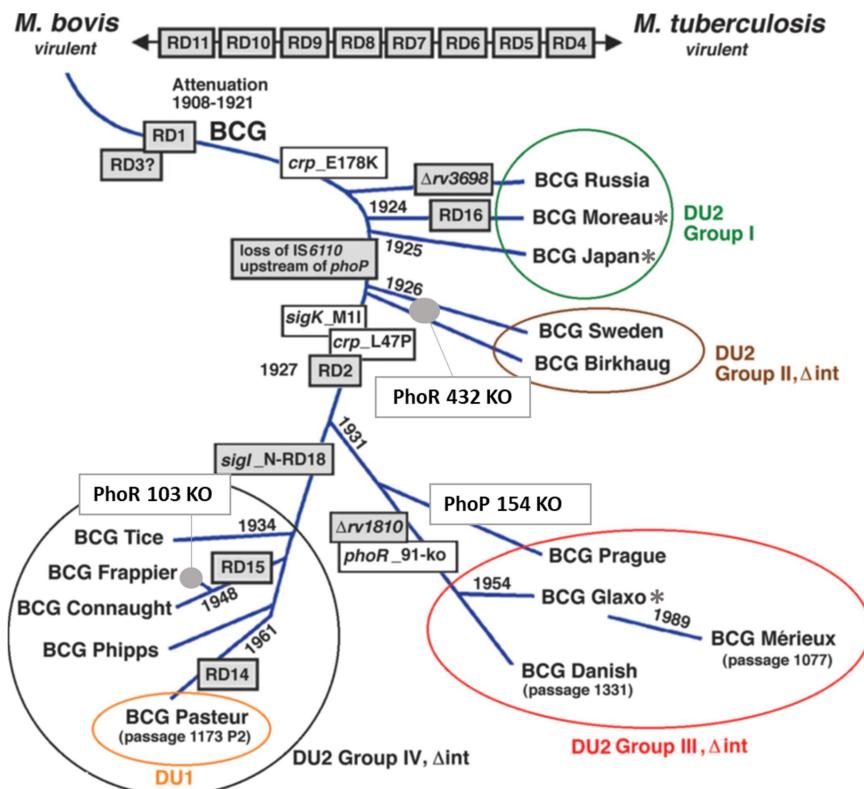


Figura 4. Filogenia entre estirpes de las diferentes cepas de BCG. Distribución de BCG según la duplicación en tandem DU2 en cuatro grupos diferentes. Se representan RD, SNP y polimorfismos en el sistema de dos componentes PhoP/PhoR. * indica deficiencia de PDIM. Adaptado de [14][25].

Las variaciones genéticas y fenotípicas entre las distintas cepas de BCG pueden producir diferencias en la atenuación y la reactogenicidad, aunque no se ha encontrado correlación con la eficacia protectora [26].

Los estudios comparativos entre el genoma de *M. bovis* y BCG revelaron que la causa principal de la atenuación sobre BCG es la pérdida de la región RD1 [27]. Esta región está ausente en todas las cepas de BCG y comprende una región importante del genoma previo a la atenuación consistiendo en una región de 9,5 kilo-bases (kb) que contiene 9 genes codificantes. Estos genes incluyen los codificantes para el sistema de secreción ESX-1, así como los antígenos inmuno dominantes de células T, las proteínas secretadas ESAT-6 y CFP-10 [28](Figura 5).

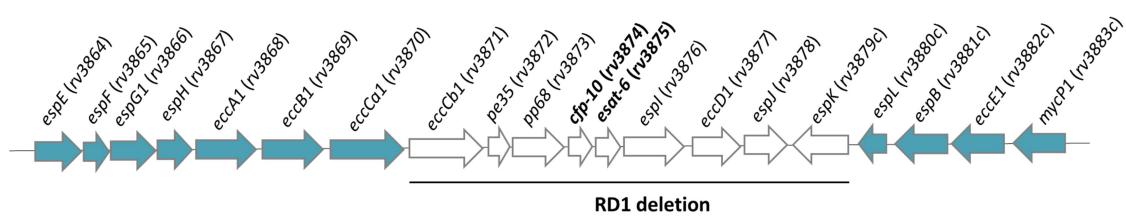


Figura 5. Organización genómica de los genes en ESX-1 en *M. tuberculosis*. Representación esquemática de los genes de *M. tuberculosis* en la región ESX-1. Las proteínas ESAT-6 y CFP-10 son antígenos inmunodominantes secretados a través del sistema ESX-1 con funciones principales en la infección por *M. tuberculosis*. Los genes *ecc* y *espB* codifican componentes conservados del sistema de secreción ESX-1 y proteínas asociadas a la secreción y son necesarios para la exportación de ESAT-6 / CFP-10. Se indica la región RD1, eliminada en todas las cepas variantes de BCG. Adaptado de [29].

Actualmente con las nuevas herramientas de manipulación genética se ha demostrado que la eliminación específica de RD1 en *M. tuberculosis* y *M. bovis* conduce a un fenotipo atenuado en modelo de ratón, confirmando a las cepa una atenuación similar a BCG [30]. A la inversa, la complementación de BCG con la región RD1 aumenta su virulencia en ratones en comparación con BCG original. La complementación de RD1 en BCG no restaura completamente la virulencia que mantiene el bacilo patógeno, lo que sugiere que además de la pérdida de RD1 otras de las múltiples variaciones genéticas presentes en las cepas de BCG respecto de *M. bovis* pueden contribuir también a la atenuación [27].

Se han propuesto varias hipótesis para explicar la protección variable de BCG contra la TB pulmonar, incluida la pérdida de antígenos inmunodominantes durante los pases continuos, la variabilidad de las cepas de BCG, la falta de células T de memoria central o la pre-exposición de los vacunados a micobacterias ambientales [17][31].

Con el fin de controlar eficazmente la TB y lograr el objetivo de la estrategia de la OMS para poner fin a la TB en el horizonte de 2050, se establece como una necesidad urgente el desarrollo de nuevas vacunas con estrategias de inmunización más eficaces, así como terapias antibióticas también más eficientes.

Nuevas estrategias de vacunación contra la tuberculosis.

Una de las principales desventajas para el desarrollo de nuevas vacunas contra la TB es la falta de conocimiento de las respuestas inmunitarias necesarias que se puedan correlacionar con la protección (correlación de protección), lo que dificulta la cuantificación o el seguimiento de la eficacia de la vacuna.

Se están desarrollando nuevos candidatos a vacunas basados en diferentes estrategias. El objetivo para las vacunas contra la TB que se encuentran actualmente en ensayos clínicos es la sustitución de BCG o la mejora de la protección de BCG a través de estrategias de inmunización con pautas de refuerzo utilizando vacunas de subunidades [17][32]. Además, existen vacunas terapéuticas en desarrollo clínico cuyo objetivo es reducir la duración de los tratamientos o prevenir la recurrencia de la TB latente, utilizando micobacterias fragmentadas o células completas inactivadas [33].

Por un lado, las vacunas de subunidades se basan en uno o unos pocos antígenos específicos de *M. tuberculosis* y se desarrollan para potenciar la respuesta inmune a BCG en individuos previamente vacunados, utilizando vectores virales o adyuvantes como sistema de administración. Los antígenos más empleados para la construcción de vacunas de subunidades de TB son el antígeno 85A, el antígeno 85B y ESAT-6.

Las estrategias para sustituir a BCG implican el desarrollo de vacunas con cepas vivas atenuadas para inducir una respuesta de memoria inmunitaria específica y

duradera cuando se administren al nacimiento [34]. Actualmente, existen dos candidatos a vacunas vivas atenuadas se encuentran en ensayos clínicos. Una es una vacuna BCG recombinante denominada VPM1002, que lleva una delección en el gen *ureC* y expresa la proteína listeriolisina-O de *Listeria monocytogenes*. Como consecuencia de estas alteraciones genéticas, el BCG recombinante puede llegar al citosol, mejorando su presentación antigénica [35][36]. El segundo candidato a vacuna viva atenuada es MTBVAC, una cepa recombinante de *M. tuberculosis* construida con dos delecciones independientes en los genes *phoP* y *fadD26*, ambos involucrados en la virulencia.

MTBVAC. ¿La vacuna del relevo?

El desarrollo de herramientas de manipulación genética durante los años 80 del siglo pasado permitió, entre otros avances, la modificación racional de determinados genes de la bacteria *M. tuberculosis* y el desarrollo de nuevas vacunas candidatas a mejorar la protección parcial de la veterana BCG.

Dentro del consorcio de científicos europeos coordinados por TBVI (Tuberculosis Vaccine Initiative), el grupo de la Universidad de Zaragoza liderado por Carlos Martín, ha desarrollado durante las últimas décadas la vacuna MTBVAC. Ésta está basada en la atenuación racional de un aislado clínico de *M. tuberculosis*, denominado Mt103, mediante la eliminación por ingeniería genética de dos genes implicados en virulencia, el factor de transcripción *phoP* y el gen *fadD26* que codifica para la síntesis de PDIM.

Durante los últimos años, diferentes grupos de investigación independientes han realizado experimentos en distintos modelos animales (ratón, cobaya, macaco), que han demostrado que MTBVAC tiene un perfil de atenuación comparable al de la vacuna actual BCG. Además, presenta un mayor perfil inmunogénico y confiere una mayor protección frente a una infección experimental con *M. tuberculosis* que la protección observada con BCG. Todos estos resultados positivos han permitido la entrada de MTBVAC en ensayos clínicos, siendo la primera vacuna viva basada en la atenuación del agente causante de la TB en humanos. El estudio de seguridad e inmunogenicidad en adultos sanos, Fase 1a, se llevó a cabo durante el año 2013 y la primera mitad del año 2014 en Lausanne (Suiza), por el profesor François Spertini,

(ClinicalTrials.gov: Dose-Escalation Study to Evaluate the Safety and Immunogenicity of MTBVAC Vaccine in Comparison With BCG Vaccine, Identifier: NCT02013245), mostrando que MTBVAC tiene una inmunogenicidad que aumenta con la dosis administrada y un perfil de seguridad similar a BCG. El estudio de seguridad e inmunogenicidad en recién nacido, Fase 1b, se realizó en Sudáfrica, país endémico con una alta incidencia de TB, siendo la Profesora Michele Tameris la investigadora principal del estudio (ClinicalTrials.gov: Dose-escalation Safety and Immunogenicity Study to Compare MTBVAC to BCG in Newborns With a Safety Arm in Adults NCT02729571). MTBVAC mostro un perfil de seguridad en bebés similar a BCG y una inmunogenicidad celular frente a antígenos de TB superior a BCG. El desarrollo industrial de esta vacuna se realiza en colaboración con la farmacéutica Biofabri. Actualmente esta candidata a vacuna se encuentra en distintos ensayos clínicos; por un lado están recién finalizados dos estudios, Fase II-a, en Sudáfrica, un estudio en adultos con y sin infección tuberculosa y otro estudio en recién nacidos (NCT03536117 y NCT02933281) [37], todavía sin resultados analizados. Por otro lado, se está iniciando la Fase III de eficacia en neonatos, en Sudáfrica, Senegal y Madagascar.

Además del desarrollo de nuevos candidatos a vacunas, los enfoques novedosos para mejorar la eficacia de la vacuna contra la TB pulmonar incluyen nuevas vías de inmunización, con un interés creciente en la vía de vacunación de la mucosa pulmonar como un enfoque racional para dar una respuesta inmune local mejorada inducida por la vacuna en el sitio primario de infección.

TUBERCULOSIS Y EXPERIMENTACIÓN ANIMAL

Tras el estudio *in vitro* de los parámetros que afectan a un agente patógeno y antes de realizar una valoración experimental de nuevos tratamientos o vacunas en las especies hospedadoras naturales o definitivas, debemos realizar estudios *in vivo* utilizando modelos animales adecuados, ya que así evitaremos importantes costes sociales y económicos.

Una vez que conocemos el agente causante de una enfermedad y hemos estudiado su patogenia, morfología, composición, desarrollo en medios de cultivo, su genoma, la posible replicación en cultivos celulares...etc, y para estudiar en detalle la patogénesis, las respuestas protectoras del huésped contra este patógeno, así como la tolerancia y protección de tratamientos y vacunas, el siguiente paso que realizamos es provocar infecciones experimentales del agente causal en especies lo más afines posibles a los hospedadores definitivos.

Actualmente existe una creciente preocupación de la población general por la experimentación con animales, pero también se da un compromiso general entre los comités científicos internacionales que en la experimentación animal hay que seguir el “principio de las 3 erres”, que hacen referencia a: Reemplazar, Reducir y Refinar. Reemplazar alude a evitar totalmente los experimentos con animales sustituyendo por ejemplo por modelos matemáticos, modelos con cultivos celulares, modelos por organoides en 3 D, modelos por biorreactores de fibra hueca (hollow fiber) etc, o por otro lado los reemplazos pueden ser relativos sustituyendo por ejemplo modelos con mamíferos por modelos con animales con una menor percepción del dolor, como peces o invertebrados. Reducir supone utilizar estrategias para bajar el número de animales de experimentación aplicando estadísticas que optimicen los datos obtenidos en el procedimiento, o maximizando las muestras a obtener por individuo. Refinar implica modificar las condiciones de estabulación y cría, o de los procedimientos y técnicas de trabajo para minimizar el dolor y la angustia, así como para mejorar el bienestar de los animales desde su nacimiento hasta su muerte.

En el caso de *M. tuberculosis*, imitar la TB humana en animales de experimentación tiene sus limitaciones y dificultades [38]. Actualmente para las pruebas preclínicas de nuevas vacunas se sigue una secuencia común entre distintos laboratorios de investigación con el uso de varias especies animales consecutivas. Se

realizan primero estudios en ratones que luego se correlacionan con estudios en cobayas y finalmente en primates no humanos (PNH) [39] [40] [41].

Tuberculosis y experimentación en ratones.

El modelo de ratón sigue siendo actualmente el más utilizado entre todos los modelos de experimentación animal. Desde 1950 el ratón doméstico, *Mus musculus*, es el actor protagonista dentro de la investigación biomédica como animal de experimentación. En España actualmente, el 75% de todos los animales utilizados en la investigación científica son roedores (Ministerio de Agricultura Pesca y Alimentación 2020), principalmente ratones. Las razones son evidentes, trabajar con ratones es barato, son fáciles de mantener pues no tienen necesidades nutritivas exigentes, ni precisan, debido a su tamaño, de mucha superficie o espacio para su desarrollo. Se pueden comprar en grandes cantidades a productores certificados, su ritmo de replicación es muy corto (en tres meses, ya hay una nueva generación), las hembras son multíparas (con unas 7 crías por parto de media) y también es corta su vida útil, lo que permite un análisis completo de sus procesos biológicos frente al agente estudiado en todos los ciclos de la vida del animal [42] [43].

Debido a la fuerte selección y endogamia que han sufrido los ratones de laboratorio, tenemos ahora diversas líneas o cepas que son genéticamente homogéneas y están adaptadas a la manipulación y al entorno de los animalarios de experimentación. Esto por un lado limita los errores de experimentación derivados de la variabilidad genética, y por otro lado significa que los estudios se pueden repetir y verificar en diferentes laboratorios con alta reproducibilidad.

Desde los años 80 se han producido además toda una serie de avances científicos que han favorecido enormemente el desarrollo del modelo de ratón como referente en la experimentación animal. Primero se produjo un ratón transgénico viable tras insertar ADN ajeno a su genoma, después se obtuvieron células madre embrionarias con genoma modificado, más adelante se crearon los primeros ratones *knockout*, que permiten la eliminación de genes no deseados. En 2002 se terminó de secuenciar el genoma completo del ratón, lo que permitió compararlo con el genoma humano, descubriendo una asombrosa cercanía entre las dos especies, más del 90% del

genoma del ratón podría estar alineado con las regiones correspondientes del genoma humano. La capacidad de poner, quitar y modificar genes individuales del ratón da a los investigadores una herramienta sin precedentes a la hora de diseñar experimentos muy dirigidos en la investigación biomédica, y con todas estas adaptaciones parece que los ratones pueden servir como un buen modelo experimental para enfermedades humanas. Existen algunos investigadores que postulan su preocupación sobre el uso de ratones como protagonista casi exclusivo de los ensayos *in vivo* de enfermedades humanas, ya que este hecho puede estar impidiendo que se descubran nuevas vías de innovación y desarrollo científico.

En el caso de los ratones frente a la TB humana hay investigaciones que determinan que a pesar de ese más de 90% de identidad genética, las pequeñas diferencias que mantienen entre sus genes producen importantes diferencias moleculares, inmunológicas y celulares entre las dos especies, que impiden que este animal sirva como un referente eficaz para buscar curas para la TB [44].

Como ya se ha descrito anteriormente, cuando *M. tuberculosis* llega a los pulmones humanos se desarrollan una serie de conformaciones anatómicas como resultado de la respuesta inmunológica a *M. tuberculosis*, denominadas granulomas. Estos granulomas pueden no progresar y permanecer inactivos durante un largo período, pero también pueden activarse (ocurre en un bajo porcentaje de los individuos infectados) crecer y romperse expulsando nuevas bacterias que inundan los alveolos pulmonares y pueden salir al exterior del individuo por el aire espirado y propagarse infectando a nuevos hospedadores, mientras tanto el enfermo con infección activa puede llegar a morir si no es tratado. Cuando infectamos ratones con TB de forma experimental, no desarrollan las mismas respuestas fisiológicas, ni siquiera los granulomas tienen la misma estructura anatomo-patológica (no hay necrosis, ni caseificación, ni tampoco cavitación), y además no hay evidencias claras de que la TB se transmita por vía aérea entre las poblaciones de ratones infectados a los ratones sanos. Y a pesar de todo ello hay una gran cantidad de estudios científicos donde son los ratones los animales de experimentación elegidos para estudiar distintos aspectos esta patología. La suma de resultados de los distintos aspectos parciales estudiados en los ratones permitirá disponer de conclusiones objetivas para tratar y prevenir esta enfermedad.

Las principales razones para trabajar con esta especie son la economía de manejo, la disponibilidad de reactivos específicos y la posibilidad de combinar distintas líneas de ratones, ratones inmuno-competentes, ratones inmuno-deficientes, ratones *knockout* para distintos genes, o incluso ratones humanizados (con la inclusión de algún gen específico humano en el genoma del ratón) [45].

Tuberculosis y experimentación en cobayas.

Los cobayas, *Cavia porcellus*, o conejillos de indias son notablemente más susceptibles a TB que los ratones, un solo microrganismo puede causar la muerte. En infección experimental desarrollan granulomas primarios en los que se puede apreciar necrosis y caseificación, similares a los granulomas humanos [46].

En el pasado, cuando aún no se conocían los medios de cultivo específicos para las micobacterias patógenas; los conejos y las cobayas se utilizaron como método de diagnóstico diferencial entre *M. bovis* y *M. tuberculosis* por la respectiva alta susceptibilidad de estas especies, se les inoculaban muestras de pacientes sospechosos de la enfermedad. También se utilizaron cobayas para estudiar la transmisión por aerosoles en hospitales. En los primeros años de descubrimiento y desarrollo de antibióticos contra la TB se usaron cobayas de forma preferente como modelo preclínico debido a su alta susceptibilidad a la infección por *M. tuberculosis* [47] [48].

En relación a los ratones, los cobayas tienen mayores necesidades de espacio (por su tamaño) y son más exigentes en su dieta ya que precisan el aporte continuo de vitamina C, lo cual dificulta y encarece la experimentación en animalarios de contención para patógenos de transmisión aérea (laboratorio de seguridad biológica tipo 3, o BSL3) [47].

Si por un lado el tamaño de los cobayas es una desventaja por su manejo en animalarios en comparación a los ratones, por otro lado, este tamaño puede ser una ventaja a la hora de obtener y manipular muestras para su análisis en laboratorio, ya que no estamos tan limitados en cantidad como con los ratones a la hora de extraer muestras de suero *in vivo* o de precisar dosis de tratamientos aportados, etc. De un solo individuo podemos obtener datos y análisis más precisos que de un ratón, pero una gran desventaja es la escasa disponibilidad de reactivos específicos de cobaya para

poder realizar análisis inmunológicos por citometría de flujo, estudios *in vivo* por luminiscencia o fluorescencia, inmunohistoquímica, etc. [39] [40]. Otra posibilidad ventajosa de los conejillos de indias es que podemos estudiar la forma de infección latente de TB, tras provocar una infección con patógeno a baja dosis y tratar a las 4 semanas post infección con terapia antibiótica [49] [50].

Actualmente los cobayas se utilizan en infecciones experimentales por *M. tuberculosis* para la confirmación o discriminación de la seguridad y eficacia de los nuevos medicamentos y vacunas ensayadas ya en ratones y en los que se ha demostrado la posible validez [51] [52]. Si posteriormente tenemos demostrada también la eficacia en cobayas podemos avanzar los estudios dando el salto a primates no humanos y acercándonos a los ensayos clínicos definitivos. En principio un medicamento o vacuna contra la TB con buena protección en cobayas es indicativo que la protección en primates y en el hombre será alta.

Tuberculosis y experimentación en primates no humanos (PNH)

Los PNH comparten una estrecha relación evolutiva con los humanos y desarrollan la TB con un cuadro clínico similar. Otro aspecto interesante al trabajar con monos como animales de experimentación es la amplia gama de reactivos bioquímicos disponible para estudios inmunológicos debido a la considerable reactividad cruzada con humanos [43], y además también son similares los perfiles farmacocinéticos entre PNH y humanos, lo que permite sencillos cálculos para la aplicación de dosis, que serán proporcionales a las diferencias de pesos entre especies [53]. Con todo ello parece que este es el modelo animal ideal para estudiar la TB. Pero el problema en el uso de PNH en experimentación tiene varios frentes. El primero es ético, ya que existe un fuerte rechazo social a experimentar con especies tan parecidas a los humanos, de hecho, actualmente en España no se experimenta con PNH y se tienen que contratar los estudios a laboratorios externos. Por otro lado, el problema es económico, la disponibilidad de animales es baja y son estudios muy caros. El tamaño de los monos, su baja tasa reproductiva y las necesidades de seguridad biológica implican grandes infraestructuras para los animalarios. Hay grandes gastos de construcción de bioterios: por la gran superficie demandada por cada animal, por las barreras de contención, la climatización en presión negativa, el esfuerzo para el control y mantenimiento de las

estancias libres de patógenos específicos (SPF), y los gastos de personal especializado. Todo ello hace que la experimentación de TB con monos sea muy costosa.

En general, los estudios con PNH se aplican para las fases previas a los estudios clínicos, principalmente para estudios de eficacia y estudios de dosis de medicamentos y vacunas.

Las dos especies de macacos más comúnmente utilizadas para investigación en TB proceden de Asia: *Macaca mulatta* (macaco Rhesus) y *Macaca fascicularis* (macaco cynomolgus o de cola larga). El macaco Rhesus es muy susceptible a *M. tuberculosis* y tiene aplicación en estudios de infección por baja dosis, desarrollo de enfermedad aguda o crónica, estudios de protección de nuevas rutas, y nuevos tratamientos y vacunas [54],[55]. Los macacos Cynomolgus tienen una relativa resistencia a *M. tuberculosis*, son buenos como modelo para el estudio de nuevos fármacos frente a altas dosis de infección, o para el estudio de la TB latente y también para generar información sobre la persistencia microbiana [56]. Otro aspecto interesante al trabajar con macacos es la posibilidad de estudiar la coinfección TB y VIH ya que el virus de la inmunodeficiencia del simio (SIV) actúa en monos de manera similar al VIH en humanos, vacunas o medicamentos polivalentes podrían ser ensayados en macacos esperando resultados paralelos para el hombre.

Una estrategia interesante, trabajando con PNH, para reducir el número de animales, favoreciendo el principio de las 3 erres, es aplicar las tecnologías emergentes de imagen como la tomografía de emisión de positrones (PET/CT) [57], que nos permiten determinar *in vivo* la progresión del número de granulomas en los pulmones a intervalos temporales determinados, o la posible reactivación de la infección latente, estudiando el desarrollo progresivo de la patología con la obtención de muchos datos, sin tener que llegar al punto final del experimento y determinar la patogenia en un solo tramo experimental tras necropsia.

Tuberculosis y la experimentación en el pez cebra.

Siguiendo con el principio de las “3 erres” en la experimentación animal y dentro de la estrategia del reemplazo nos encontramos con el pez cebra, *Danio rerio*, como una de las principales especies candidatas como alternativa para remplazar a

modelos animales clásicos (mamíferos en su mayoría) y cumplir con el primer objetivo del principio ético en experimentación animal.

La posibilidad de utilización del pez cebra en el campo de la TB surge debido a su sensibilidad frente a *Mycobacterium marinum* [58]. Esta bacteria produce una enfermedad sistémica en el pez cebra con formación de granulomas, que son estructuralmente similares a los granulomas de tuberculosis humana [59]. *M. marinum* no es una especie que pertenezca a MTBC pero mantiene un 85% de similitud genética con *M. tuberculosis* [60].

A pesar de las diferencias anatómicas y fisiológicas entre el pez cebra y los humanos, el modelo de pez cebra puede tener su aplicación en las primeras fases de estudio *in vivo* de la patogénesis de la enfermedad de TB, y en el desarrollo de nuevos fármacos y vacunas, ya que el sistema inmune del pez cebra tiene componentes primarios similares al sistema inmune de los humanos.

Trabajar con pez cebra tiene una serie de ventajas. La necesidad de espacio es reducida y tienen una alta tasa reproductiva lo cual permite experimentos con alto número de individuos por cada grupo experimental. Las instalaciones son medianamente complejas, pero *M. marinum* es un patógeno de contención BSL2, en personas inmuno-comprometidas puede producir lesiones cutáneas. Tanto los embriones del pez como los adultos son sensibles a la infección [61] [62]. El pez cebra admite manipulaciones genéticas que permiten estudios dirigidos a mecanismos moleculares específicos.

Entre las desventajas del modelo de pez cebra está la adaptación que hay que realizar de los animalarios convencionales a unas nuevas instalaciones con baterías de peceras y nuevos métodos de aplicación y dosificación de los inóculos patógenos y de fármacos, y por otro lado el medio acuático conlleva la presencia frecuente de otras patologías que pueden desvirtuar los resultados de los estudios propios que estamos realizando.

Tuberculosis y experimentación en ganado bovino/caprino.

En principio no podemos considerar al ganado bovino o caprino como animales de experimentación para TB a pesar de la similitud entre la TB humana y la TB del ganado, debido principalmente al alto coste de las instalaciones de contención BSL3 para animales de ese tamaño y longevidad. Pero para el caso de la TB bovina/caprina son los animales de elección al ser estas especies las hospedadoras naturales.

La prevención de la TB en bovino/caprino mediante la vacunación es una perspectiva interesante, principalmente para el control de la enfermedad en estas especies económicamente importantes, pero también para la eliminación de una de las vías de transmisión zoonótica de TB causada por *M. bovis* en el hombre [39] [40] [63]. El problema actual de la vacunación, con BCG, tanto para el ganado como para el hombre es que con los métodos actuales de diagnóstico (tuberculina intradérmica o IGRA) no somos capaces de discriminar la infección de la vacunación.

Una ventaja de la experimentación con bovino/caprino es la disponibilidad de reactivos comerciales para estas especies. La otra gran ventaja es que debido a la similitud entre los dos procesos patológicos tanto los avances en la investigación (nuevos métodos de diagnóstico, tratamientos o vacunas) por vía de la TB bovina como por vía de la TB humana podrían ser cruzados y confirmados entre sí con experimentos relativamente simples.

RUTAS ALTERNATIVAS DE VACUNACIÓN CON BCG.

Las rutas de vacunación son las vías o los sistemas que utilizamos para inocular el agente o antígeno vacunal. Estas vías de administración de vacunas deben ser adecuadas para lograr la inmunización que prevenga la enfermedad en el futuro. La vía correcta tiene que dar lugar a una absorción suficiente de la vacuna minimizando el riesgo de reacciones adversas.

En el caso de la vacuna BCG, aunque inicialmente fue administrada por vía oral (18 de julio de 1921), actualmente, la vía indicada es la intradérmica. La administración intradérmica consiste en inocular el producto justo debajo de la parte más superficial de la piel (epidermis), es una vía poco frecuente, pero ya sólo de BCG se administran unos 100 millones de dosis anuales por esta vía.

Este tipo de inoculación de lenta absorción persigue evitar efectos adversos, que se podrían producir tras inyectar una bacteria, aunque atenuada, por otras rutas sistémicas como la vía intramuscular o endovenosa, donde podrían llegar a producir infección generalizada en individuos inmunodeprimidos. Así, la lesión típica que se produce en la vacunación intradérmica de BCG es un eritema o nódulo rojizo en el sitio de inoculación, con afección del ganglio regional, que aparece hacia la segunda semana de la vacunación y desaparece hacia los 3 meses dejando una ligera cicatriz circular.

En los inicios de nuestros estudios de seguridad y eficacia comparada entre BCG y MTBVAC en modelos experimentales con ratón frente a patógeno de TB, vimos que tanto en la ruta de vacunación intradérmica como en la ruta subcutánea se obtenían resultados similares de recuentos de bacterias en pulmón y bazo, con lo cual adoptamos la inoculación subcutánea como ruta de elección para los posteriores experimentos de eficacia de la vacuna, por ser una técnica mucho más sencilla y rápida de aplicar que la vía intradérmica.

Existe un interés creciente en nuevos enfoques para la administración de vacunas que exploran rutas no convencionales con el fin de activar nuevos mecanismos de inmunización. Uno de estos enfoques está basado en la aplicación de las vías naturales de la infección de la enfermedad. En el caso de TB la vía natural de infección es la vía respiratoria, la mucosa respiratoria es el tejido principal para el

establecimiento de la infección. Entonces, ¿por qué no vacunar por la vía respiratoria?

Se ha descrito ya, en diferentes modelos preclínicos, que la vacunación con BCG por vía respiratoria confiere una protección sustancialmente mejorada en comparación con la inmunización subcutánea o la intradérmica [64] [65] [66]. La justificación de esta mejora parece ser que está basada en el tipo de inmunidad generada en la mucosa pulmonar. Hasta ahora los investigadores que trabajan en vacunas contra la TB han estado tratando de identificar parámetros inmunológicos basados en respuestas celulares, ahora existe un interés creciente en el papel de posibles respuestas humorales para la protección contra la TB. Las respuestas inmunitarias provocadas por BCG pulmonar son de tipo celular, pero también humoral, se observa producción de inmunoglobulinas específicas contra TB en la mucosa alveolar, así como respuesta celular a nivel sistémico.

La adaptación de la ruta respiratoria a los modelos preclínicos no tiene gran dificultad ya que se puede aplicar en roedores y PNH por vía intranasal o intratraqueal en animales previamente anestesiados, o por vía aerosol en cabinas controladas en animales no anestesiados. Sin embargo, parece algo más complicado la adopción de la vía intranasal como ruta de vacunación para BCG u otras vacunas vivas atenuadas en clínica. Existe algún precedente con problemas de seguridad con una vacuna contra la gripe administrada por vía intranasal que causó algunos casos de episodios transitorios de parálisis facial [67]. Además, la proximidad de la cavidad nasal al cerebro puede ser una condición anatómica que cause reticencias a las autoridades médicas reguladoras. Por el contrario, la vía del aerosol parece más factible para el hombre porque llega más directa a la mucosa alveolar [68]. Estudios recientes en PNH han demostrado que BCG se dispersa bien por los pulmones después de la aerosolización con nebulizadores clínicos [69]. La vía aerosol se ha utilizado ya en algún estudio para administrar BCG en humanos sin mayores problemas de toxicidad [70],[71], y en la actualidad hay al menos dos ensayos clínicos en curso para evaluar la seguridad de BCG administrada por vía aerosol (NCT03912207, NCT02709278).

Otro enfoque novedoso de ruta de vacunación para BCG es la aplicación de la vía endovenosa. Recientemente se ha demostrado que la vacunación con BCG intravenosa induce protección en ratones y PNH y esta protección mejora la

inmunidad producida por la vacunación clásica por vía intradérmica o subcutánea [72]. Existen evidencias de que la inoculación endovenosa de BCG puede fortalecer la protección contra TB a través de la inducción de cambios epigenéticos y metabólicos, produciendo la reprogramación de las células inmunes innatas, un proceso llamado inmunidad entrenada [73]. Será necesario confirmar estos resultados en modelos animales estudiando no solo la eficacia frente al patógeno sino la seguridad a largo plazo, antes de realizar pruebas de seguridad por esta vía en humanos. La inoculación endovenosa de bacterias vivas, aunque atenuadas, se asocia con la idea de una posible septicemia y no se podrá aplicar en individuos inmunocomprometidos o con patologías previas. Al igual que la ruta intranasal para vacunas vivas aplicadas en humanos, la ruta endovenosa posiblemente causará reticencias en las autoridades médicas reguladoras que aprueban el paso de las fases preclínicas a las fases clínicas.

Aplicaciones de BCG para el tratamiento de otras patologías más allá de la tuberculosis.

Aunque BCG se concibió inicialmente como una vacuna contra la TB, también tiene otras aplicaciones clínicas muy diferentes, como el tratamiento del cáncer de vejiga por ejemplo, que fue el primer tratamiento de inmunoterapia aplicado contra el cáncer y actualmente el tratamiento de elección en el cáncer de vejiga no músculo invasivo. Otras propiedades de BCG se han descubierto a partir de estudios epidemiológicos que se han realizado entre la masiva población que ha sido vacunada con BCG. Uno de los efectos beneficiosos no específicos es el descenso de la mortalidad infantil por causas diferentes de TB, principalmente neumonías, gastroenteritis o meningitis. Recientemente hemos descrito en un modelo murino experimental de neumonía que la vacunación intranasal de BCG y MTBVAC confiere protección heteróloga frente a *Streptococcus pneumoniae* [74]. Entre los efectos heterólogos beneficiosos sobre los que trabajamos en esta tesis están la aplicación de BCG y MTBVAC en la terapia del cáncer de vejiga, así como en la terapia del asma.

Tratamiento del cáncer de vejiga.

Una de las aplicaciones de BCG, fuera de su indicación principal, es la de tratar el cáncer de vejiga superficial o no musculo invasivo. Estos cánceres sólo se encuentran en el revestimiento de la luz de la vejiga, sin propagación a la capa muscular de la pared vesical ni a otros órganos. La inmunoterapia por BCG se aplica ya desde hace 4 décadas en este tipo concreto de cáncer [75]. El mecanismo de acción se relaciona con la respuesta inmunitaria generada contra las micobacterias, puesto que se reclutan las células inmunitarias que atacarán también a las células cancerosas que están en contacto con las bacterias de BCG. Actualmente es el tratamiento de elección con resultados relativamente buenos, pero no exentos de efectos secundarios entre los que se encuentran la infección sistémica por BCG o la aparición de recidivas. Para mejorar los tratamientos y evitar estos efectos secundarios se llevan a cabo nuevos ensayos en modelos animales con otras especies del género *Mycobacterium*, como la vacuna MTBVAC o *Mycobacterium brumae* [76], que puedan ser más efectivas y también más atenuadas que la cepa de BCG que se utiliza actualmente.

Una vez realizada la extirpación del tumor por cirugía intravesical, el tratamiento posterior se realiza administrando directamente BCG dentro de la vejiga del paciente por instilación del inóculo bacteriano a través de un catéter blando por vía uretral. El objetivo es destruir las células cancerosas que puedan quedar tras la resección. La ventaja de esta ruta de administración, en esta localización específica, es que el instilado terapéutico afecta a las células que revisten el interior de la vejiga sin afectar a otras capas u órganos anejos. El instilado se mantiene durante 2 horas como máximo tras lo cual se retira la cánula, la pauta habitual del tratamiento es realizar un instilado semanal durante 6 semanas y después de un periodo de descanso realizar otras pautas de tratamiento de recuerdo, según criterio de los especialistas, hasta completar un año.

Tratamiento contra el asma.

El asma bronquial es una enfermedad del aparato respiratorio caracterizada por una obstrucción de los bronquios y que se acompaña de una inflamación crónica de las vías respiratorias inferiores. Actualmente ha alcanzado niveles de pandemia, con más de 300 millones de personas afectadas en todo el mundo. La etiología del asma puede

ser diversa, las causas principales suelen ser: infecciones víricas, ejercicio intenso, antiinflamatorios no esteroideos, y alérgenos. Pero destaca esta última causa como la más prevalente con diferencia. Se estima que los alérgenos producen el 80% del asma bronquial en niños y adolescentes, así como en un 40% de los casos en adultos jóvenes. Es especialmente frecuente en países desarrollados. Una de las explicaciones más aceptadas para estas diferencias proviene de la denominada "hipótesis de la higiene", que sugiere que el desarrollo del asma se ve favorecido por la menor exposición en la infancia a determinados factores ambientales [77]. En este sentido, la exposición a ciertos microorganismos o ácaros (como los presentes en las granjas) durante etapas tempranas de la vida podría contribuir a la educación del sistema inmunológico lo que conllevaría la adquisición de una mayor tolerancia a los alérgenos. Esta teoría nos anima a que podamos aplicar micobacterias atenuadas como posible profilaxis y también como terapia frente al asma alérgica.

Recientemente hemos descrito en modelo murino una protección heteróloga por infección intranasal con el patógeno *M. tuberculosis* frente al asma alérgica por ovoalbúmina (OVA). La presencia de TB implica una reducción de eosinófilos en médula ósea y en el infiltrado alveolar de los pulmones [78].

El modelo de asma en ratones está bien definido, principalmente con ovoalbúmina como agente alérgeno y también por ácaros del polvo doméstico (HDM), o por el hongo *Alternaria*.

La ruta de administración tanto para el reto con el alérgeno como para el tratamiento por micobacterias se realiza directamente por vía respiratoria, que puede ser por vía intratraqueal o por vía intranasal. Nuestra experiencia previa en ratones entre estas 2 vías nos indica que la vía intranasal es más fácil de administrar y produce resultados más homogéneos y fiables.

La inoculación por vía intranasal no tiene especial dificultad, se realiza por instilado lento (gota a gota) del inóculo correspondiente en los orificios nasales, con micropipeta de punta fina (puntas blancas de hasta 20 µl de volumen). Los ratones deben estar anestesiados para evitar el rechazo del inóculo por la tos, y hay que tapar la boca para evitar la respiración bucal del animal. El instilado se mantiene en el hocico hasta que el ratón va inspirando cada gotita hasta completar un volumen equivalente a unos 40 µl. La anestesia respiratoria por isoflurano funciona muy bien ya que el ratón

inoculado se despierta espontáneamente en su cubeta en menos de un minuto tras el procedimiento.

Las pautas de inducción y tratamiento del asma son diferentes para cada tipo de alérgeno que utilicemos y el curso de la enfermedad que queremos producir, curso agudo o curso crónico. Una pauta tipo para inducir asma aguda por OVA por ejemplo, conlleva 2 sensibilizaciones intraperitoneales, separadas de una semana, con OVA y con hidróxido de aluminio (Al(OH)_3) como adyuvante, Hacia las cuatro semanas de la 1^a sensibilización se reta al animal con altas dosis de OVA por vía intranasal durante 3 días consecutivos, al 4º día podemos sacrificar los animales para observar la eosinofilia por lavados broncoalveolares (BAL), o también comprobando la patogenia producida por estudio histológico de pulmón, acúmulo de moco en bronquiolos que se puede observar por tinción diferencial PAS.

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OBJETIVOS

MODELOS ANIMALES

El objetivo global de esta tesis doctoral fue optimizar los procedimientos para inocular cepas patógenas y atenuadas de *M. tuberculosis* por diferentes rutas de administración en ratones, y de este modo estudiar distintas aplicaciones para vacunas vivas contra TB. En concreto hemos trabajado en las vías intravesical, intranasal, subcutánea (en ratones adultos y neonatos) e intraperitoneal. Además, también se optimizaron modelos experimentales para patologías de asma, cáncer de vejiga y TB, para evaluar la eficacia de las vacunas vivas BCG y MTBVAC como terapia frente a estas enfermedades. Todos estos procedimientos han producido distintos resultados que han sido publicados en los 4 artículos que se presentan en esta tesis, y los modelos aplicados se resumen a continuación.

1er Modelo: Puesta a punto de un modelo preclínico de cáncer de vejiga en ratón para el estudio de la eficacia terapéutica de MTBVAC.

Adaptar la ruta intravesical al modelo de ratón tiene sus dificultades. Es un modelo complejo, con muchos factores variables a tener en cuenta. Las diferencias de tamaño entre hombre y ratón son evidentes, la relación entre masas es 1/3000. Las características anatómicas hacen que sólo se haya podido adoptar el modelo en ratones hembra, el acceso a la vejiga a través de la uretra con una cánula de silicona no ha sido posible en ratones macho debido al fino diámetro uretral y su trayectoria sinuosa. La inducción tumoral se realiza inoculando células tumorales de vejiga de ratón que proceden de una línea celular estable, la línea MB-49. Para que aniden o se adhieran las células tumorales a la vejiga, previamente tenemos que aplicar un tratamiento que irrite o altere la capa epitelial del lumen vesical, por acción de pequeñas descargas con un electrodo interno o por irritado químico, con instilado de poli-l-lisina por ejemplo. Como los tratamientos son de larga duración, hasta 2 horas, los ratones tienen que estar inmovilizados durante todo ese tiempo, o sea, anestesiados, lo que supone el continuo control del ritmo respiratorio y temperatura corporal. La anestesia intraperitoneal supone la repetición de 3 o 4 dosis mientras dura el tratamiento, la anestesia gaseosa por Isoflurano es continua pero precisa de un equipo adaptado con

impulsión de mezcla de gases y recuperación y neutralización de gases residuales. Todos estos factores hacen que la adaptación del modelo a un determinado laboratorio precise de un tiempo considerable hasta que los técnicos y/o investigadores consigan resultados satisfactorios en la implantación de los tumores y la sincronización de tratamientos. En este trabajo, estudiamos por primera vez el potencial de MTBVAC como terapia frente al cáncer de vejiga en modelo de ratón.

Modelo experimental aplicado.

Para los estudios de tratamiento antitumoral se utilizaron ratones de la cepa C57BL/6, suministrados por Janvier Biolabs, hembras de 8-10 semanas de vida al inicio de los experimentos. Las instilaciones intravesicales se realizaron con los animales anestesiadas con isoflurano, primero se drenó la orina presente en la vejiga por una ligera presión en la parte inferior del abdomen, después lubricamos un catéter endovenoso de calibre 24G (BD InsiteAutoguard, Becton Dickinson) conectado a una jeringa de 1 ml y lo insertamos cuidadosamente a través de la uretra hasta la vejiga. El catéter y la jeringa se mantuvieron mientras duraron los diferentes procedimientos de inducción tumoral o de tratamiento. Para la implantación del tumor, las vejigas de ratón se pretrataron con 100 µl de poli-l-lisina (0,1 mg/ml) durante 20 minutos. Luego se instilaron 100.000 células MB49-Luc por ratón y se mantuvo el instilado durante 60 minutos. Tres días después de la inducción del tumor de vejiga los ratones se dividieron aleatoriamente en 3 grupos, 6-9 animales/grupo, y se trataron intravesicalmente durante 1 hora, un grupo con 100 µl de BCG Pasteur (10^7 UFC /animal), otro grupo con MTBVAC 100 µl de (10^7 UFC /animal), el tercer grupo con el mismo volumen de PBS como control. Se comprobó el número de UFC bacterianas instiladas en todos los tratamientos mediante recuento por crecimiento en medio 7H10 con enriquecimiento de ADC.

La pauta de tratamiento se basó en la administración de instilados bacterianos descritos a los días 3, 9 y 16 después de la implantación del tumor. Para la visualización del tumor *in vivo*, los ratones se inocularon por vía intraperitoneal, los días 7 y 14 después de la implantación tumoral, con 200 µl de luciferina (15 mg/ml) (Perkin Elmer), y la luminiscencia se analizó con un dispositivo de imagen IVIS (Xenogen). Los animales que no mostraron ninguna señal de luminiscencia ni presencia de hematuria en las etapas iniciales del experimento fueron descartados del estudio, ya que consideramos que las células tumorales no habían sido implantadas

con éxito en estos animales. La ausencia de tumores en estos ratones fue corroborada por examen anatomico-patológico de las vejigas.

Se estudió la evolución de los ratones durante 60 días y se revisaron 3 veces por semana anotando el porcentaje de pérdida de peso, la presencia de hematuria, la presencia de tumores y el comportamiento general. Se fueron sacrificando los ratones en función de los criterios de punto final humanitario preestablecidos (anotados en una tabla de supervisión con clasificación sintomatológica individual), y se extrajeron las vejigas para el estudio histológico, mediante fijación en formol para la evaluación de la presencia de tumores por tinción con hematoxilina-eosina.

Inóculos bacterianos:

El stock de BCG utilizado en el presente estudio se preparó a partir de un vial comercial de BCG-MEDAC, que es una de las formulaciones de BCG autorizadas para el tratamiento del cáncer de vejiga. BCG-MEDAC proviene de una siembra de BCG Pasteur (cepa 1173P2; Institut Pasteur, París, Francia).

La vacuna MTBVAC se generó en nuestro laboratorio como hemos descrito anteriormente mediante la eliminación de los factores de virulencia *PhoP* y *FadD26* de un aislado clínico *M. tuberculosis* Mt103. Ambas vacunas se cultivaron a 37°C en medio líquido Middlebrook 7H9 (Difco) suplementado con 10% de ADC (Difco) y 0,05% (v/v) Tween80 (Sigma) o en medio sólido Middlebrook 7H10 (Difco) suplementado con 10% de ADC.

2º Modelo: Puesta a punto de los modelos de ratón para el estudio de seguridad y de eficacia de diferentes candidatos de vacunas vivas atenuadas de diferentes linajes de *M. tuberculosis*.

Como ya se ha descrito, los linajes 2, 3 y 4 de *M. tuberculosis*, se consideran ramas "modernas" dentro de las variantes del patógeno humano, y son las responsables de la mayoría de la TB actual en todo el mundo. Dado que la vacuna BCG confiere una protección variable contra la TB pulmonar se están investigando nuevos candidatos. Uno de estos candidatos es MTBVAC, que se construyó originalmente mediante delecciones en *phoP* y *fadD26* no marcadas y partiendo de un aislado clínico perteneciente al linaje L4. En este trabajo se han utilizado otras dos nuevas candidatas

basadas en delecciones idénticas a las de MTBVAC pero procedentes de aislados clínicos de *M. tuberculosis* de los linajes modernos L2 y L3. Se han comparado estas tres vacunas candidatas, primero su caracterización a nivel molecular *in vitro* y después también en experimentos de seguridad y protección con animales de experimentación.

Modelo experimental aplicado.

Para el experimento de valoración de la **atenuación**, se inocularon las 3 vacunas en ratones inmunodeprimidos SCID, hembras de ocho semanas de edad, por vía intraperitoneal con 10^6 bacterias en 100 µl de PBS. Los ratones fueron controlados revisando sintomatología y peso durante todo el experimento, realizando inicialmente tres controles semanales y posteriormente controles diarios en función del puntaje en la tabla de supervisión. El criterio de valoración del punto final humanitario se determinó cuando el peso individual llega al 80% del peso al inicio del experimento.

Para los experimentos de **eficacia protectora**, se vacunaron por vía subcutánea ratones inmunocompetentes C3H/HeNRj, hembras de ocho semanas de edad, con 10^6 UFC en 100 µl de PBS. Los ratones se vacunaron con BCG Pasteur, MTBVAC, MTBVAC-L2::hig, MTBVAC-L3::hig, y un grupo no vacunado como grupo de control de cada línea de patógeno. Ocho semanas después, los ratones fueron infectados por vía intranasal con 200 UFC en 40 µl de PBS de cada cepa (W4, HCU3524 o H37Rv) de *M. tuberculosis* perteneciente a cada uno de los linajes modernos 2, 3 y 4, respectivamente. Cuatro semanas después de la infección, se evaluó la carga bacteriana en los pulmones y en bazo. Diluciones seriadas de los homogenizados de estos pulmones y bazos se cultivaron en medio 7H10-ADC, en estufa a 37°C, y se determinó el número de colonias a las 3 semanas.

3er Modelo: Puesta a punto del modelo de ratón para el estudio de la vacuna inactivada por calor MTBVAC HK en inmunización por vía respiratoria.

La vacunación por la vía natural, la vía en la que se produce una infección de una enfermedad de forma habitual, representa una atractiva estrategia de inmunización. En el caso de la tuberculosis corresponde con la administración de la vacuna por vía respiratoria. El interés de utilizar esta ruta ha aumentado en los últimos años, demostrando su eficacia en diferentes modelos animales. En este contexto, la vacunación por vía respiratoria desencadena mecanismos inmunológicos pulmonares

que no están presentes cuando las vacunas se administran por vía parenteral. La contribución de la inmunidad de la mucosa alveolar en la protección inducida por las vacunas administradas por vía respiratoria ha sido poco estudiada. En este trabajo evaluamos, en ratones y PNH, la vacuna inactivada por calor MTBVAC (HK), por administración natural.

Modelo experimental aplicado.

Para los experimentos de **eficacia protectora** se utilizaron ratones hembras, de 8 a 10 semanas de edad. Cuatro estirpes: ratones C57BL/6 (Janvier Biolabs), ratones pIgR -/- (una donación de Gerard Eberl, Institut Pasteur Paris), ratones IgA -/- (repositorio MMRRC), y ratones DBA/2J (Janvier Biolabs). Todos los ratones fueron vacunados por vía subcutánea con 5×10^5 UFC de vacuna BCG en 100 µl de PBS. Cuatro semanas después de la vacunación, los ratones se revacunaron por vía intranasal con 10^7 bacterias MTBVAC HK en 40 µl de PBS. Cuatro semanas más tarde los ratones se infectaron por vía intranasal con 150 UFC del patógeno H37Rv. La carga bacteriana se evaluó cuatro semanas después de la infección cultivando los homogenizados triturados de pulmones y bazo, que fueron sembrados en medio sólido. Se sacrificó un grupo de ratones infectados un día después de la infección para determinar la carga bacteriana inicial en los pulmones, lo que resultó en aproximadamente 20 UFC en todos los experimentos.

Para los experimentos con **animales neonatos**: los ratones se vacunaron por vía subcutánea con $2,5 \times 10^5$ UFC de BCG en 50 µl de PBS en los primeros tres días después del nacimiento. Los controles negativos de vacunación se inocularon con 50 µl de PBS. Se administraron 10^7 bacilos de MTBVAC HK por vía intranasal ocho semanas después. Cuatro semanas más tarde, se expuso a los ratones a 150 UFC de H37Rv para la determinación de la carga bacteriana, o con 10^4 UFC para la evaluación de la supervivencia. En este último caso, los síntomas asociados a la enfermedad (incluido el peso, el aspecto y el comportamiento individual/social) se controlaron 3 veces por semana y los ratones se sacrificaron de forma humanitaria de acuerdo con los criterios de valoración preestablecidos.

4º Modelo: Puesta a punto de diferentes modelos en ratón para el estudio de la eficacia terapéutica de BCG y MTBVAC, administradas por vía pulmonar, contra el asma alérgica.

Las vacunas vivas atenuadas, como la vacuna BCG, se han relacionado con un tipo de respuesta de inmunidad celular tipo TH1, y han sido propuestas como posibles moduladores de la respuesta tipo TH2, que es la respuesta asociada con alergias y enfermedades hiperreactivas como el asma alérgica. En nuestro estudio utilizamos diferentes modelos murinos de asma, agudos y crónicos. Para estudiar el posible efecto o la eficacia que produce la administración intranasal de las vacunas vivas BCG y MTBVAC en ratones con asma, inducimos primero la alergia a través de la sensibilización a ovoalbúmina (OVA) y posteriormente tratamos los ratones con las vacunas antituberculosas por vía intranasal. El objetivo es regular del entorno inmunológico del pulmón asociado con la hiperreactividad de las vías respiratorias producida en el asma.

Modelo experimental aplicado.

Para la inducción de la hiperreactividad de las vías respiratorias específica por OVA, utilizamos ratones hembra de la estirpe C57BL/6 (Janvier Biolabs), de 8 a 10 semanas de edad. Los animales fueron sensibilizados con dos inoculaciones intraperitoneales de 50 µg de OVA de huevo de gallina (polvo liofilizado, 98% Sigma) con 2 mg de hidróxido de aluminio (Sigma), con una semana de diferencia entre las 2 sensibilizaciones.

En el modelo de **asma aguda**, una semana después de la segunda sensibilización, los ratones fueron vacunados con una única administración intranasal de BCG o MTBVAC, con 10^6 o 10^7 UFC respectivamente en 40 µl de inóculo por ratón. Cuatro semanas después de la vacunación, los animales se enfrentaron al alérgeno por vía intranasal con 100 µg OVA suspendida en PBS durante 3 días consecutivos, sacrificando a los animales un día después del último desafío con antígeno. En alguno de los experimentos la exposición a la OVA se retrasó hasta 4 meses después de la vacunación con BCG para evaluar la persistencia del tratamiento a largo plazo.

En el modelo de **asma crónica**, tres semanas después de las sensibilizaciones, los ratones se expusieron a 10 µg de OVA dos veces por semana durante ocho semanas por vía intranasal. En este caso, las vacunas se administraron en la semana 9 del inicio del procedimiento, en mitad del proceso del desarrollo asmático.

Para el modelo de **asma crónica por hipersensibilidad al polvo de ácaros doméstico (HDM)**, los ratones fueron sensibilizados por instilación intranasal dos veces a la semana durante tres semanas consecutivas con 10 µg/ratón de HDM. Las vacunas fueron administradas en la semana 4 del experimento (es decir, una semana después del último desafío con HDM), y en la semana 8 del experimento los ratones fueron desafiados intranasalmente con 10 µg/ratón de HDM durante tres días consecutivos. Un día después de la última administración de HDM, los animales se sacrificaron para valorar la patología en pulmones.

En el modelo de **asma y tratamiento con dexametasona** los ratones fueron sensibilizados con OVA y posteriormente fueron tratados por vía intraperitoneal con 5 mg/kg de dexametasona (DEX) (Dexametasona soluble en agua, Sigma-Aldrich). La pauta de tratamiento comenzó el día antes de iniciar la fase de desafío, y luego tres inoculaciones más con DEX administrada 1 hora antes de cada desafío con OVA intranasal.

NORMATIVA COMUN APLICADA a los procedimientos con animales de experimentación.

En todos los modelos experimentales los ratones se mantuvieron bajo condiciones controladas y observando si aparecía cualquier signo de enfermedad. El trabajo experimental se realizó de acuerdo con las directivas europeas (Directiva 2010/63/UE) y nacionales (RD 53/2013) para la protección de animales de experimentación y con la aprobación del Comité de Ética y Asesoramiento para la Experimentación Animal de la Universidad de Zaragoza.

Publicación nº 1, protocolo PI46/14

Publicación nº 2, protocolo PI50/14 y PI33/15

Publicación nº 3, protocolos PI14/14, PI50/14 y PI33/15

Publicación nº 4, protocolo PI22/15

TRABAJOS PUBLICADOS

PUBLICACIÓN 1:

Therapeutic efficacy of the live-attenuated *Mycobacterium tuberculosis* vaccine, MTBVAC, in a preclinical model of bladder cancer.

Therapeutic efficacy of the live-attenuated *Mycobacterium tuberculosis* vaccine, MTBVAC, in a preclinical model of bladder cancer



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ZARAGOZA, MADRID AND BELLATERRA, BARCELONA, SPAIN AND LAUSANNE, SWITZERLAND

Intravesical instillation of bacillus Calmette-Guérin (BCG) has been a first-line therapy for non-muscle-invasive bladder cancer for the last 4 decades. However, this treatment causes serious adverse events in a significant number of patients and a substantial percentage of recurrence episodes. MTBVAC is a live-attenuated vaccine derived from a *Mycobacterium tuberculosis* clinical isolate and is currently under evaluation in clinical trials to replace BCG as a tuberculosis vaccine. Here, we describe for the first time the potential of MTBVAC as a bladder cancer therapy *in vitro* and *in vivo* in a preclinical model. MTBVAC colonized human bladder tumor cells to a much greater extent than BCG via a mechanism mediated by macropinocytosis and induced cell growth inhibition after internalization. *In vivo* testing in an orthotopic murine model of bladder cancer demonstrated a higher antitumor effect of MTBVAC in experimental conditions in which BCG did not work. Our data encourage further studies to support the possible application of MTBVAC as a new immunotherapeutic agent for bladder cancer. (Translational Research 2018;197:32–42)

Abbreviations: BC = Bladder cancer; BCG = Bacillus Calmette-Guérin; MTBVAC = Mycobacterium tuberculosis vaccine

INTRODUCTION

Bladder carcinoma (BC) is one of the most frequently occurring types of cancer worldwide, especially in developed countries.¹ Established more than 4 decades ago,² intravesical instillation of the tuberculosis vaccine *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) after tumor resection is still a first-line therapy for high-risk non-muscle-invasive bladder cancer to prevent tumor progression and recurrence.² The current generally accepted regimen consists of an induction phase of 6 weekly instillations of BCG, followed by a maintenance regimen of 3 weekly instillations every 3 and 6 months for more than 3 years.³ Recurrence-free survival at 5 years with this regimen ranges from 60% to approximately 90%, depending on the study.^{4,5} In the case of patients who received only BCG at the induction phase, this percentage decreases to 40%.³ BCG is considered a therapy that is well tolerated by many patients. However,

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AT A GLANCE COMMENTARY

Alvarez-Arguedas S, et al.

Background

Live-attenuated bacillus Calmette-Guérin (BCG) is a first-line treatment for non-muscle-invasive bladder cancer. Nevertheless, BCG treatment fails in a significant percentage of patients. Additionally, periodic shortages of BCG supply represent a serious problem that obligates the use of less efficient alternative chemotherapeutic treatments.

Translational Significance

Our data demonstrate an antitumor effect of the novel live-attenuated *Mycobacterium tuberculosis* vaccine, MTBVAC, in an orthotopic murine model of bladder cancer. Notably, MTBVAC is currently under clinical evaluation as tuberculosis vaccine. Our results suggest that MTBVAC could be a promising candidate and support its further exploration as a novel bladder cancer immunotherapy.

there are still a significant number of people who must discontinue this treatment because of severe adverse events.⁶

The mechanism of action of BCG against BC has not been completely elucidated. The possible involvement of BC cells includes fibronectin-mediated attachment and internalization of BCG,⁷ secretion of cytokines and chemokines by tumor cells, and presentation of BCG and cancer cell antigens to the immune system.⁸ There is a broad consensus that a competent immune system is required to obtain an effective response, and many cell types from both the innate and the adaptive systems, including CD4+ and CD8+ T cell lymphocytes, as well as natural killer cells, granulocytes, macrophages, and dendritic cells, have been implicated in this process.⁸

MTBVAC is a live vaccine based on rational attenuation of a clinical isolate of *Mycobacterium tuberculosis* that conserves the whole gene repertoire absent in BCG (derived from *Mycobacterium bovis* originally isolated from cattle).⁹ MTBVAC attenuation is conferred by 2 independent unmarked deletions in the *phoP* and *fadD26* virulence genes. PhoP is a transcription factor that controls approximately 2% of the coding capacity of the *M. tuberculosis* genome and is mainly involved in virulence. Deletion of *fadD26* leads to complete abolishment of phthiocerol dimycocerosate synthesis, known to be essential for virulence.¹⁰ Intradermal MTBVAC administration has shown an excellent safety profile in healthy

human adults,¹¹ and currently, it is being evaluated in newborns (clinical trial identifier: NCT02729571).

MTBVAC has shown better immunogenic properties than BCG in different preclinical models.¹⁰ As a result, we hypothesize that this live vaccine could be a good approach to treat BC. In the present study, we studied the ability of MTBVAC to infect human bladder cancer cells and its cytotoxic effect in vitro compared with BCG. In addition, we evaluated the efficacy of MTBVAC in vivo in an orthotopic mouse model of BC. To our knowledge, this study is the first in which a live-attenuated *M. tuberculosis* strain has been evaluated as a BC treatment.

METHODS

Bacteria. BCG stock used in the present study was prepared from a commercial vial of BCG-MEDAC, which is one of the BCG formulations licensed for bladder cancer treatment. As indicated by the manufacturer, BCG-MEDAC was obtained from a seed of BCG Pasteur (strain 1173P2; Institut Pasteur, Paris, France). MTBVAC vaccine was generated in our laboratory by deletion of the virulence factors *phoP* and *fadD26* in the clinical isolate *M. tuberculosis* Mt103.⁹ Both vaccine strains were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC (Difco) and 0.05% (v/v) Tween-80 (Sigma) or on solid Middlebrook 7H10 (Difco) supplemented with 10% ADC. For in vitro experiments, BCG and MTBVAC were transformed by electroporation with an integrative pMV361 plasmid encoding green fluorescent protein (GFP) (a kind gift from Christophe Guilhot, Toulouse, France).

Cells and infections. Human J82 and T24 cells,¹² murine MB49-luc cells,¹³ and murine MH-S cells (HPA Culture Collections, catalog number 95090612) were used in the present study. All experiments were performed with cells thawed from the original frozen stocks prepared after cell line acquisition. Cells were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% inactivated fetal bovine serum (Biological Industries), 2 mM glutamine (Biological Industries), and antibiotics (penicillin/streptomycin/ciprofloxacin) (Sigma). All experiments were performed using cells with less than 5 passages from its thawing. Cells were routinely cultured in the presence of ciprofloxacin (10 µg/mL) to prevent mycoplasma contamination. Mycoplasma absence was confirmed after finalizing the experiments using the MycoAlert PLUS Mycoplasma Detection Kit (Lonza).

For in vitro experiments, cells were seeded in complete medium without antibiotics the day before infection. A total of 10⁴ cells were seeded in 96-well plates and allowed to attach to plastic overnight. Log-phase bacterial cultures were centrifuged at 100 g to remove clumps,

and bacterial density in the supernatant was determined by optical density. Then, bacterial suspensions for the indicated multiplicity of infection (MOI) were prepared in complete medium (without antibiotics) and added to cell cultures during the indicated times. The inhibitors wortmannin (Millipore), IPA-3 (Millipore), ethyl-isopropyl amiloride (EIPA) (Sigma), and nystatin (Sigma) were added to the cultures at the indicated concentrations 1 hour before the bacterial suspensions.

To monitor infected cells by flow cytometry, infections were performed with GFP-expressing BCG and MTBVAC. At the indicated time points, cells were detached with trypsin and fixed with 4% paraformaldehyde. Infections were analyzed with a FACS Aria flow cytometer (BD Biosciences).

Mice and in vivo experiments. All mice were kept under controlled conditions and observed for any sign of disease. Experimental work was conducted in agreement with European and national directives for protection of experimental animals and with approval from the Ethics Committee of the University of Zaragoza (approved protocol PI46/12).

Intravesical instillations were performed following the protocols described previously.^{14,15} C57BL/6 female mice were anesthetized with isoflurane, and urine was drained by slight pressure to the lower abdomen. After disinfecting the urethral orifice with iodine, a 24-gauge catheter (BD Insyte Autoguard, Becton Dickinson) connected to a 1-mL syringe was carefully inserted through the urethra. The catheter and syringe were maintained during the different procedures.

For tumor implantation, mouse bladders were pretreated with 100 µL of poly-L-lysine (0.1 mg/mL) for 20 minutes. Then, 100,000 MB49-Luc cells were instilled per mouse and retained for 60 minutes. Three days after bladder tumor induction, mice were randomly divided into 3 groups (6–9 animals/group) and intravesically treated with 100 µL of BCG Pasteur or MTBVAC in PBS (10^7 CFU (colony-forming unit)/animal) for 1 hour or with the same volume of PBS as a control. The number of bacterial CFUs was determined for all treatments by plating on 7H10 ADC. Treatment schedule was based on a previously described protocol.¹⁴ Mycobacteria were administered at days 3, 9, and 16 after tumor implantation. For in vivo tumor visualization, mice were inoculated intraperitoneally at days 7 and 14 after inoculation with 200 µL of 15 mg/mL d-luciferin potassium salt (Perkin Elmer), and luminescence was analyzed with an IVIS lumina imaging device (Xenogen). The animals that did not show any luminescence signal and presence of hematuria at the initial stages of the experiment were discarded from the study as we considered that tumor cells had not been successfully implanted in these animals. Absence

of tumors in these mice was corroborated by pathologic examination of the bladders.

Animals were monitored for 60 days and scored 3 times a week based on weight loss percentage, presence of hematuria, tumor presence, and general behavior. Mice were sacrificed based on pre-established end point criteria, and the bladders were harvested and fixed for hematoxylin-eosin staining and tumor presence evaluation.

Confocal microscopy. For confocal microscopy studies, 4×10^5 cells of the indicated cell lines were seeded in 24-well plates containing sterile 12-mm-round cover glasses. At the time points indicated, the cells were fixed, and the nuclei were stained with Hoechst 33342 (Invitrogen) for 15 minutes at room temperature. In the indicated experiments, cells were incubated with Alexa Fluor₅₉₄-labeled dextran (10,000 MW) (Invitrogen) at a final concentration of 0.5 mg/mL and incubated for 30 minutes. In another set of experiments, an anti-Mtb antibody (Acris GmbH) (1/500) followed by phycoerythrin (PE)-conjugated anti-rabbit IgG (1/500) was added to nonpermeabilized cells or cells permeabilized using a Cytofix/Cytoperm kit (BD Biosciences). Following a washing step with deionized water, microscope preparations were performed by placing the cover glass over a slide with a 3-µL drop of Prolong Gold Antifade reagent (Invitrogen). Images were acquired with a Fluoview FV10i confocal microscope (Olympus).

Cell growth inhibition assay. Cell growth was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma). After culture supernatants were removed, and the cells were washed 3 times with PBS, 1 mg/mL MTT in complete medium was added and incubated with the cells for 3 hours at 37°C. After the medium was removed, water-insoluble dark blue formazan was dissolved in 6 N HCl in 1-propanol (acidic isopropanol) for 1 hour at room temperature. Finally, absorbance was measured at 570 nm (MTT) and 690 nm (background) using a plate reader (Biotek Synergy HT).

To assess cell death, plasma membrane integrity was evaluated with 7-actinomycin D (BD Biosciences) staining according to the manufacturer's instructions, and stained cells were analyzed using flow cytometry.

Statistics. Sample size calculation was performed with the StatsToDo online tool (https://www.statstodo.com/SSizSurvival_Pgm.php). GraphPrism software (GraphPad Software) was used for the rest of statistical analysis. Sample size used in the animal experiments was powered enough to demonstrate statistical differences between MTBVAC-treated and nontreated groups ($\alpha = 0.05$; $[1 - \beta] = 0.8$). The statistical tests chosen for each experiment are indicated in the figure legends. All statistical tests used were two-tailed. Differences were considered

significant at $P < 0.05$. Analysis of the results of this study was not blinded.

RESULTS

Increased capacity of MTBVAC to infect human bladder tumor cells.

The need for close contact between BCG and urothelial tumor cells to trigger an efficient antitumor response is well accepted, and strategies to enhance this contact have been shown to improve treatment efficacy.¹⁶ Thus, we first evaluated in vitro MTBVAC internalization by bladder tumor cells. We performed these studies using the cell lines T24 and J82, which have been widely used for this type of experiment. We incubated cells with GFP-expressing MTBVAC at a low (10:1) and high (100:1) multiplicity of infection (MOI), and percentage of infected cells was monitored daily by flow cytometry for 1 week. The differences between both MOIs were substantial (Fig. 1, A). MTBVAC infection at a high MOI

reached 80% of the cells before day 3. At MOI 10, the infection kinetics increased progressively until reaching 40% and 80% of J82 and T24 cells, respectively, at day 7. Comparison of MTBVAC and BCG showed dramatic differences between both strains. Even with an MOI of 100:1, our results indicated the limited infectivity of BCG, with an infectivity lower than 20% at 7 days after infection (Fig. 1, B), a value that is in agreement with previous study.¹⁷ MH-S murine macrophage infection results were comparable for both strains, indicating that the BCG used in this study did not present any alterations that led to lower infection capacity (Supplementary Fig. S1).

Because flow cytometry did not allow for the discernment of whether bacteria were internalized or attached to the plasma membrane, we visualized infected cultures using confocal microscopy. These data confirmed a general cytosolic localization of the bacteria and corroborated the flow cytometry results, showing a substantial

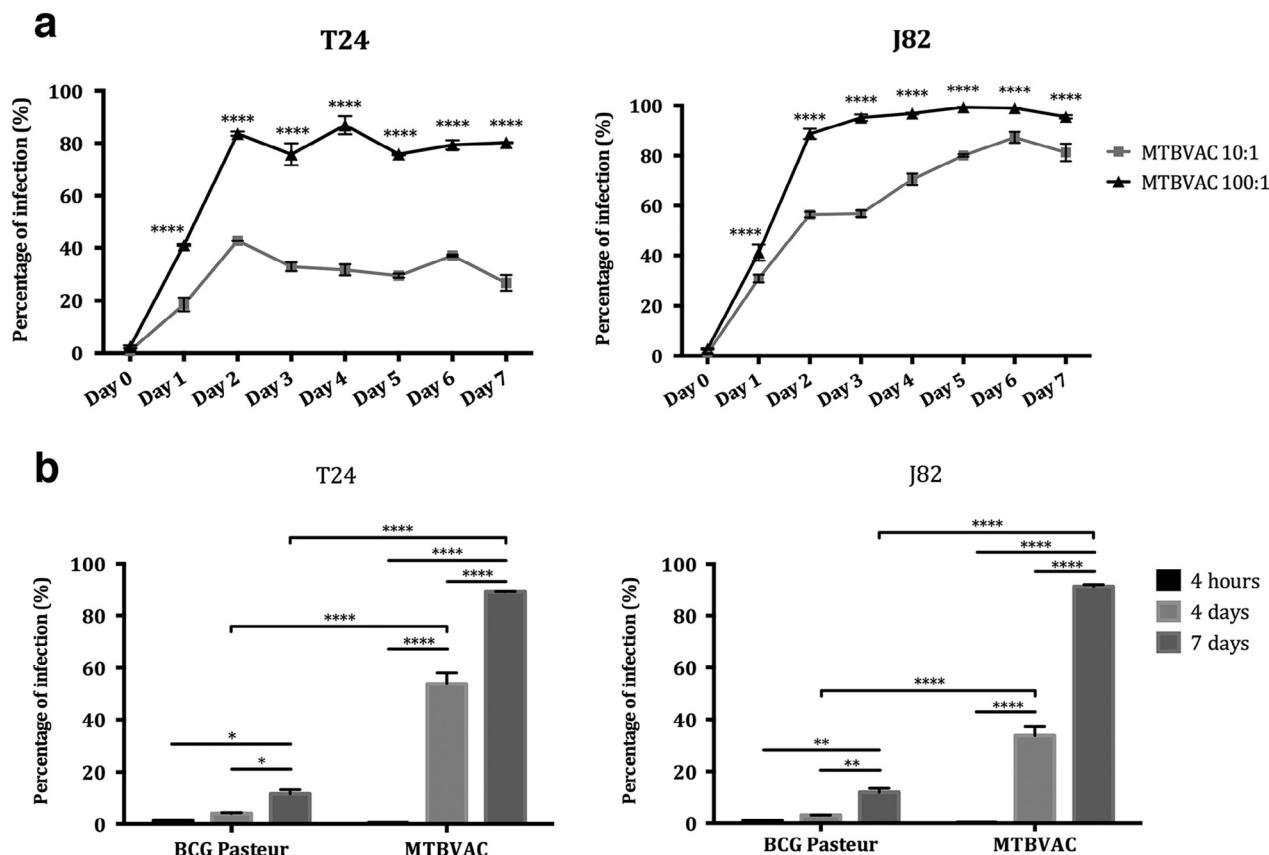


Fig 1. MTBVAC and bacillus Calmette-Guérin (BCG) infectivity in J82 and T24 cells. J82 and T24 cells were infected with green fluorescent protein-expressing BCG Pasteur or MTBVAC strains, and the percentage of infection was analyzed using flow cytometry at the indicated time points. **A**, MTBVAC infection kinetics over 7 days. **B**, BCG and MTBVAC infectivity comparison at multiplicity of infection of 20:1. The data in the graphs are presented as mean \pm standard deviation. A representative experiment of 2 (A) and 3 (B) experiments is shown in the figure. Statistical analysis was performed using two-way analysis of variance and Bonferroni's post-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

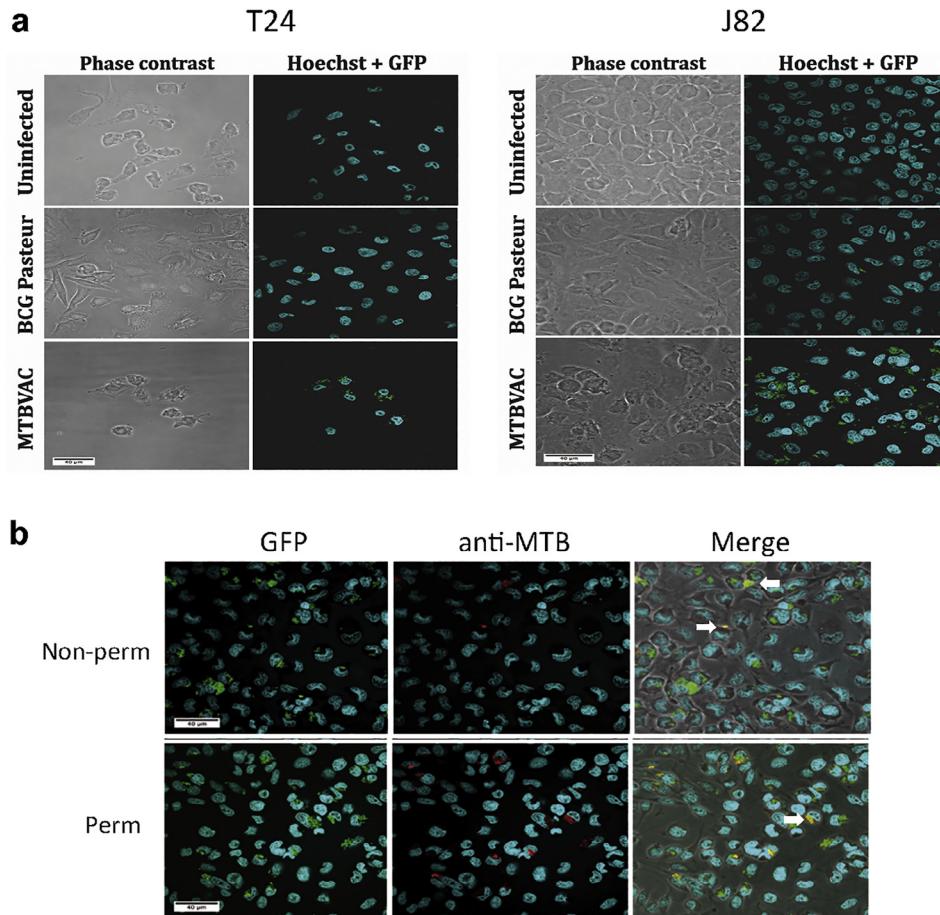


Fig 2. MTBVAC and bacillus Calmette-Guérin (BCG) are internalized by J82 and T24 cells. **A**, T24 (left) or J82 (right) cells were infected with BCG Pasteur-GFP or MTBVAC-GFP (multiplicity of infection [MOI] 50:1) for 4 days, and bacterial localization was analyzed with confocal microscopy. Phase contrast (PC) images (left) and merged images of Hoechst 33342 (blue) and green fluorescent protein (GFP) (green) fluorescence signals (right) are shown. **B**, Infected cells (MOI 10:1) were labeled with an anti-Mtb followed by phycoerythrin (PE)-conjugated secondary antibody in the absence of permeabilization (upper panels) or after permeabilization with saponin (lower panels). White arrows indicate PE-labeled bacteria attached to the plasma membrane. The images are representatives of 2 independent experiments. Scale bar: 40 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

qualitative increase in the number of infected cells in the case of MTBVAC compared with the results for BCG (Fig. 2, A). In addition, we added a PE-conjugated antibody specific for mycobacteria to the culture. Thus, bacteria attached to cells were labeled with the antibody, whereas internalized bacteria were unaffected by the presence of the antibody. This experiment confirmed the predominant intracellular localization of MTBVAC and BCG after infection (Fig. 2, B).

MTBVAC infection inhibits human bladder cancer cell growth. Even though it is controversial, some authors have reported that BCG is able to exert cell growth inhibition on tumor cells.¹² To evaluate this claim, we used an MTT assay to assess cellular growth of T24 and J82 cultures previously infected with BCG or MTBVAC. Our

results showed a strong dose-response profile in the case of MTBVAC between the MOI and the rate of inhibition. At low MOI (10:1), the inhibitory effect of MTBVAC was limited, but was close to 80% at an MOI of 100:1. Remarkably, in the case of BCG, we did not detect any inhibitory effect under the conditions tested, even at an MOI of 100 bacteria per cell (Fig. 3, A). Microscopy images confirmed these results, showing a lower number of cells after MTBVAC long-term infection (Fig. 3, B). Altogether, these data showed that MTBVAC was able to inhibit cell growth in experimental conditions in which BCG did not exert direct cell growth inhibition.

Macropinocytosis contributes to MTBVAC internalization. With the aim of elucidating the internalization mechanism of MTBVAC, we infected cells in the presence of

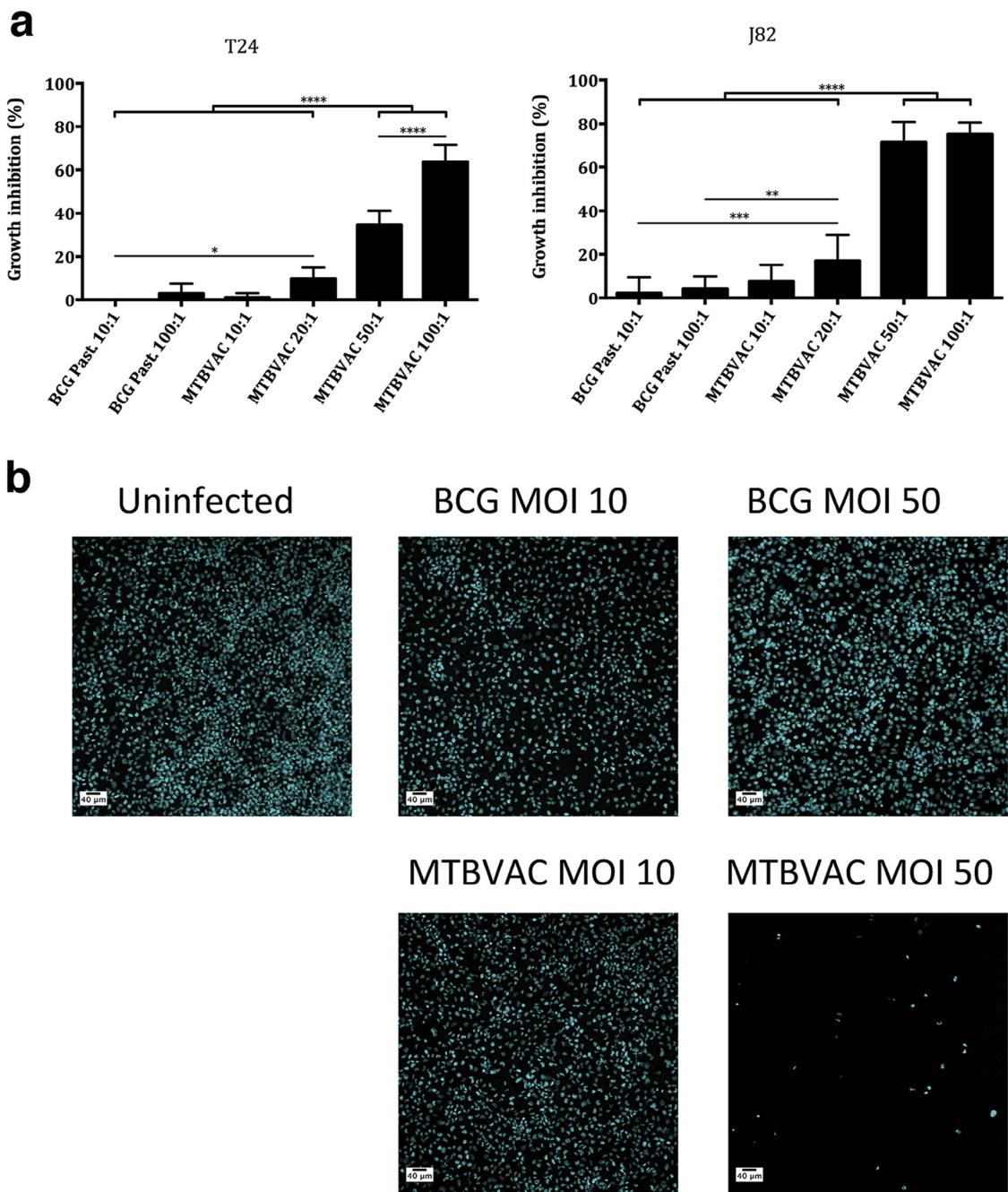


Fig 3. MTBVAC infection inhibits cell growth. **A**, T24 and J82 cells were infected with green fluorescent protein-expressing BCG Pasteur or MTBVAC strains at the indicated multiplicity of infection (MOI) for 4 days, and cell growth was analyzed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The data in the graphs represent mean \pm standard deviation. A representative experiment of 2 experiments is shown in the figure. Statistical analysis was performed with one-way analysis of variance and Bonferroni's post-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. **B**, Representative confocal images of Hoechst 33342-stained T24 cells infected with BCG and MTBVAC at the indicated MOI for 4 days. A representative experiment of 2 experiments is shown in the figure.

different inhibitors described to be involved in the mechanism of BCG engulfment¹⁷: nystatin, a cholesterol-depleting agent that disrupts lipid rafts and endocytosis; wortmannin, an inhibitor of the PI3K pathway; IPA-3, an inhibitor of p21-activated kinase (PAK); and finally, EIPA, an inhibitor of Na^+/H^+ exchangers and a well-known inhibitor of macropinocytosis. We first analyzed the toxicity of these molecules over J82 and T24 cells at different concentrations, with the aim of identifying nontoxic concentrations for subsequent experiments (*Supplementary Fig. S2*).

Once nontoxic concentrations were determined, we added the inhibitors to MTBVAC-infected cultures. Results showed no effect in the case of IPA-3, nystatin and wortmannin, whereas they demonstrated a wide inhibition of MTBVAC entry after incubation with EIPA, suggesting a strong contribution of macropinocytosis to MTBVAC internalization (*Fig. 4, A*). Inhibition by EIPA was corroborated with confocal microscopy (*Fig. 4, B*). As a control group, we tested the capacity of EIPA to inhibit dextran internalization, a molecule previously described to enter cells through macropinocytosis¹⁸ (*Supplementary Fig. S3*). EIPA also impaired BCG engulfment (*Fig. 4, C*), demonstrating that macropinocytosis was also involved in BCG engulfment, in accordance with published results.¹⁷

In addition, we performed an MTT assay of infected cultures in the presence of EIPA. The results showed a partial reduction in the growth inhibition induced by MTBVAC in the presence of EIPA (*Fig. 4, D*). These results suggest that bacterial entry into tumor cells is required at least in part to impair cell growth.

Antitumor effect of intravesical treatment with MTBVAC in an orthotopic mouse model of BC. Finally, we aimed to compare the *in vivo* efficacy of MTBVAC with that of BCG in a physiological model of BC. To this end, we used a well-accepted murine orthotopic model induced by intravesical instillation of MB49 cells. Before carrying out this study, we performed some *in vitro* experiments to confirm that MB49 cells' behavior was comparable with that previously observed in the case of human cells. Both flow cytometry and confocal microscopy experiments indicated a higher infectivity of MTBVAC in MB49 murine cells in comparison with BCG (*Fig. 5*).

For *in vivo* experiments, we administered 3 mycobacteria treatments, with the first instillation at 3 days after tumor implantation (*Fig. 6, A*). Animals were followed-up and euthanized when required according to predefined end point criteria. After tumor cell administration, hematuria events were observed in all mice included in the study in the following days after instillation, indicating that the cells had been implanted successfully in the bladder. In addition, as we used a luciferase-expressing version of MB49 cells, we assessed tumor

implantation by visualization of the luminescence produced by tumor cells in the bladder area (*Supplementary Fig. S4*). Animals that did not show any luminescence signal and hematuria at the initial stages of the experiment were discarded from the study as we considered that tumor cells had not been successfully implanted in these mice.

The results revealed 60% (7/12) mortality in the PBS-treated group, 40% (7/18) in the BCG-treated group, and less than 10% (1/13) in the MTBVAC-treated group (*Fig. 6, B*). Deceased animals showed bladder positivity for tumor presence in all cases (*Supplementary Fig. S5*). At day 60, the remaining mice were sacrificed and found to be tumor-free in all cases. Statistical analysis indicated a significant effect in the MTBVAC group compared with that in the PBS-treated mice ($P < 0.05$). When both vaccine treatments were compared, despite the higher survival rate in the case of MTBVAC, we did not find significant differences between the groups ($P = 0.079$). Finally, comparison of BCG- and PBS-treated animals showed no significant difference ($P > 0.05$) (*Fig. 6, B*).

DISCUSSION

Live-attenuated BCG is the first choice to treat non-muscle-invasive BC and is one of the most successful biotherapies for cancer. Nonetheless, there is a wide margin for BCG improvement because this treatment fails in up to 50% of patients, considering recurrence episodes and patients who must withdraw from treatment because of severe adverse events, who represent approximately 10% of BC cases.⁴ In addition, a high proportion of patients experience side effects less serious but that affect their lifestyle, with more than 60% and 30% reporting local and systemic effects, respectively.⁶ Moreover, BCG is not a defined strain but a diverse family of substrains with genetic heterogeneity. As a result, there are phenotypic differences among BCG substrains with respect to antigenicity and reactogenicity.¹⁹ In addition, we should consider the periodic shortages of BCG supply, which represent a serious problem that necessitates the use of suboptimal BCG doses or alternative chemotherapeutic treatments with lower efficacy.²⁰ Thus, search for novel treatments that are more efficient and less toxic than BCG is justified.

It is assumed that close contact between BCG and tumor cells is necessary for optimal therapeutic effects,⁸ and it has been observed that *in vivo* blocking of BCG internalization in mouse models impairs treatment efficacy.²¹ In this regard, we have demonstrated *in vitro* the superior ability of MTBVAC to colonize bladder tumor cells (human and murine) compared with that of BCG, and found a strong contribution of macropinocytosis in this process (although other

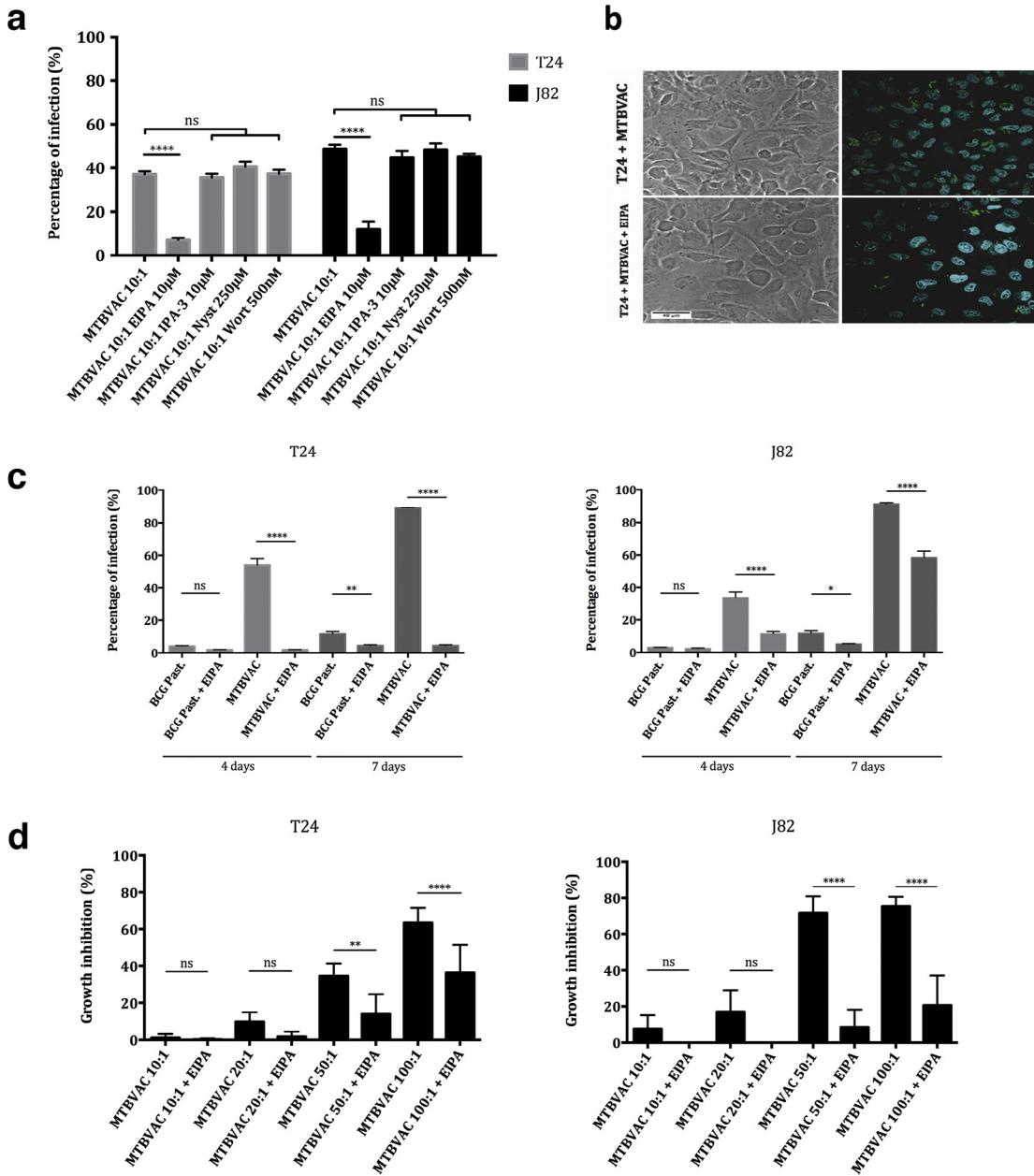


Fig 4. MTBVAC is internalized by macropinocytosis. **A, B**, T24 and J82 cells were infected with green fluorescent protein-expressing MTBVAC strain at multiplicity of infection (MOI) 10:1 in the presence of ethylisopropyl amiloride (EIPA), IPA-3, nystatin, or wortmannin at the concentrations indicated, and the percentage of infection was analyzed with flow cytometry or confocal microscopy at 4 days. **C**, T24 and J82 cells were infected with BCG or MTBVAC at MOI 20:1 in the presence of 10 µM EIPA, and the percentage of infection was analyzed by cytometry. **D**, T24 and J82 cell growth inhibition was analyzed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay 4 days after infection. The data in the graphs are presented as mean ± standard deviation. A representative experiment out of 2 experiments is shown in the figure. Statistical analysis was performed with one-way analysis of variance (ANOVA) (A) or two-way ANOVA (C, D) and Bonferroni's post-test. ns, nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

mechanisms of internalization cannot be excluded to participate). We could speculate that this higher capacity of infection could be a concern in terms of safety. However, according to our data, this risk seems unlikely. MTBVAC

has been extensively characterized in different animal models, and safety has been evaluated in a human cohort. In all cases, the persistence and reactogenicity of MTBVAC was similar to or lower than that observed with

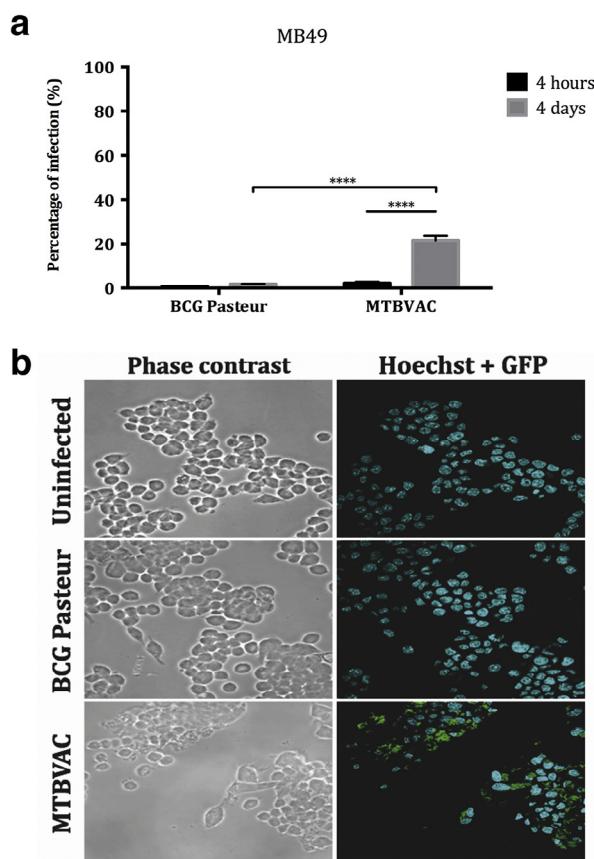


Fig 5. MTBVAC and bacillus Calmette-Guérin (BCG) infectivity in MB49 cells. MB49 murine cells were infected with green fluorescent protein (GFP)-expressing BCG Pasteur or MTBVAC strains (multiplicity of infection 20:1), and the percentage of infected cells was analyzed with flow cytometry (**A**) and confocal microscopy (**B**). The data in the graph represent mean value \pm standard deviation of 1 representative of 3 independently performed experiments. *** P < 0.0001, determined by two-way analysis of variance and Bonferroni's post-test.

BCG, and when we studied vaccine biodistribution in mice, presence of bacteria was restricted in all cases to lymph nodes and spleen, and MTBVAC was never detected in nonlymphoid organs.^{9,11}

BCG internalization is mediated by direct binding of bacteria to fibronectin through fibronectin-attachment proteins.⁷ Two main protein complexes have been described to bind directly to fibronectin: fibronectin-attachment protein, which corresponds to the secreted antigen MPT32 from *M. tuberculosis*,²¹ and Ag85A, Ag85B, and Ag85C from the Ag85 complex.²² MPT32, Ag85A, and Ag85C have been reported to be substrates of the twin-arginine translocation (TAT) system.^{23,24} Interestingly, our previous study revealed that deletion of *phoP* in MTBVAC leads to profound downregulation of the noncoding RNA *mcr7*, which is a negative regulator of the TAT system, and therefore, MTBVAC demonstrates a greater capacity to secrete TAT substrates

(including Ag85A and Ag85C).²⁴ In addition, BCG expresses a polymorphism in the *ag85b* gene that drives the expression of an unstable protein²⁵ that is indeed absent in the extracellular fraction of BCG cultures.²⁶ Together, these processes could result in a higher capacity of MTBVAC to bind fibronectin, with the subsequent attachment to urothelial cells and internalization.

The significance of BCG-induced direct cytotoxicity to treatment outcome is controversial,⁸ with several studies showing different findings that might be attributed to the differences in experimental conditions, or the BCG strains used in each work.^{12,17,27,28} Under our experimental conditions, we did not detect any antiproliferative effect associated with BCG incubation, even at high MOI values over 7 days. However, we observed significant cellular growth inhibition after MTBVAC infection. Our results indicate that MTBVAC entry into host cells is directly related to this effect and suggest that the antiproliferative effect triggered by MTBVAC could contribute to the antitumor response.

MTBVAC has been demonstrated to induce a strong Th1 response in different animal models and in humans.^{10,11} The Th1 response is normally associated with tumor immunosurveillance, which could also explain the therapeutic efficacy of MTBVAC in the present study. In addition, incubation of MTBVAC with human dendritic cells leads to increased production of inflammatory cytokines, such as TNF- α or IL-6, which are important for triggering the inflammatory cascade responsible for the BCG antitumor effect.²⁹ Interestingly, the higher infectivity of MTBVAC might drive higher presentation of mycobacteria-derived antigenic peptides by MHC molecules on the surface of infected tumor cells, and therefore, T cells specific for mycobacteria could also participate in the antitumor response. In this regard, BCG has demonstrated a higher efficacy as BC therapy in individuals with an immune response specific to mycobacterial antigens.¹⁴

In the present study, we have compared the antitumor efficacy of MTBVAC and the BCG substrain Pasteur. However, possible differences between BCG substrains in terms of clinical efficacy have been reported. Concretely, a previous study showed a lower level of recurrence in patients treated with BCG Connaught in comparison with BCG Tice, when maintenance regimen was not administered.³⁰ Thus, even though BCG Pasteur has shown comparable clinical benefits with respect to other BCG,³¹ we find it crucial to assess in the future the MTBVAC antitumor effect in comparison with other BCG substrains licensed as bladder cancer therapies, including the widely used Tice and Connaught.

In the present study, we have demonstrated MTBVAC efficacy in a relevant orthotopic mouse model of BC. In addition, our data indicate substantial differences between

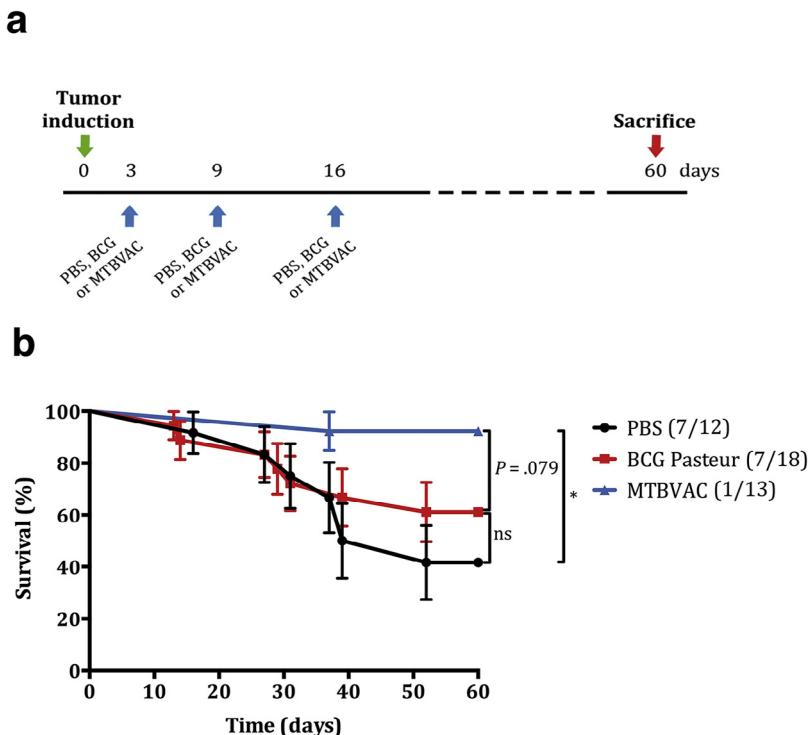


Fig 6. Intravesical treatment with MTBVAC increases the survival of tumor-bearing mice. **A**, Schedule of in vivo experiments. Groups of mice were instilled with 10^5 MB49-luc cells and treated intravesically at 3, 6, and 9 days after tumor induction with PBS or 10^7 CFU of BCG Pasteur or MTBVAC. **B**, Animals were followed up for 60 days, and survival was determined according to pre-established end point criteria approved by the ethics committee. Pooled data from 2 independent experiments are plotted in a survival curve. Data represent the percentage of surviving animals \pm standard deviation. Death or total events are represented in brackets. Statistical significance was calculated with a log-rank test after Benjamini-Hochberg multiple testing adjustment. ns, nonsignificant; $*P < 0.05$.

MTBVAC and BCG in terms of in vitro vaccine interaction with bladder tumor cells. Notably, GMP (Good Manufacturing Practices) production of MTBVAC as tuberculosis vaccine has been developed, and the vaccine has demonstrated an excellent safety profile and immunogenicity in humans, when delivered by the intradermal route. Altogether, our results support further exploration of MTBVAC as a potential novel bladder cancer immunotherapy.

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Conflicts of Interest: All authors have read the journal's authorship agreement. All authors have read and approved the manuscript for this submission. All authors have read the journal's policy on conflicts of interest. Carlos Martín is inventor of the patent "tuberculosis vaccine" filled by the University of Zaragoza (Application number: PCT/ES 2007/070051). The remaining authors declare no competing financial interests.

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Supplementary Data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.trsl.2018.03.004>.

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PUBLICACIÓN 2:

Live attenuated TB vaccines representing the three modern
Mycobacterium tuberculosis lineages reveal that the Euro-American genetic
background confers optimal vaccine potential.



Research paper

Live attenuated TB vaccines representing the three modern *Mycobacterium tuberculosis* lineages reveal that the Euro–American genetic background confers optimal vaccine potential



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ABSTRACT

Background: Human tuberculosis (TB) is caused by a plethora of *Mycobacterium tuberculosis* complex (MTBC) strains belonging to seven phylogenetic branches. Lineages 2, 3 and 4 are considered “modern” branches of the MTBC responsible for the majority of worldwide TB. Since the current BCG vaccine confers variable protection against pulmonary TB, new candidates are investigated. MTBVAC is the unique live attenuated vaccine based on *M. tuberculosis* in human clinical trials.

Methods: MTBVAC was originally constructed by unmarked *phoP* and *fadD26* deletions in a clinical isolate belonging to L4. Here we construct new vaccines based on isogenic gene deletions in clinical isolates of the L2 and L3 modern lineages. These three vaccine candidates were characterized at molecular level and also in animal experiments of protection and safety.

Findings: Safety studies in immunocompromised mice showed that MTBVAC-L2 was less attenuated than BCG Pasteur, while the original MTBVAC was found even more attenuated than BCG and MTBVAC-L3 showed an intermediate phenotype. The three MTBVAC candidates showed similar or superior protection compared to BCG in immunocompetent mice vaccinated with each MTBVAC candidate and challenged with three representative strains of the modern lineages.

Interpretation: MTBVAC vaccines, based on double *phoP* and *fadD26* deletions, protect against TB independently of the phylogenetic lineage used as template strain for their construction. Nevertheless, lineage L4 confers the best safety profile.

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1. Introduction

Tuberculosis (TB) is the most devastating disease caused by a single infectious agent over the last 200 years [1]. In 2018, the WHO estimated 10 million new TB cases and more than 1.4 million deaths caused by TB [2].

TB is principally caused in humans by *Mycobacterium tuberculosis* and *Mycobacterium africanum*, classified within the *M. tuberculosis*

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Research in context

Evidence before this study

Current vaccines against pneumococci, meningococci, influenza or polio are designed taking into account the existing pathogen variability. However, to our knowledge, none of the existing TB vaccine candidates that aim to improve the efficacy of the current vaccine BCG, have addressed vaccine efficacy taking into consideration the evolutionary landscape of TB-causing bacteria. Indeed, TB vaccine candidates under clinical development have exclusively demonstrated protection against *M. tuberculosis* strains belonging to a single lineage, of the seven existing phylogenetic lineages, of this pathogen. Since 2012, a live vaccine based on attenuated *M. tuberculosis* (MTBVAC) is in Phase 1 and Phase 2 clinical trials in newborns and adults and in 2021 will be ready for efficacy studies.

Added value of this study

In this manuscript, we reappraise TB vaccine efficacy in the context of the evolutionary genomics of *M. tuberculosis*. Here, we construct and characterize live attenuated vaccines based on MTBVAC in the three modern lineages of *M. tuberculosis*, which represent the majority of circulating strains worldwide. This MTBVAC set is subsequently tested in mouse models of safety and protective efficacy against challenge with three representative strains from each modern lineage. Notably, this is the first time demonstrating that a live attenuated vaccine in clinical development is able to protect mice against pathogens from the most prevalent lineages, a result translationally relevant for the future efficacy trials with MTBVAC. Additionally, this is the first time to study how the genetic background impacts on safety and protective efficacy of live attenuated TB vaccines.

Implications of all the available evidence

With this study, we aim to anticipate future TB efficacy trials. This study provides proof-of-concept with MTBVAC and we aim to establish recommendations for other vaccine candidates in the pipeline.

complex (MTBC) as human-adapted mycobacteria [3–5]. Occasionally, zoonotic TB in humans caused by animal-adapted members of the MTBC is also observed [6]. Human-adapted mycobacteria can be classified in “ancestral” or “modern” lineages based on the presence or absence of the *M. tuberculosis* specific deletion region TbD1 [7,8]. Lineages that harbor the TbD1 region include lineages 1 and 7 of *M. tuberculosis*, and lineages 5 and 6 of *M. africanum*. These are referred as ancient lineages and they are geographically restricted to specific areas, except for lineage 1, which shows an intermediate distribution, with main prevalence in South India and South East Asia [4,9]. By contrast, *M. tuberculosis* lineage 2, which are also known as representing Beijing strains, lineage 3 strains, known also as CAS/Dehli strains and lineage 4, corresponding to the Euro-American strain families are considered as modern lineages and they include worldwide distributed strains [9]. The widespread distribution of modern lineages probably reflects the adaptive evolution of the MTBC to transmit and cause disease in crowded, dense and urbanized populations, a hypothesis that was recently supported by data on increased resistance of modern lineages to oxidative stress and hypoxia relative to L1 strains [8]. As such, there is converging evidence from epidemiologic and experimental data, which suggests that strains from lineages 2, 3 and 4, have become more successful in terms of their geographical distribution, being responsible for a large proportion of the global TB burden [9].

The only vaccine licensed against TB is the Bacille Calmette and Guerin (BCG), which was obtained after *in vitro* passaging of *Mycobacterium bovis* –the causative agent of bovine TB– for 13 years at the beginning of the 20th century [10]. Loss of Region of Difference 1 (RD1) with respect to the MTBC members, substantially contributes to BCG attenuation [11,12]. This region codes for genes of the ESX-1 protein complex responsible for the co-secretion of ESAT-6 and CFP-10. Both proteins are major antigens of *M. tuberculosis*. Specifically, ESAT-6, secreted by ESX-1 type VII secretion system, has been extensively documented as a putative virulence and immunogenicity factor of MTBC pathogens. Among the various putative functions of ESAT-6, its implication in phagosomal rupture within infected phagocytes, is of particular interest, and enables ESX-1 proficient strains to get in contact with the host cytosol [13,14]. BCG was administered for the first time in 1921 and during the next decades BCG sub-strains emerged as a consequence of the parallel *in vitro* sub-cultivation of the original BCG in different laboratories [10,15]. BCG confers protection from the severe forms of the disease in children although protection against pulmonary TB in adolescents and adults is considerably variable [16]. Consequently, this main limitation of BCG to stop TB transmission imposes a priority in the research of new vaccines candidates. A rationale vaccine research requires to understand the MTBC virulence factors and immunogenicity conferred by mycobacterial components [17]. Today, a wide pipeline of vaccine candidates targeting different populations is currently in clinical trials including live attenuated vaccines, adjuvanted protein subunit vaccines, viral-vectorized vaccines and whole cell inactivated vaccines [18].

MTBVAC is currently the unique live vaccine candidate in clinical development which is based on an attenuated strain of the human pathogen *M. tuberculosis*. MTBVAC consists on the unmarked deletion of *phoP* and *fadD26* genes in the clinical *M. tuberculosis* isolate Mt103, representing a lineage 4 strain [19].

The gene *phoP* encodes the transcription factor of the Two-Component System PhoPR, which controls approximately 2% of the genome content of *M. tuberculosis* [20–23]. The PhoP regulon orchestrates diverse networks in *M. tuberculosis*, including the secretion of the ESAT-6 [24], the synthesis of sulfolipid and di- and polyacyltrehaloses [25], the expression of the non-coding RNA *mcr7*, impacting the activity of the Twin Arginine Translocation (Tat) secretion apparatus [23] and production of phosphatidylinositol mannosides [26]. The gene *fadD26* is the first gene in an operon required for the synthesis of phthiocerol dimycocerosates (PDIM) [27], mycobacterial virulence lipids that are involved in phagosomal rupture in concert with ESAT-6 [28–30]. Since MTBVAC is derived from an *M. tuberculosis* clinical isolate, it conserves the whole T cell epitope repertoire described for MTBC pathogens including the major immunodominant antigens ESAT-6, CFP-10 and PPE68 absent in BCG as a consequence of the RD1 deletion. These three proteins, albeit small, are unusually immunogenic and contain 285 of the total 1.603 epitopes described in *M. tuberculosis* [31]. In line with this observation, our recent preclinical studies have demonstrated that immunity against ESAT-6 and CFP-10 conferred after MTBVAC vaccination remarkably correlate with improved vaccine efficacy relative to BCG Danish [32].

After an extensive preclinical testing in animal models [19,33–36], MTBVAC was the first and unique live attenuated *M. tuberculosis* vaccine approved to enter into clinical trials in 2012 [37]. After successfully completed phases Ia (NCT02013245) [38] and Ib (NCT02729571) [39], currently, two phases IIa trials are ongoing in South Africa, one in newborns using MTBVAC as a prime vaccine (NCT03536117) and the other on in adults where MTBVAC is tested as a boost for BCG (NCT02933281).

In this study, we reappraise vaccine efficacy of newly constructed MTBVAC variants in the context of the global genetic diversity of the MTBC. The main aims of the study are linked to the question whether the original MTBVAC is able to protect against the three modern lineages of the MTBC or whether a vaccine candidate constructed in a specific lineage might lead to lineage-dependent improved protection.

2. Material and methods

2.1. Bacteria and culture conditions

M. tuberculosis strains used in this work are detailed in Table S1. Briefly, GC1237 [40], HMS13037 (a clinical isolate from our laboratory collection belonging to lineage 3 of *M. tuberculosis*), Mt103 [41], their respective *phoP-fadD26* mutants, H37Rv [42], W4 (kindly provided by Gill Kaplan) [43], HCU3524 (a clinical isolate from our laboratory collection belonging to lineage 3) and BCG Pasteur 1173P2, were grown in liquid media at 37 °C with Middlebrook 7H9 broth (Difco) supplemented with 10% (vol/vol) of ADC (0.5% bovine serum albumin, 0.2% dextrose, 0.085% NaCl and 0.0003% beef catalase) and 0.05% (vol/vol) Tween-80. For solid media, Middlebrook 7H10 (Difco) supplemented with ADC, except for the HMS13037 strain, supplemented with OADC (ADC with 0.005% (vol/vol) oleic acid) was used. When required, hygromycin (Hyg) 20 µg/ml or Kanamycin (Km) 20 µg/ml were added. *Escherichia coli* strains (MC1061, DH5α, DH10B, XL1-BLUE or BW25141) were grown in liquid media at 37 °C in Luria-Bertani (LB) broth. When required, ampicillin (Amp) 100 µg/ml, Km 20 µg/ml, Hyg, 50 µg/ml or chloramphenicol (Cm), 12.5 µg/ml were added. On solid media, LB-agar was used.

2.2. Construction of *fadD26* and *phoP* mutants

Isogenic deletions in *fadD26* and *phoP* genes present in the original MTBVAC vaccine [19] were obtained in GC1237 and HMS13037 strains using two different approaches: suicide plasmids and BAC-rec (see Table S2 for extended information about the vectors used). Suicide plasmid pAZ5 was used to obtain the *fadD26* deletion in GC1237 and pAZ18 was used to construct the *phoP* deletion in GC1237 and HMS13037 as described in Arbués and colleagues [19]. pAZ5 and pAZ18 harbor *fadD26* and *phoP* genes interrupted with the *res-Hyg-res* cassette respectively, Gentamicin resistance gene cassette, reporter gene *xylE* and the counter selectable marker *sacB*. Single recombinants were selected by plating transformants in Hyg-containing plates and testing positive *XylE* activity by addition of a catechol solution (0.55 g of catechol dissolved in 47.5 ml of water and 2.5 ml of PBS). Single recombinants were grown in liquid media and serial dilutions were plated onto 7H10 (ADC or OADC) containing 2% of sucrose (Suc) and Hyg to select double recombinants. *XylE* negative clones were confirmed by PCR using primers 5'upstream of 3'downstream of the region cloned in the plasmid, in *res* sites or in the gene deleted region. To remove the Hyg cassette, pAZ20 replicative plasmid harbouring γδ-resolvase, Km- and Gentamicin (Gm)-cassette resistance and the counter selectable marker *sacB* was used [19]. Unmarked deletion was confirmed by replica plating in Km, Hyg and w/o antibiotic plates and by PCR using the appropriate primers (Table S3). BAC-rec strategy was used to obtain *fadD26* deletion in HMS13037. A *M. tuberculosis* H37Rv bacterial artificial chromosome (BAC) library obtained in the pBelobAC11 vector contained in *E. coli* DH10B was used [44]. The clone DH10B carrying the Rv209 BAC containing the *fadD26* (Rv2930) gene was selected and the thermosensitive plasmid pKD46 carrying red recombinase from lambda phage was co-transformed [45]. DH10 Rv209 pKD46 induced with arabinose 0.15% was transformed with a PCR product (using *fadD26-P1-Fw* and *fadD26-P2* primers) containing the FRT-Km-FRT-cassette from pKD4 flanked with 40 bp of identity arms to target the *fadD26* gene. Gene deletion in the BAC was confirmed by PCR amplification using specific primers. BAC Rv209-Δ*fadD26*:Km was used as template to obtain the allelic exchange substrate (AES) using KO-*fadD26-Fw* and KO-*fadD26-Rv* primers to transform in mycobacteria. AES consists on the FRT-Km-FRT cassette flanked with 700 pb of identity arms of the specific site of recombination and was transformed in HMS13037 strain harbouring pJV53H (induced with 0.2% of acetamide) [46]. Recombinants were plated onto Km-containing plates

and were confirmed using appropriate primers. Loss of pJV53H plasmid was confirmed by replica plating onto Hyg and Km. To unmark the deletion, pRES-FLP-Mtb plasmid (Hyg^R) harbouring the *flp* recombinase from *Saccharomyces cerevisiae* with adapted codons for *M. tuberculosis* [47]. Transformants were selected in Hyg-containing plates and the removal of the cassette was confirmed by replica plating and PCR amplification using *fadD26F* and *fadD26R* primers. Loss of pRES-FLP-Mtb plasmid was confirmed by replica plating.

2.3. Protein extraction and Western blotting

Cultures were grown in 7H9-0.05% Tween-80 supplemented with dextrose, NaCl and catalase to avoid albumin contamination in the secreted fraction. Bacteria were grown until an OD of 0.6–0.8 at 37 °C and pelleted. Supernatant fractions were filtered through a 0.22 µm-pore-size filter and incubated on ice for 2 h with 10% (vol/vol) of trichloroacetic acid. Samples were centrifuged for 1 h at 4 °C and pellets were washed with acetone. Supernatants were discarded after centrifugation and pellets were air dried and dissolved in Tris 150 mM pH 8.8. For whole-cell protein extractions, bacteria were resuspended in PBS containing 1% Triton X-100 and transferred into tubes containing glass beads (MP Biomedicals). Suspensions were disrupted by Fast-Prep (6.5 m/s, 45 s) twice and samples were cooled on ice between the cycles. Supernatants containing soluble proteins were filtered through a 0.22 µm-pore-size filter were after centrifugation. Protein extractions were quantified using QuantiPro BCA assay (Sigma Aldrich). Samples were heated for 10 min at 100 °C after adding Laemmli buffer and samples were loaded in 12–17% polyacrylamide gels containing 0.1% SDS. Then, proteins were transferred to a PVDF membrane using a semi-dry electrophoretic transfer cell (Trans-Blot® Semi-Dry Transfer cell, Bio-Rad). Proteins were blocked with 5% (w/v) skimmed milk in TBS-T buffer (25 mM Tris pH = 7.5, 150 mM NaCl, 0.05% Tween 20) for 30 min and incubated overnight with the primary antibody. Membranes were then washed with TBS-T buffer before incubation with secondary antibodies for 1 h. Membranes were washed and signals were detected using chemiluminescent substrates (Western Bright™ Quantum, Advansta). Immunodetection was carried out using PhoP-antisera (1:5000), antibodies anti-sigA and anti-CFP-10 (Thermo Scientific) (both at 1:5000) followed with the incubation with secondary antibody anti-rabbit IgG human serum adsorbed conjugate (1:5000) (KPL) or incubation with monoclonal antibody anti-ESAT-6 (1:2500) (abcam), anti-GroEL2 (Hsp65) (1:2500) (Invitrogen) or anti-PE_PGRS (1:2000) (described in [48,49]), followed by incubation with an anti-mouse IgG human serum adsorbed conjugate (1:5000) (KPL). To reprobe blots, ReBlot Plus Strong Antibody Stripping Solution (Millipore) was used following the specification sheet.

2.4. RNA extraction and qRT-PCR

Bacteria were pelleted and resuspended in 250 µl in wash buffer (aqueous solution containing 0.137 M NaCl and 0.5% Tween 80) and 500 µl of RNA protect reagent (Qiagen) to avoid RNA degradation. Suspensions were centrifuged after 5-minute incubation at room temperature. Pellets were dissolved in lysis buffer (20 mM sodium acetate, 0.5% SDS, 0.1 mM EDTA) and 1 ml of phenol:chloroform (5:1), pH = 4.5. Bacterial suspensions were transferred into tubes containing glass beads (MP Biomedicals) and cells were lysed by Fast-Prep (2 cycles, 45 s at speed 6.5 m/s, samples were cooled on ice between cycles). Tubes were centrifuged and aqueous phases were transferred to a tube containing chloroform:isoamylic alcohol (24:1). After centrifugation upper phases were transferred to a tube containing isopropanol and 0.3 M sodium acetate (pH = 5.5) and tubes were incubated at -20 °C overnight. After centrifugation at 4 °C, precipitated nucleic acids were collected and pellets were washed with ethanol 70%. Samples were again centrifuged and pellets were dissolved in diethylpyrocarbonate (DEPC)-treated water (RNase free). DNA was

removed by two consecutive incubations of 1 h at 37 °C with Turbo DNA-free (Ambion) by addition of 1 µl of DNase. Later, RNA was purified by adding phenol:chloroform (5:1) pH = 4.5, and previous steps were repeated to precipitate, collect, dry and dissolve the RNA in DEPC-treated water. RNA integrity was confirmed by agarose gel electrophoresis and absence of DNA was confirmed by absence of amplification products after PCR. Reverse transcription was performed using PrimeScript™ RT Reagent Kit as detailed in the product manual. qRT-PCR reaction was performed in the StepOne Plus Real Time PCR System (Applied Biosystems) using TB Green Premix Ex Taq™ (Tli Rnase H Plus) (Takara) kit. For the reaction, primers at a final concentration of 0.25 µM and cDNA diluted 1:10 were used. Normalization was calculated using the *sigA* housekeeping gene in each sample. Absence of unspecific PCR products were confirmed after examination of melting curves in each sample.

2.5. Neutral red staining

Bacteria grown on solid media were transferred to a tube containing 50% of methanol in water. Supernatants were discarded after 1 h of incubation. Pellets were resuspended in 750 µl of barbital buffer (1% sodium barbital in 5% NaCl, pH 9.8). After centrifugation, bacteria were resuspended in 4 ml of barbital buffer. 150 µl of a solution of 0.05% neutral red in barbital buffer were added and incubated at 37 °C for 1 h. After incubation, supernatants were discarded to evaluate the staining of the pelleted bacteria.

2.6. Mouse infection

All mice were observed and kept under controlled conditions. For protective efficacy experiments, eight-week old immunocompetent C3H/HeNRj female mice were vaccinated subcutaneously with 10⁶ CFU in 100 µl of PBS. Mice were vaccinated with BCG, MTBVAC, MTBVAC-L2::hyg or MTBVAC-L3::hyg, or unvaccinated as control group. Eight weeks later, mice were challenged by intranasal route with 200 CFU in 40 µl of PBS of each *M. tuberculosis* strain belonging to one of the modern lineages (W4, HCU3524 or H37Rv belonging to lineages 2, 3 and 4 respectively). Four weeks post-challenge, bacterial burden was evaluated in lungs and spleen. Serial dilutions were plated onto 7H10-ADC. For attenuation experiment, eight-week old immunocompromised SCID female mice were inoculated by intraperitoneal route with 10⁶ bacteria in 100 µl PBS, equivalent to 2 times the dose of BCG recommended for humans. Mice were controlled and their weight was followed during the experiment. Experimental endpoint was defined when loss of weight was more than 20%.

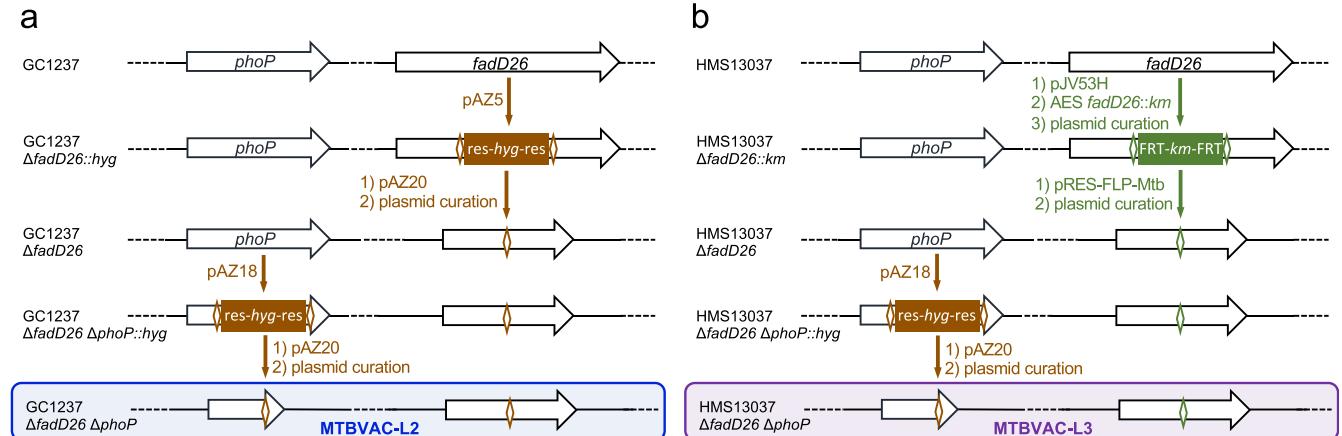


Fig. 1. Sequential genetic steps to construct unmarked *ΔphoP ΔfadD26* mutants in *M. tuberculosis* strains from lineages 2 and 3. (a) Construction of MTBVAC-L2. (b) Construction of MTBVAC-L3. Those steps involving the use of suicide plasmids pAZ5 or pAZ18 are colored in brown and utilization of the BAC-rec strategy is indicated by green colors. The *res* and FRT sites used for resolution of the hygromycin and kanamycin cassettes are indicated by brown and green open diamonds respectively. Each genetic step was confirmed by PCR and/or Western-Blot (see Supplementary Material).

2.7. Statistical analysis

For protection experiments, statistical analysis of CFUs in the organs was performed using One-way ANOVA, Bonferroni post-test. For safety experiments, the statistical analysis was calculated applying a Log-rank (Mantel-Cox) test.

2.8. Ethics statement

Experimental animal studies were performed in agreement with European and national directives for the protection of animal for experimental purposes. All procedures were carried out under Project Licenses PI50/14 (protective efficacy) and PI33/15 (attenuation) approved by the Ethic Committee for Animal Experiments from the University of Zaragoza.

2.9. Role of the funding source

The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

3. Results

3.1. Construction of two new vaccine candidates based on isogenic *phoP* and *fadD26* deletions present in MTBVAC

Based on previous knowledge acquired during the preclinical and clinical development of MTBVAC [37–39], a live attenuated *M. tuberculosis* strain belonging to lineage 4 [19], we have introduced simultaneous *phoP* and *fadD26* deletions in two clinical isolates belonging to lineages 2 and 3 of *M. tuberculosis*. Our objective was to obtain a set of isogenic MTBVAC-like vaccine candidates constructed in the genetic backgrounds of the three modern lineages of *M. tuberculosis*. *M. tuberculosis* GC1237 [40], which is a Beijing strain classified to the Asian-Ancestral 3 sub-branch of lineage 2 strains [50] and the HMS13037 clinical isolate, a lineage 3 isolate showing a CAS-1 Delhi spoligotype, were selected as parental strains for the genetic constructions (Figure S1 and Table S1). To obtain double unmarked gene deletions, two alternative and complementary strategies based on suicide plasmids [19] or BAC-rec [32] were used. In the GC1237 strain, the *fadD26* deletion was obtained using the suicide plasmid pAZ5 followed by the *phoP* deletion using pAZ18 (Fig. 1a). In the HMS13037 strain, BAC-rec was used to delete *fadD26* and the suicide

plasmid pAZ18 was used to construct the *phoP* deletion (Fig. 1b). The antibiotic markers were subsequently eliminated using $\gamma\delta$ and FLP recombinases suitable for the suicide plasmids and BAC-rec strategies respectively (Fig. 1). Gene deletions were confirmed by PCR using two pairs of primers flanking the mutation (Figure S2, S3 and S4, Table S2), and the *phoP* deletion was additionally confirmed by Western-blot (Figure S4). Altogether, two new double unmarked *phoP* and *fadD26* mutants were obtained and named MTBVAC-L2 and MTBVAC-L3, for lineage 2 and 3 respectively (Fig. 1).

3.2. Molecular characterization of MTBVAC-L2 and MTBVAC-L3 demonstrate equivalent phenotypes described in MTBVAC

At first, PhoP-dependent phenotypes previously described for MTBVAC were evaluated in the new vaccine candidates. A transcriptomics approach was used to evaluate whether specific downregulation of representative transcripts of the PhoP-regulon (*mcr7*, *pks2* and *pks3*) [20,23] existed in MTBVAC-L2, MTBVAC-L3 and MTBVAC, compared to their parental strains. For *mcr7* and *pks2* we observed the expected downregulation in the three MTBVAC variants. Exceptionally, expression of *pks3* in MTBVAC-L3 was slightly increased compared to the parental HMS13037 strain (Fig. 2a), which might indicate that subtle inter-lineage differences could exist in the PhoP regulon. We also confirmed that MTBVAC, MTBVAC-L2 and MTBVAC-L3 are unable to fix neutral red, contrasting to their parental strains (Figure S5a). This phenotype is putatively related to the altered cell envelope absent in PDIM and acyltrehalose-derived lipids, whose synthesis involves *fadD26* and *phoP*, respectively [19,25].

One of the best characterized phenotypes linked to PhoP, is its involvement in regulation of the secretion of ESAT-6 [22,24,51]. Western-blot analyses showed that ESAT-6 was only secreted in the parental strains in contrast to MTBVAC, MTBVAC-L2 and MTBVAC-L3. Nevertheless, ESAT-6 was detected in the whole-cell lysate of both the parental strains and the *fadD26*- and *phoP*-deleted strains (Fig. 2b). Despite the inability of *phoP*-deletion mutants to secrete ESAT-6, it has been recently described that MTBVAC is able to secrete CFP-10 [32], although the mechanism of the process relative to the previously defined ESAT-6 and CFP-10 co-secretion [52], still needs to be defined. Here, we demonstrate that MTBVAC-L2 and MTBVAC-L3, similar to classical MTBVAC, does secrete CFP-10 into the supernatant (Fig. 2b).

RNA-seq of MTBVAC revealed that the operon formed by *fadD26* and downstream genes (*ppsA-D*) is substantially less expressed compared to the Mt103 wild-type strain (Figure S6). We tried to confirm this result by qRT-PCR of *ppsA* and *ppsB* genes in MTBVAC-L2 and MTBVAC-L3 compared to their parental strains. The expected absence of the *ppsAB* transcripts was observed in MTBVAC and MTBVAC-L2; however, we observed similar levels of *ppsAB* in MTBVAC-L3 relative to the HMS13037 parental strain (Fig. 2d). This unexpected result might be related to the genetic scar left in the chromosome after resolution of the antibiotic resistance marker and will be discussed below.

3.3. MTBVAC-L2 produces but does not secrete PE_PGRS proteins and this phenotype is related to the lineage background used in genetic constructions

The *ppe38* locus is polymorphic in the Beijing family resulting in deletion of the *ppe38* gene at the branching point of the “modern” Beijing sub-strains [53]. Deletion of *ppe38* completely blocks the ability to secrete PE_PGRS proteins (a subfamily of PE proteins). Interestingly, lack of secretion of these proteins has been associated with an increased virulence of *M. tuberculosis* strains in mouse models [53]. Moreover, BCG strains were also shown to lack PE_PGRS secretion due to the deletion of the RD5 region, although this absence had little impact on the protective efficacy of BCG strains in mice [49]. To further characterize the vaccine candidates MTBVAC, MTBVAC-L2 and MTBVAC-L3, the organization of the *ppe38* locus was studied

according to McEvoy and colleagues [54], indicating the putative absence of the *ppe38* gene in GC1237 (Figure S5). Indeed, PE_PGRS proteins were detected by Western-blotting in the whole-cell fractions of the three strains (Fig. 2c) using a specific antibody, whereas PE_PGRS proteins were only detected in the supernatants of MTBVAC and MTBVAC-L3 strains, and not in MTBVAC-L2 supernatant fractions. Accordingly, here we confirm a differential phenotype between MTBVAC-L2 and MTBVAC/MTBVAC-L3 strains, which is related to the specific lineage used as background for vaccine construction.

3.4. MTBVAC-L2 and MTBVAC-L3 are attenuated in SCID mice although both showed less attenuation than MTBVAC

To evaluate the safety profile of the new MTBVAC candidates, a survival experiment was performed in immunocompromised SCID mice. To discard differences due to *in vitro* fitness of different vaccines, we first confirmed that MTBVAC-L2 and MTBVAC-L3 as well as their parental strains exhibited comparable *in vitro* growth curves (Figure S7).

Next, mice were inoculated with MTBVAC-L2 or MTBVAC-L3. BCG Pasteur and the original MTBVAC strains were used as comparators. MTBVAC-L2 and MTBVAC-L3 showed an attenuated profile in SCID mice (mean of survival of 85.5 days in the former and 93.5 days in the latter). Thus, MTBVAC-L3 exhibits comparable attenuation to BCG Pasteur but MTBVAC-L2 is less attenuated than the currently licensed BCG. Notably, of the three double *phoP*- and *fadD26*-deletion mutants tested, the original MTBVAC was the most attenuated and even safer than BCG Pasteur (Fig. 3).

3.5. MTBVAC, MTBVAC-L2 and MTBVAC-L3 confer protection in mice against modern strains of *M. tuberculosis* and this phenotype is unrelated to the lineage used for vaccine construction

According to previous research, greater protection of MTBVAC relative to BCG Danish was observed in C3H/HeNRj mice with respect to other commonly used mouse strains such as C57BL/6 or BALB/c³². This phenotype is likely attributable to the H-2^k haplotype of the Major Histocompatibility Complex present in the C3H/HeNRj mouse strain, which is able to recognize both ESAT-6 and CFP-10 immunogens, in contrast to the H-2^b haplotype in C57BL/6 mice that only recognizes ESAT-6, and/or the H-2^d haplotype in BALB/c mice which does neither recognizes ESAT-6, nor CFP-10. Accordingly, the C3H/HeNRj model was considered as useful to discriminate the protective efficacy of MTBVAC compared to BCG [32]. In this study, we used the C3H/HeNRj mouse model to compare protective efficacy of MTBVAC, MTBVAC-L2 and MTBVAC-L3 against challenge with *M. tuberculosis* strains belonging to the modern lineages of the MTBC. For intranasal challenge, *M. tuberculosis* strains H37Rv (lineage 4), W4-Beijing (lineage 2) and HCU3524 (lineage 3) were used. The licensed vaccine BCG Pasteur 1173P2 was used as comparator, since this strain is widely used in animal experiments. Enumeration of bacterial burden in lungs and spleens showed that the three MTBVAC strains conferred similar protection against challenge with isolates belonging to modern lineages. After challenge with H37Rv, all vaccines tested conferred similar protection against lineage 4 (Fig. 4a). Challenge with a Beijing strain resulted in significant increased protection of animals previously vaccinated with MTBVAC and MTBVAC-L2 compared to BCG Pasteur (Fig. 4b). Variable protection was observed against challenge with lineage 3, the lowest bacterial burden in lungs was observed for vaccination with MTBVAC and MTBVAC-L3, followed by MTBVAC-L2 and BCG Pasteur (Fig. 4c). Noteworthy, the three MTBVAC vaccines exhibited comparable protection against each challenge strain, indicating that protective efficacy phenotypes are independent of the lineage used as MTBVAC background.

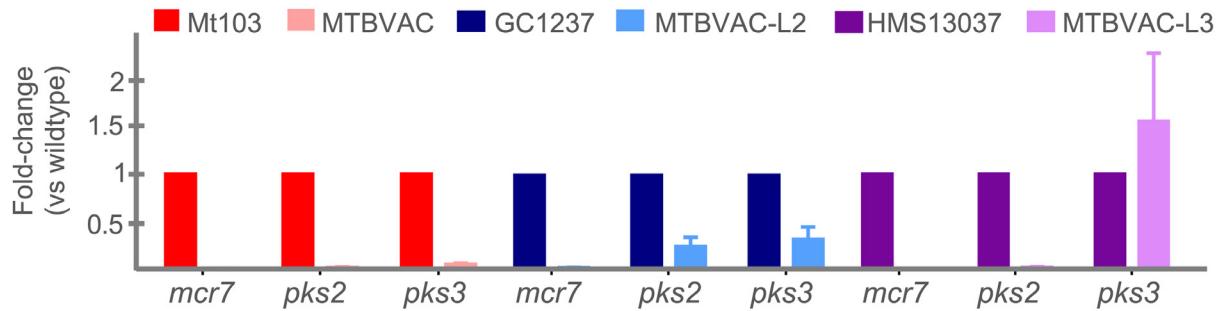
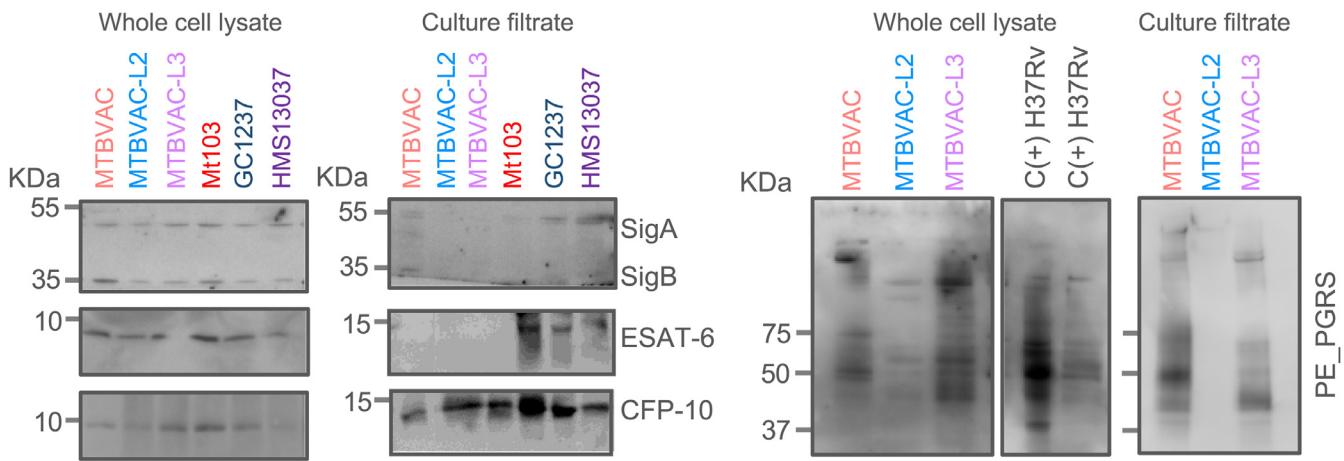
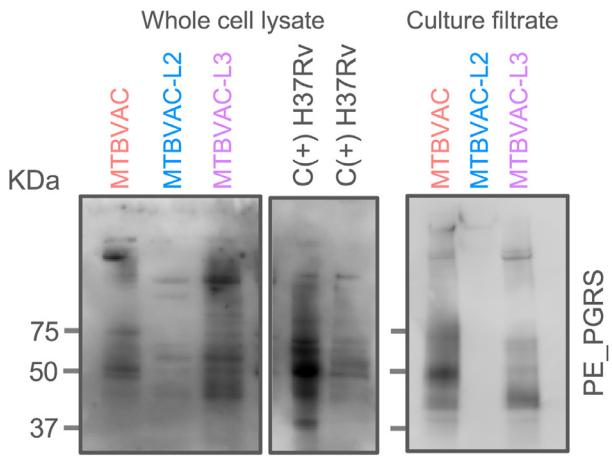
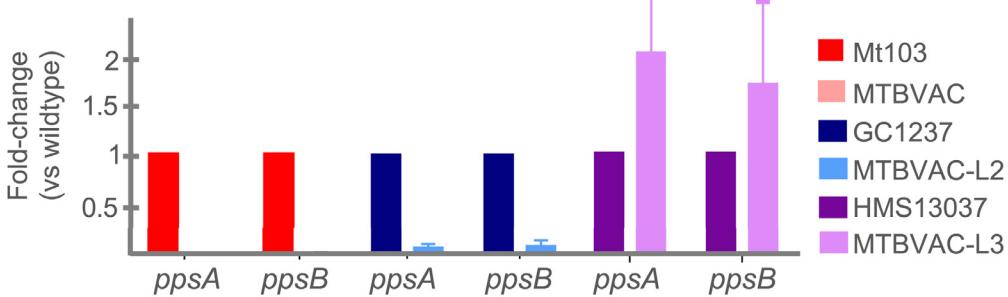
a**b****c****d**

Fig. 2. Molecular characterization of MTBVAC, MTBVAC-L2 and MTBVAC-L3. (a) Expression of representative genes from the PhoP-regulon (*mcr7*, *pks2* and *pks3*) measured by qRT-PCR in MTBVAC, MTBVAC-L2 and MTBVAC-L3 compared to their respective parental strains. Each gene was normalized against *sigA* expression in each sample. Bars represent the mean and standard deviation from three independent experiments. (b) Western-blot of ESAT-6 and CFP-10 proteins in whole-cell lysates (left panel) and secreted fractions (right panel) in the MTBVAC vaccines and their wild type strains. Note the absence of ESAT-6 secretion as a consequence of the *phoP* mutation in all vaccine strains. Also note the presence of CFP-10 in the secreted fraction in all MTBVAC strains. Detection of SigA and SigB serves as loading control in the whole-cell lysate and also as cell integrity control in the secreted fraction. (c) Western-blot of PE_PGRS in total (left panels) and secreted fractions (right panel) from the MTBVAC vaccine set. Note the differential absence of PE_PGRS secretion in MTBVAC-L2. (d) Expression of the *ppsAB* genes belonging to the PDIM biosynthetic operon measured by qRT-PCR. Bars represent the mean and standard deviation from three independent experiments. Each sample was normalized relative to the endogenous control *sigA*.

4. Discussion

To achieve the end of TB, new tools including new effective vaccines, better diagnostics and new drugs are needed [55]. Despite the diversity in vaccine strategies currently in clinical trials, preclinical research on new live vaccines continues in order to keep the vaccine pipeline filled. Recently, live vaccine candidates based on BCG [56,57]

or attenuated *M. tuberculosis* [58] have been reported. Live attenuated *M. tuberculosis* vaccines are expected to confer an effective T-cell immune response since they maintain those antigens missing in RD regions from BCG [31]. MTBVAC is one of the two live attenuated vaccine candidates currently in clinical trials, representing the unique candidate based on genetic background of the human pathogen *M. tuberculosis*. MTBVAC confer higher and more durable T-cell

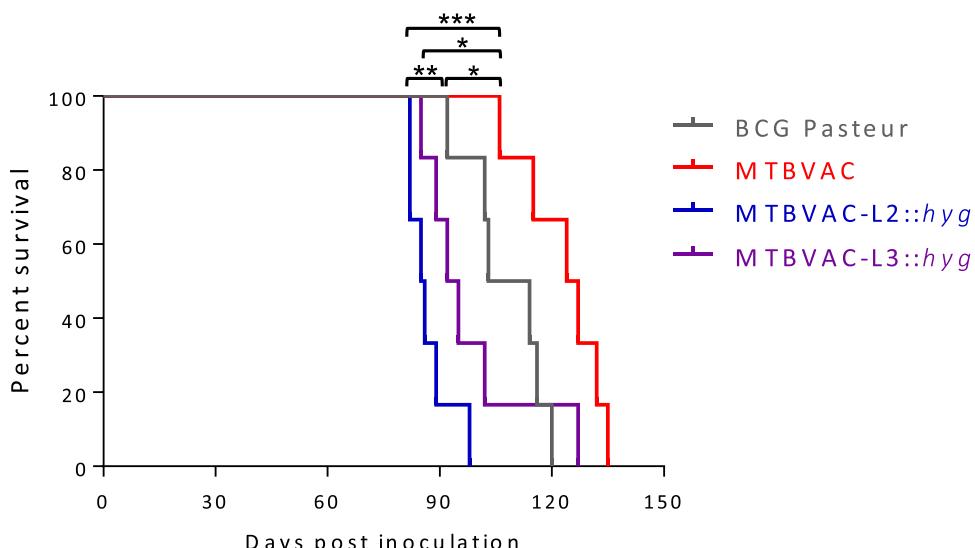


Fig. 3. Attenuation of MTBVAC, MTBVAC-L2 and MTBVAC-L3 in the SCID mouse model. Survival curves from groups of 6 SCID mice inoculated by intraperitoneal route with MTBVAC, MTBVAC-L2, MTBVAC-L3 and BCG Pasteur (as control) are shown. Asterisks indicate * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ [Log-rank (Mantel-Cox) test].

responses than BCG in a vaccinated human cohort [39] and this finding might be related to the fact that MTBVAC contains approximately a 50% broader antigenic repertoire of T-cell epitopes compared to BCG [31]. However, the possible lineage-dependent protection of MTBVAC remains unknown and its protective efficacy in animal models has been exclusively evaluated against *M. tuberculosis* H37Rv or *M. tuberculosis* Erdman strains, both belonging to lineage 4 [19,36].

To evaluate lineage-dependent protection, we have constructed two new vaccine candidates based on isogenic deletions of MTBVAC in the lineages 2 and 3 of *M. tuberculosis* (MTBVAC-L2 and MTBVAC-L3). As a result, we have obtained the complete set of MTBVAC vaccines in the three modern lineages of *M. tuberculosis*. Molecular characterization of this vaccine set revealed that most PhoP- and FadD26-dependent phenotypes are maintained across lineages, although some differences were observed. The most prominent PhoP-regulated genes (*mcr7*, *pks2* and *pks3*) are overall downregulated in the MTBVAC set. However, *pks3* expression remained unchanged in MTBVAC-L3 compared to the parental strain. This unexpected finding opens new perspectives to decipher whether the PhoP-regulon might be lineage-dependent. Indeed, similar inter-strain differences have been recently observed in the PhoP regulon. The *whiB6* gene, which belongs to the PhoP regulatory network, is positively regulated by PhoP in Mt103 and GC1237 strains while negatively regulated in H37Rv. This differential regulation is caused by a single nucleotide insertion exclusively present in H37Rv and results in lower ESAT-6 secretion in H37Rv relative to other *M. tuberculosis* strains [51].

Another marked differential phenotype was observed in the expression of the *fadD26-ppsAD* operon. The *ppsAB* genes are similarly expressed in MTBVAC-L3 relative to the HMS13037 parental strain in contrast to MTBVAC and MTBVAC-L2 when compared to their respective parental strains. A closer inspection of the genetic constructions suggests that the chromosomal “scar” after resolution of the antibiotic markers is different between MTBVAC-L3 (FRT site) and MTBVAC/MTBVAC-L2 (res site) (Fig. 1). Thus, the res site would cause a polar effect in expression of the 3' *fadD26* downstream region. By contrast, the FRT site contained in the *fadD26* deleted gene in MTBVAC-L3 is designed to create nonpolar effects, as described by Datsenko and colleagues [45].

The two MTBVAC derivatives constructed here robustly reproduce the presence of CFP-10 and the absence of ESAT-6 in the secreted protein fraction, previously documented for MTBVAC [32]. It is important to mention that this phenotype could interfere with the diagnostic potential of current interferon gamma release assays (IGRA)-which contain ESAT-6 and CFP-10- as diagnostic antigens, as observed for

MTBVAC-vaccinated newborns [39] and consequently, specific diagnostic tools able to differentiate between MTBVAC-immunized and *M. tuberculosis*-infected individuals are currently under study.

Preliminary safety experiments in immunodeficient mice revealed that albeit the three MTBVAC derivatives were attenuated, inter-lineage differences were found. Beijing-strain-derived MTBVAC-L2 was found to be the least attenuated candidate and only MTBVAC constructed from lineage 4 was more attenuated than BCG Pasteur. Some phenotypes specific to Beijing strains might have contributed to this finding. The presence of phenolic glycolipids in Beijing strains, which is different to L4 strains due to a frameshift mutation in the *pks15/1* gene [59], are linked to inhibition of innate immune responses and higher virulence of the Beijing family [60]. Moreover, in this work, we explored another differential phenotype between the three vaccine candidates, linked to PE_PGRS secretion. Indeed, MTBVAC-L2 failed to secrete PE_PGRS proteins in contrast to MTBVAC and MTBVAC-L3. Since lack of secretion of these proteins has been associated with increased virulence in mice [53], this phenotype might also contribute to the lower attenuation of MTBVAC-L2 observed in SCID mice.

Zhang and colleagues recently demonstrated a marked heterogeneity in attenuation and protective efficacy among 13 different BCG sub-strains in mice [61]. Results from this study suggest a correlation between a higher protection conferred by those BCG strains which were less attenuated and vice versa [61]. Supporting differences in protective efficacy across BCG strains, we also observed that BCG Danish conferred less protection than MTBVAC in the C3H/HeNRj strain [32], albeit BCG Pasteur induces equivalent protection as MTBVAC vaccines in this model (Fig. 4a). However, our results obtained with the three MTBVAC derivatives, show that different levels of attenuation in SCID mice do not necessarily predict vaccine efficacy in immune-competent mice, as no clear association between attenuation and protection was found. It is important to mention nevertheless, that the dosage and inoculation routes differ between both studies, which might explain the differences in the time-to-human endpoint durations after vaccination with BCG sub-strains or MTBVAC derivatives.

Our protective efficacy experiments against modern *M. tuberculosis* strains suggest no lineage-dependent protection in C3H/HeNRj mice, although additional protection experiments using different vaccination regimes or additional animal models might provide a more detailed insight on this topic. This similar protective efficacy might be related to the presence of the complete antigenic repertoire of *M. tuberculosis* across MTBVAC vaccines, which is thought to result

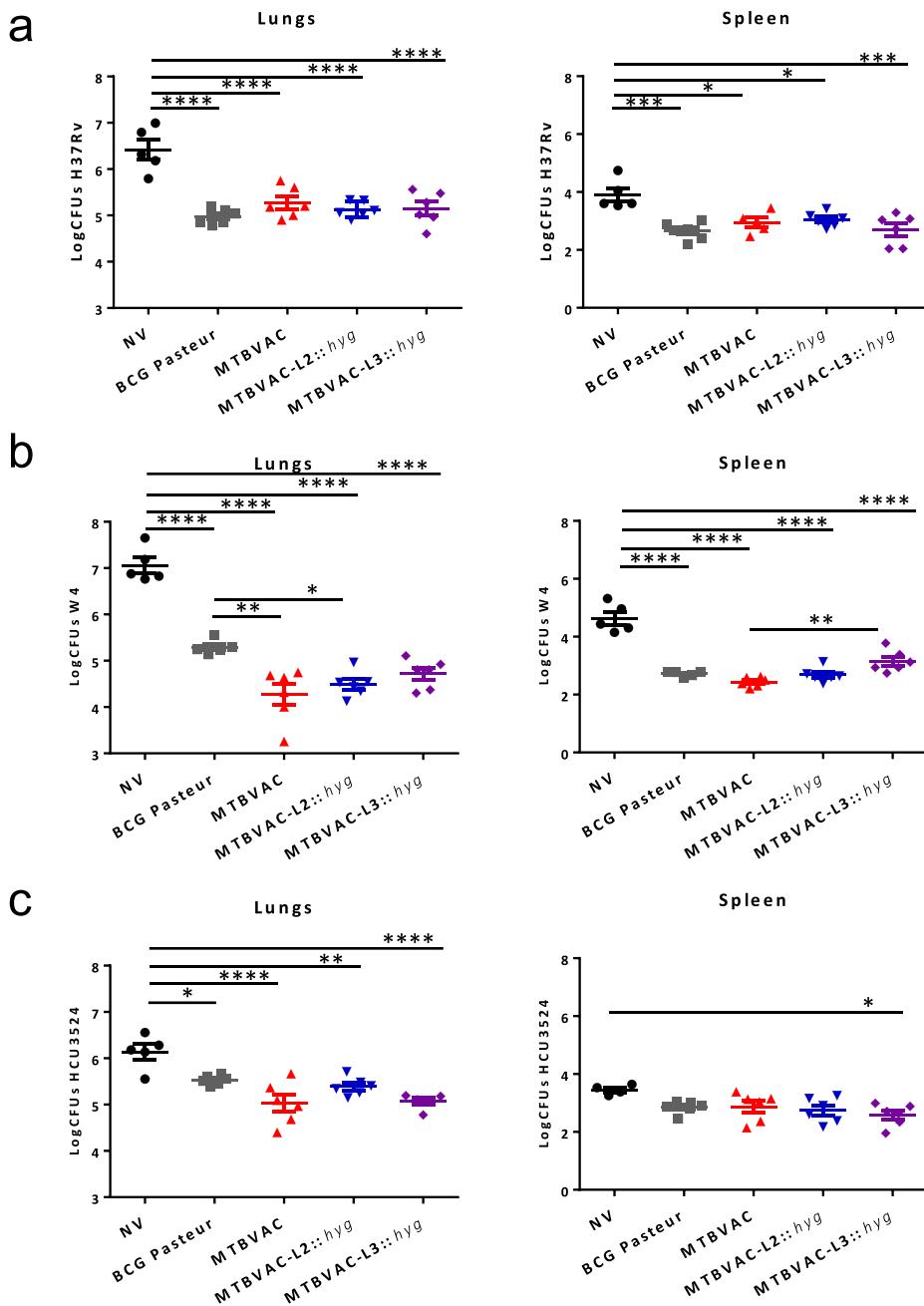


Fig. 4. Protective efficacy of MTBVAC, MTBVAC-L2 and MTBVAC-L3 in C3H/HeNRj mice against challenge with strains from modern lineages of *M. tuberculosis*. The graphs represent bacterial burden in lungs (left panels) and spleen (right panels) from groups of 6 mice subcutaneously immunized with the corresponding vaccine and after a 4 weeks intranasal challenge against (a) H37Rv (lineage 4 strain), (b) W4-Beijing (lineage 2 strain) and (c) HCU3524 (lineage 3 strain). All data are mean \pm SEM. Asterisks indicate * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ [One-way ANOVA, Bonferroni post-test].

in a wide and complete immune stimulation. Remarkably, higher protection was conferred by MTBVAC and MTBVAC-L2 compared to BCG Pasteur against challenge with W4-Beijing strain. Beijing strains have been associated to recent TB outbreaks, hyper-virulent phenotypes and drug resistance [62]. One hypothesis for this successful emergence of L2 strains could be the ability of these strains to escape from BCG vaccination, although this remains elusive because of the controversial conclusions in different epidemiological studies [63]. It is noteworthy that MTBVAC vaccines conferred improved protection against a challenge with the W4-Beijing strain, which is an important finding, even in the light that the MTBVAC-L2 vaccine strain appeared slightly less attenuated than other vaccines in mouse experiments.

Our previous preclinical studies demonstrated that MTBVAC protected against challenge with H37Rv in mice [19] or with Erdman in non-human primates [36], both strains belonging to the lineage 4. Importantly, here we show for the first time that MTBVAC vaccine candidates are able to protect against different representatives of the modern lineages of *M. tuberculosis* in mice. This finding represents an important detail for the clinical evaluation of MTBVAC and putative new live attenuated vaccines based on *phoP* and *fadD26* deletions.

Since the three MTBVAC vaccine candidates provide different relative levels of protection against challenge with modern *M. tuberculosis* strains, this suggests that the properties of the infecting strain or their distribution in different parts of the world may determine the

efficacy of the vaccine used in humans. MTBVAC is today in Phase IIa in adults and newborns and the results presented here further support its progress into clinical efficacy trials. In such efficacy studies, we should be prepared for two possible scenarios. Either the original MTBVAC constructed in the lineage 4 background might confer equivalent protection independently of the vaccinated population; or alternatively, MTBVAC or the new MTBVAC-L2 and MTBVAC-L3 constructed in this work might confer efficacious protection in populations where strains from lineages 4, 2 or 3 are the most prevalent, respectively. In this latter scenario, the three MTBVAC vaccines could be combined into a polyvalent vaccine that affords a worldwide protection against TB. Altogether, our results not only expand the current knowledge of the MTBVAC vaccine but anticipate novel MTBVAC-based strategies for the future.

Author contribution

Conceptualization, J.G.-A., C.M., and I.P.; Methodology, J.G.-A., C.M., R.B., N.A., W.F., F.S., S.U., and I.P.; Reagents, A.A., S.S.; Investigation, W.F., F.S., S.U. and I.P.; Writing – Original Draft, J.G.-A., C.M. and I.P.; Writing – Review & Editing, J.G.-A., C.M. and R.B.; Funding Acquisition, C.M. and R.B.; Supervision, J.G.-A., C.M., R.B., N.A., W.F., and F.S. All authors reviewed the final report.

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Declaration Competing of Interest

C.M., A.A. and J.G.-A. are co-inventors on the patent “Tuberculosis vaccine”. N.A., C.M. and J.G.-A. are co-inventors on the patent “Compositions for use as a prophylactic agent to those at risk of infection of tuberculosis, or as secondary agents for treating infected tuberculosis patients”. Both patents were filled by the University of Zaragoza

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.ebiom.2020.102761](https://doi.org/10.1016/j.ebiom.2020.102761).

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Respiratory Immunization with a whole cell inactivated vaccine induces functional mucosal immunoglobulins against tuberculosis in mice and non-human primates.



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Respiratory Immunization With a Whole Cell Inactivated Vaccine Induces Functional Mucosal Immunoglobulins Against Tuberculosis in Mice and Non-human Primates

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Vaccination through the natural route of infection represents an attractive immunization strategy in vaccinology. In the case of tuberculosis, vaccine delivery by the respiratory route has regained interest in recent years, showing efficacy in different animal models. In this context, respiratory vaccination triggers lung immunological mechanisms which are omitted when vaccines are administered by parenteral route. However, contribution of mucosal antibodies to vaccine-induced protection has been poorly studied. In the present study, we evaluated in mice and non-human primates (NHP) a novel whole cell inactivated vaccine (MTBVAC HK), by mucosal administration. MTBVAC HK given by intranasal route to BCG-primed mice substantially improved the protective efficacy conferred by subcutaneous BCG only. Interestingly, this improved protection was absent in mice lacking polymeric Ig receptor (plgR), suggesting a crucial role of mucosal secretory immunoglobulins in protective immunity. Our study in NHP confirmed the ability of MTBVAC HK to trigger mucosal immunoglobulins. Importantly, *in vitro* assays demonstrated the functionality of these immunoglobulins to induce *M. tuberculosis* opsonization in the presence of human macrophages. Altogether, our results suggest that mucosal immunoglobulins can be induced by vaccination to improve protection against tuberculosis and therefore, they represent a promising target for next generation tuberculosis vaccines.

Keywords: whole-cell vaccine, pulmonary vaccination, animal models, mucosal immunoglobulins, opsonization, tuberculosis

INTRODUCTION

Tuberculosis (TB) disease causes one and a half million deaths per year, and is one of the leading infectious diseases affecting mainly developing and underdeveloped countries. The rising spread of multidrug resistant strains with increasing human migration makes TB an alarming global health problem, according to World Health Organization (WHO). Therefore, there is an urgent need for new effective TB vaccines.

Vaccination through the natural route of infection represents an attractive strategy for priming the natural host immunity. In the case of TB, respiratory mucosal tissue is the primary site for establishment of infection. It has been well described in different preclinical models that vaccination with BCG by the respiratory route confers a substantially improved protection in comparison to subcutaneous or intradermal immunization (Lagranderie et al., 1993; Aguiló et al., 2016; Dijkman et al., 2019). Indeed, in the last few years, it has raised an interest in exploring new vaccination approaches delivered through respiratory routes of administration. These strategies include attenuated *M. tuberculosis* (Kaushal et al., 2015) in addition to BCG, as well as subunit vaccines formulated with adjuvants or non-replicative virus (Stylianou et al., 2015; Woodworth et al., 2019). In 2014, the first clinical trial of an aerosol tuberculosis vaccine was reported (Satti et al., 2014).

It is assumed that inactivation of whole-cell tuberculosis vaccines reduces their immunogenic and protective potential. Nevertheless, and likely based on safety concerns described for live BCG under specific conditions (e.g., immunodeficiencies), researchers have explored the use of inactivated vaccine approaches for tuberculosis. To overcome the loss of immunogenicity, different strategies have been conducted, such as the use of inactivated whole-cell vaccines as booster for BCG (Von Reyn et al., 2017).

The present work describes vaccination with a heat-killed (HK) version of the live attenuated *M. tuberculosis* vaccine MTBVAC (Arbues et al., 2013) both in mice and non-human primates (NHP). MTBVAC is the first and only live attenuated tuberculosis vaccine based on *M. tuberculosis* that has reached clinical stages of development, and it has shown an excellent safety profile both in adults and newborns, as well as stronger immunogenicity compared to BCG (Spertini et al., 2015; Tameris et al., 2019). Results in the present study demonstrate improved efficacy of MTBVAC HK when given by intranasal route to mice previously vaccinated with subcutaneous BCG. In addition, we interrogated lung humoral immune responses elicited by MTBVAC HK in mice and NHP, finding an induction of tuberculosis-specific mucosal immunoglobulins with functional activity against *M. tuberculosis*.

RESULTS

Intranasal MTBVAC HK Enhances Protection Conferred by Subcutaneous BCG

We and others have previously demonstrated an advantageous vaccine-induced protection of whole-cell live vaccines when

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given by respiratory route, compared to subcutaneous or intradermal administration in immunologically naïve subjects (Aguiló et al., 2016). Since respiratory airways could be a sensitive organ for exacerbated inflammatory response caused by live bacteria in the pre-exposed, we chose to evaluate the efficacy of an inactivated whole cell vaccine as a booster strategy after primary BCG. To this end, we inactivated MTBVAC building upon the promising immunogenic profile shown by the live version of this vaccine both in animal models and in humans (Marinova et al., 2017). MTBVAC was inactivated by heating the vaccine at 100°C for 30 min. Bacterial inactivation was confirmed by plating on 7H10 solid agar medium (data not shown). Bacteria visualization using electron microscopy confirmed that MTBVAC maintained their bacillary shape upon heat treatment (**Supplementary Figure S1**). Then, we characterized MTBVAC HK-conferred protection in mice under different experimental conditions. First, 10⁷ MTBVAC HK bacteria were inoculated intranasally in naïve or in BCG-primed mice. One month later, mice were challenged intranasally with a low dose (150 CFU) of the H37Rv Mtb strain. After one month, mice were sacrificed and lung bacterial load evaluated by plating in solid medium. Our data showed that MTBVAC HK alone did not confer protection compared to unvaccinated control. However, when combined with subcutaneous BCG priming, protection was about one-log higher than that provided by BCG only (**Figure 1A**), indicating the need of a BCG prime to trigger a MTBVAC HK-induced protective response.

We also evaluated protection induced by MTBVAC killed with formalin, since this method of inactivation had been shown in a previous study with other inactivated vaccines to better preserve immunogenicity compared to heating (Cryz et al., 1982). However, in this case, we did not find any difference in efficacy between both MTBVAC inactivation methods (**Supplementary Figure S2**). Since heat-inactivation is easier to implement, we continued vaccine characterization using this method of inactivation. MTBVAC HK induced better protection only when given by intranasal route, but did not improve BCG when administered subcutaneously (**Figure 1B**). Ultimately, our data show that the MTBVAC HK booster effect was dose-dependent, as we only observed improved protection with a high dose of MTBVAC HK (10⁷), whereas no effect was obtained using 10⁴ bacteria (**Figure 1C**). We and others have previously reported the lack of protection induced by BCG subcutaneous in the mouse strain DBA/2 (Aguiló et al., 2016). Interestingly, MTBVAC HK also induced protection in BCG-vaccinated DBA/2 mice, suggesting that this vaccination approach could confer protective efficacy in cases in which BCG is ineffective (**Supplementary Figure S3**). Comparison of different independent lots of MTBVAC HK provided a similar protective profile, superior to BCG only, evidencing the reproducibility of our results (**Supplementary Figure S4**).

Considering that BCG is primarily administered in the clinic in newborn populations, we used a neonatal mouse model in which BCG was inoculated at birth, and MTBVAC HK given 8 weeks later, when the immune system has reached a mature status. Protection by Mtb reduction in lungs was

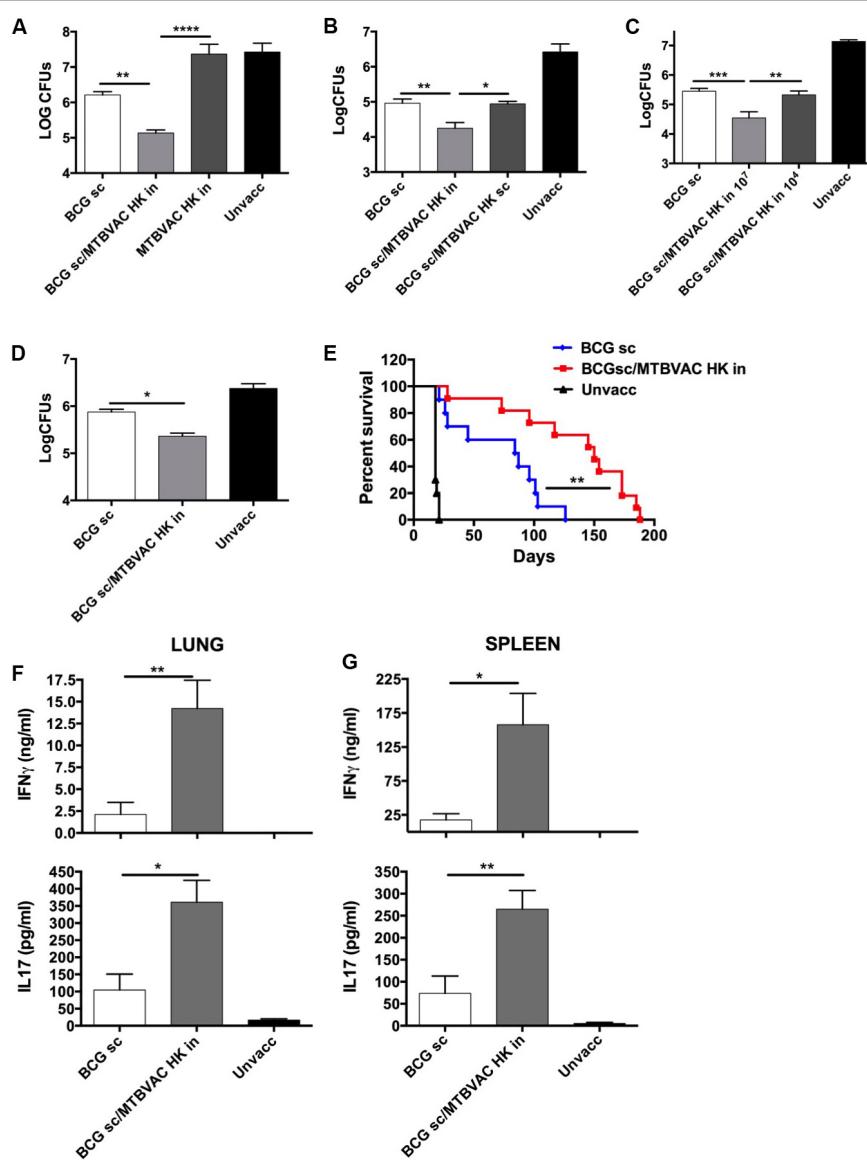


FIGURE 1 | Improved protection induced by intranasal MTBVAC HK as boost of subcutaneous BCG. **(A–C)** Groups of C57BL/6 adult mice where vaccinated with BCG and MTBVAC HK 4 weeks apart (107 MTBVAC HK dose when not specified). After one month, mice were intranasally challenged with H37Rv and lung bacterial load analyzed one month later. **(D,E)** Newborn mice were vaccinated with BCG and 8 weeks later boosted intranasally with 107 MTBVAC HK. Mice were challenged with a low-dose (**D**) or a high-dose (**E**) H37Rv inoculation and lung CFUs or survival analyzed, respectively. **(F,G)** Antigen-specific IFN γ and IL17A production one month after MTBVAC HK vaccination, following PPD stimulation of cells from lungs (**F**) and spleen (**G**). **(A–D, F,G)** Data are shown as mean \pm SEM and are representative of at least two independent experiments. ($n = 6$ mice/group). $^*p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.001$; $^{****}p < 0.0001$ by one-way ANOVA and Bonferroni post-test. **(E)** Data from one experiment ($n = 10$ mice/group) are represented in a Kaplan-Meier survival curve and statistical significance calculated by a LogRank test. $^{**}p < 0.01$.

significantly improved in the MTBVAC HK booster group (**Figure 1D**), and comparable to the protection level observed in adult mice immunized with BCG. We also evaluated vaccine efficacy by survival as a readout in a high-dose, mouse challenge model and found that intranasal MTBVAC HK boosting substantially extended mouse survival in comparison to BCG sc immunization (**Figure 1E**).

Although we did not investigate other administration routes, our results suggest that the beneficial effect of MTBVAC

HK boosting specifically depends on its interaction with the respiratory mucosal immune system. Therefore we analyzed cellular responses in lungs as well as in spleen after *ex vivo* stimulation with *M. tuberculosis* secreted antigens (Purified protein derivative: PPD). Data revealed that MTBVAC HK intranasal boosting enhanced antigen-specific IFN γ and IL17 induction in lungs (**Figure 1F**). In addition, spleen response profiling also revealed a higher IFN γ and IL17 production at a systemic level elicited by MTBVAC HK boosting (**Figure 1G**).

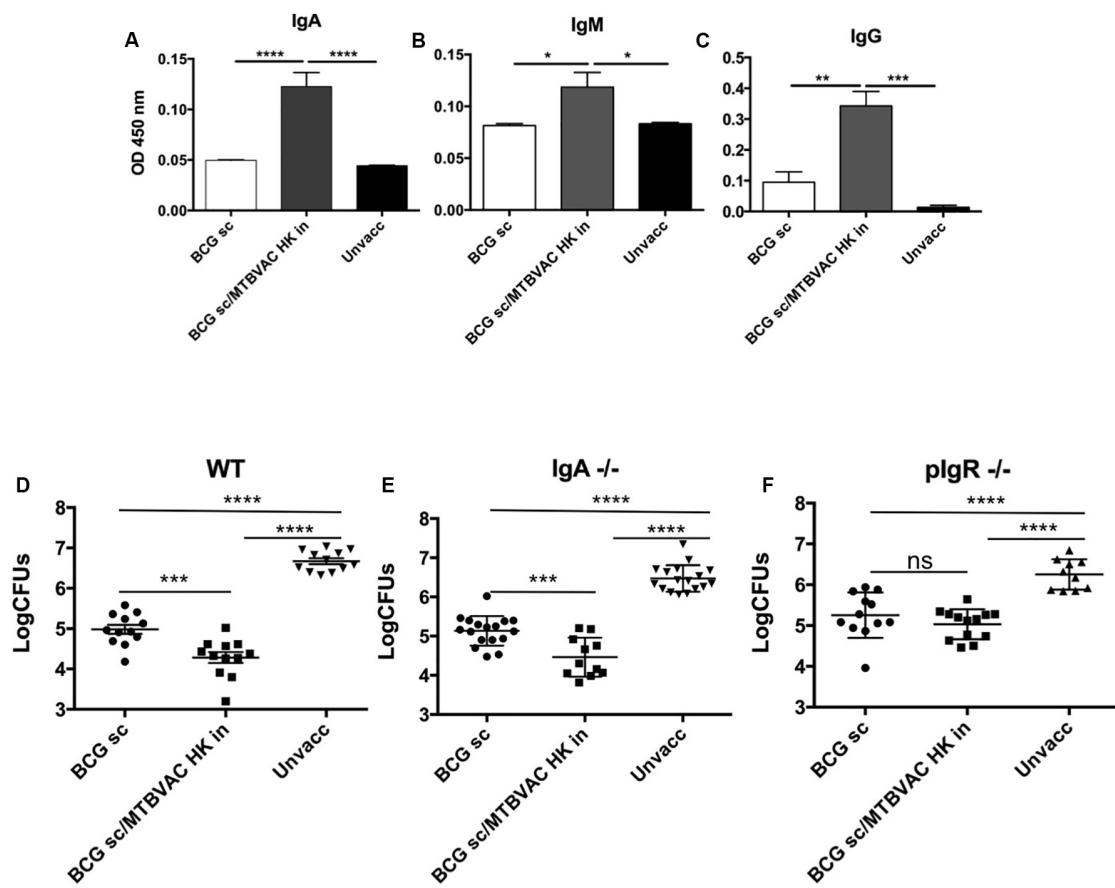


FIGURE 2 | Intranasal MTBVAC HK induces mucosal immunoglobulins in respiratory airways. **(A–C)** Groups of C57BL/6 adult mice were vaccinated with BCG and MTBVAC HK 4 weeks apart. One month later PPD-specific IgA, IgM, IgG in BAL samples. **(D–F)** One month after MTBVAC HK boosting, wild-type, IgA-/- and pIgR-/- mice were intranasally challenged with H37Rv and lung bacterial load analyzed one month later. All data are mean \pm SEM **(A–C)** Data are representative of two independent experiments ($n = 6$ mice/group). **(D–F)** Data in the graphs represent a pool of two independent experiments ($n = 12$ mice/group). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ by one-way ANOVA and Bonferroni post-test.

Intranasal MTBVAC HK Induces Protective Secretory Immunoglobulins

We have previously reported that respiratory, but not subcutaneous live BCG, triggered IgA production in respiratory airways (Aguilo et al., 2016). Thus, we next assessed whether intranasal MTBVAC HK induced PPD-specific immunoglobulins, including IgA, IgM and IgG subtypes, in bronchoalveolar lavage (BAL) (Figures 2A–C). MTBVAC HK booster vaccination triggered an increase of the three types of antibodies compared to unvaccinated and BCG only control groups.

To assess contribution of mucosal secretory immunoglobulins (sIg) to MTBVAC HK-mediated protection, we tested vaccine-conferred protective efficacy in mice deficient by genetic knockout for IgA (iga-/-) or for the polymeric immunoglobulin receptor (pIgR-/-). This latter molecule is a protein transporter highly expressed in mucosal tissues, which binds to J chain to actively translocate multimeric IgA or IgM across mucosal epithelium, and therefore lack of pIgR leads to retention of multimeric sIg in the mucosal lamina propria, preventing their

active transport over the mucosal barrier (Kaetzel et al., 1991). Our results revealed a similar protection provided by intranasal MTBVAC HK in iga-/- mice when compared to wild type (Figures 2D,E) suggesting no specific protective role of IgA. Conversely, MTBVAC HK-specific protection was completely abrogated in the absence of pIgR (Figure 2F). Altogether, this result would indicate a crucial contribution of sIg to protective efficacy mediated by MTBVAC HK, suggesting a substantial contribution of sIgM rather than sIgA. Remarkably, pIgR-/- mice did not show less protection by BCG s.c, suggesting that sIg has no role in systemic vaccine-induced protection.

Mucosal MTBVAC-HK Booster Vaccination in NHP

MTBVAC-HK was further evaluated as a booster vaccine in a non-human primate (NHP) vaccination and infection study to address tolerability, immunogenicity and protective efficacy in the primate host. Adult rhesus macaques (*Macaca mulatta*) were vaccinated either with BCG only, or with BCG followed by a MTBVAC-HK booster by pulmonary mucosal instillation

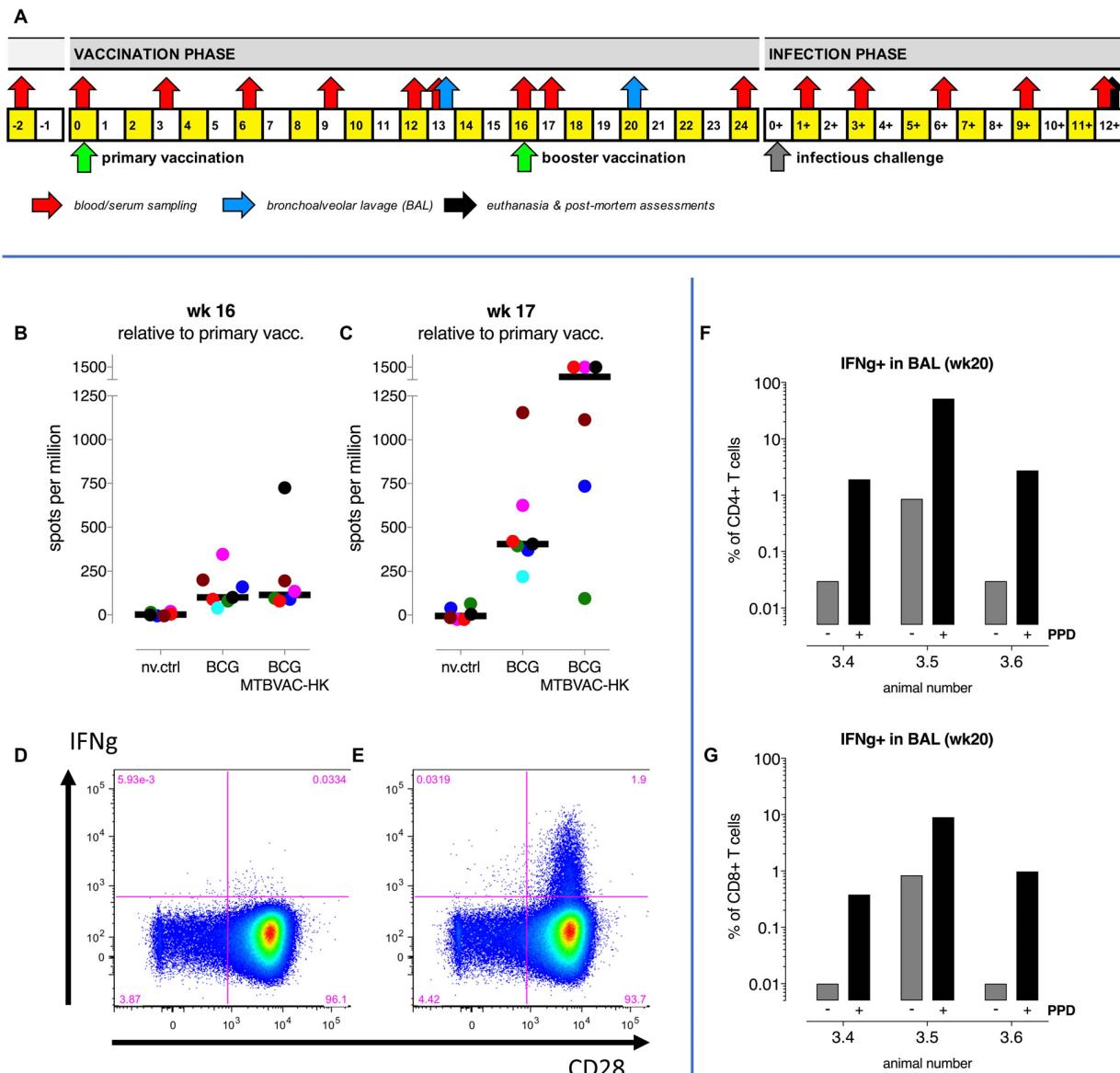


FIGURE 3 | Intrabronchial MTBVAC HK boosting induces local and systemic tuberculosis-specific cellular responses in NHP. **(A)** A study design schematic shows the time lines on a weekly basis relative to primary BCG vaccination and infectious challenge with *M. tuberculosis*, including booster immunization and biosampling events. **(B,C)** PPD-specific IFNg responses in peripheral blood were measured by ELISPOT immediately prior to and 1 week after MTBVAC HK boosting, at week 16 and 17, respectively. Individual data points are consistently colored according to the **Supplementary Table 1**; fat horizontal lines indicate group medians. Also, flow cytometry after intracellular cytokine staining was used to analyze IFNg responses. Dot plots of CD28 versus IFNg specific fluorescence signals of CD4 + T lymphocytes from a representative BAL sample illustrate local IFNg production **(D)** in the absence and **(E)** in the presence of PPD recall stimulation *in vitro*. Percentages per individual of PPD-induced IFNg-positive **(F)** CD4+ and **(G)** CD8+T lymphocytes from BAL are depicted.

16 weeks later (Figure 3A). One group was left untreated as non-vaccinated controls. Twenty-five weeks after primary vaccination all animals were challenged by endobronchial instillation of 50 colony forming units (CFU) of *Mtb* strain Erdman, with a follow-up of 12 weeks until study endpoint and post-mortem evaluation of infection and disease (Figure 3A). (See Supplementary Table 1 for an overview of animals per treatment group).

Within the limits of observation, animals tolerated mucosal MTBVAC-HK boosting well; we did not observe any adverse

events. Measuring vaccine-induced responses by specific interferon-gamma (IFNg) ELISPOT, using PBMC and antigenic recall stimulation with PPD, MTBVAC-HK boosting induced a transient elevation of the frequency of IFNg producing cells in the periphery at week 17 (one week post MTBVAC HK immunization) relative to the response induced by BCG ($p = 0.0997$ by non-parametric Mann-Whitney testing; Figures 3B,C). This increase was found to be transient since MTBVAC HK-specific T cell response dropped sharply at week

20 (**Supplementary Figure S5A**). Locally, and examining three pre-assigned animals out of six of the MTBVAC-HK boosted animals only (comparing week 13 versus week 20 post-BCG), we registered a clear influx of cells into the lung lumen upon mucosal MTBVAC-HK boosting and the induction of IFNg-positive CD4+ and CD8+ T lymphocytes (**Figures 3D–G**).

Upon infectious challenge with *Mtb*, highest pathology scores and lung bacterial counts were obtained in 3 non-vaccinated control (nv.ctrl) animals (**Figures 4A–D**, and **Supplementary Figures S5B,C**). However, there was an unexpected large spread in pathological involvement, with three out of six unvaccinated controls showing relatively mild disease levels (for which we have no explanation at this point), which altered all the statistical comparisons. Both BCG vaccinees and the MTBVAC-HK boosted animals revealed lower pathology scores by group median values (except for extrathoracic dissemination), but without statistical significance (**Figures 4A–C**). Likewise, there was no statistically significant vaccine effect by enumeration of *Mtb* at necropsy from lung (**Figure 4D**) or hilar lymph node and spleen (**Supplementary Figures 4B,C**, respectively). Despite the lack of statistically significant improvement, by various parameters there appears some positive trend by group median scores of improved TB disease outcome after MTBVAC-HK boosting relative to BCG alone: for total pathology score (**Figure 4A**), body weight development, and infection-associated anemia by hematological mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) development (**Supplementary Figures S5D,F,G**, respectively).

Most notably, MTBVAC-HK boosting significantly suppressed the PPD-specific IFNg response up until 6 weeks after infection (**Figures 4E–H**). Also, the *Mtb*-specific response against an ESAT6-CFP10 fusion protein (covering antigens which are absent from *M. bovis*-derived BCG vaccine) is significantly suppressed up until 6 weeks post-infection by MTBVAC-HK boosting (**Figures 4I–K**). While we have recently established the correlation between lower anti-*Mtb* IFNg responses following challenge and protective immunity against infection and disease in a repeated limiting dose (RLD) infection model in rhesus macaques (Dijkman et al., 2019), we consider that these unprecedented, suppressed PPD and ESAT6-CFP10 specific responses might reflect the protective effect of MTBVAC-HK boosting in this NHP model under relatively high-dose challenge condition. Most likely because of the relatively high challenge dose of 50 CFU of *Mtb*, we anticipate that, from 6 weeks post-infection onward, disease in these highly susceptible rhesus macaques can no longer be controlled. As a consequence, pathology scores and mycobacterial counts at endpoint may have converged to a similar outcome leaving a trend of improvement by MTBVAC-HK boosting only.

MTBVAC HK Vaccination Induces Mucosal Immunoglobulins in Vaccinated NHP

MTBVAC HK mucosal vaccination induced a strong cellular response both systemically and locally. However, considering the results obtained in mice we focused further analyses on systemic

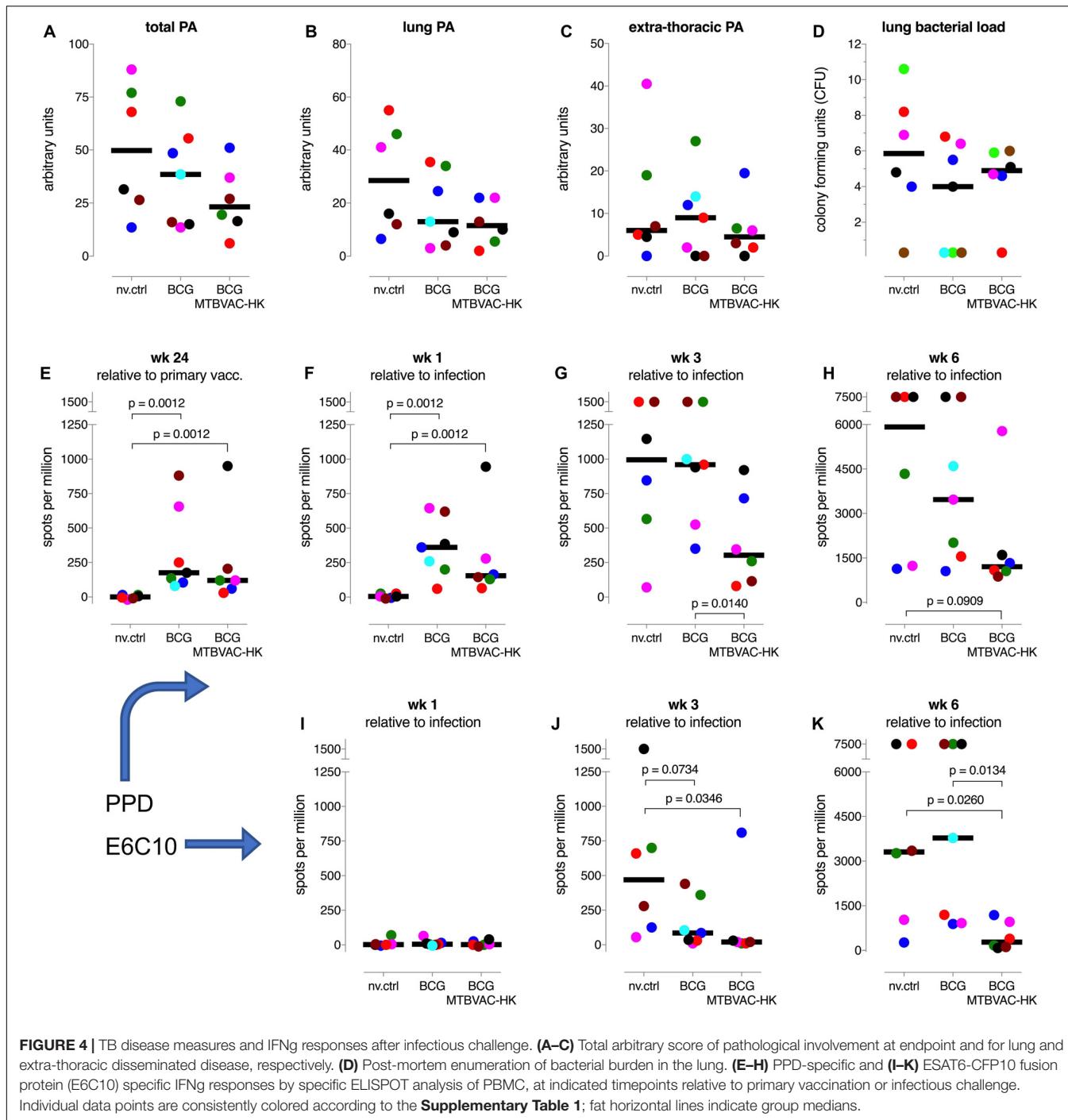
and local antibodies. To this end, we used sera and BAL samples from vaccinated monkeys obtained along the study time line to characterize humoral immune responses. PPD-specific IgG, IgM and IgA kinetics were monitored in serum samples from MTBVAC HK group during vaccination phase (from week 0 to week 20). Specific IgG showed a significant increase at week 20, which was particularly pronounced in 4 of the 6 animals, indicating a correlation between PPD-specific IgG and MTBVAC HK vaccination (**Figure 5A**). In the case of IgM, no significative changes were observed at the timepoints analyzed, although a slight transitory increase was observed in two animals at week 17, one week after MTBVAC HK inoculation (**Figure 5B**). Finally, the PPD-specific IgA profile showed a decrease that correlated with the administration of MTBVAC HK (**Figure 5C**).

We obtained BAL samples of three monkeys from the MTBVAC HK group at two different timepoints: at week 13 (before MTBVAC HK booster immunization), and at week 20 (4 weeks after MTBVAC HK administration). Our results clearly indicate that mucosal vaccination with MTBVAC HK induced a substantial increment of PPD-specific IgA, IgG, and IgM in the three animals studied, similar to the observations in mice. In addition, we also observed an increase in J chain presence, suggesting the presence of multimeric secretory IgA and IgM (**Figures 5D–G**).

Our data demonstrate the induction of PPD-specific immunoglobulins by MTBVAC HK. However, PPD comprises the secreted fraction of *M. tuberculosis* proteome. Thus, we next aimed to elucidate whether MTBVAC HK-induced mucosal Ig could bind to *M. tuberculosis* bacilli. To this end, we incubated H37Rv bacteria with BAL fluid samples, followed by biotin-conjugated secondary antibodies specific for human IgM, IgG and IgA. A final incubation step with PE-bound streptavidin allowed visualization of Ig binding by flow cytometry. Our data show a positive shift of the fluorescence signal for either of the three immunoglobulin families, IgA, IgM, and IgG, when bacteria had been incubated with BAL fluid from MTBVAC HK-vaccinated animals, indicating the presence of IgA, IgM, and IgG, able to bind to *Mtb* directly. This binding was substantially higher in the case of IgM, which would correlate with the pentameric conformation of this immunoglobulin. While for the BCG only-vaccinated animals, the binding was similar to that measured in BAL samples from unvaccinated animals, the highest signals were obtained after MTBVAC HK boosting specifically (**Figure 5H**).

H37Rv Opsonization Following Incubation With MTBVAC HK-Derived BAL

Phagocytosis mediated by antibody-dependant opsonization represents a major bactericidal mechanism triggered by immunoglobulins. To evaluate opsonization in our study, we added H37Rv to human monocyte cells THP1, after it had been incubated with the different BAL samples. We used GFP-expressing bacteria in order to monitor internalization by flow cytometry. Our data demonstrate about a two-fold increase of infected cells when H37Rv had been previously incubated with



BAL fluid from MTBVAC HK-vaccinated monkeys compared to control groups (**Figure 6A**). Opsonization has been associated with an increase in the capacity of macrophages to restrict bacteria into acidic compartments. To assess this, we analyzed intracellular colocalization of H37Rv with lysotracker, a probe that becomes fluorescent under acidic conditions. In the three monkeys analyzed, our results showed an increased bacterial colocalization following pre incubation of H37Rv with BAL from week 20 compared to those from week 13 (**Figure 6B**).

Our results showed great ability of BAL IgM and IgG to bind Mtb surface. Complement activation in respiratory airways has been previously reported, and therefore, since IgM and IgG is a very efficient complement activators, we evaluated the role of C3b, one of the major opsonins released during complement activation, for MTBVAC HK vaccination-induced opsonization. We incubated H37Rv with the different BAL samples and then we added a C3b-specific antibody to detect binding of this protein to Mtb surface by flow cytometry. In

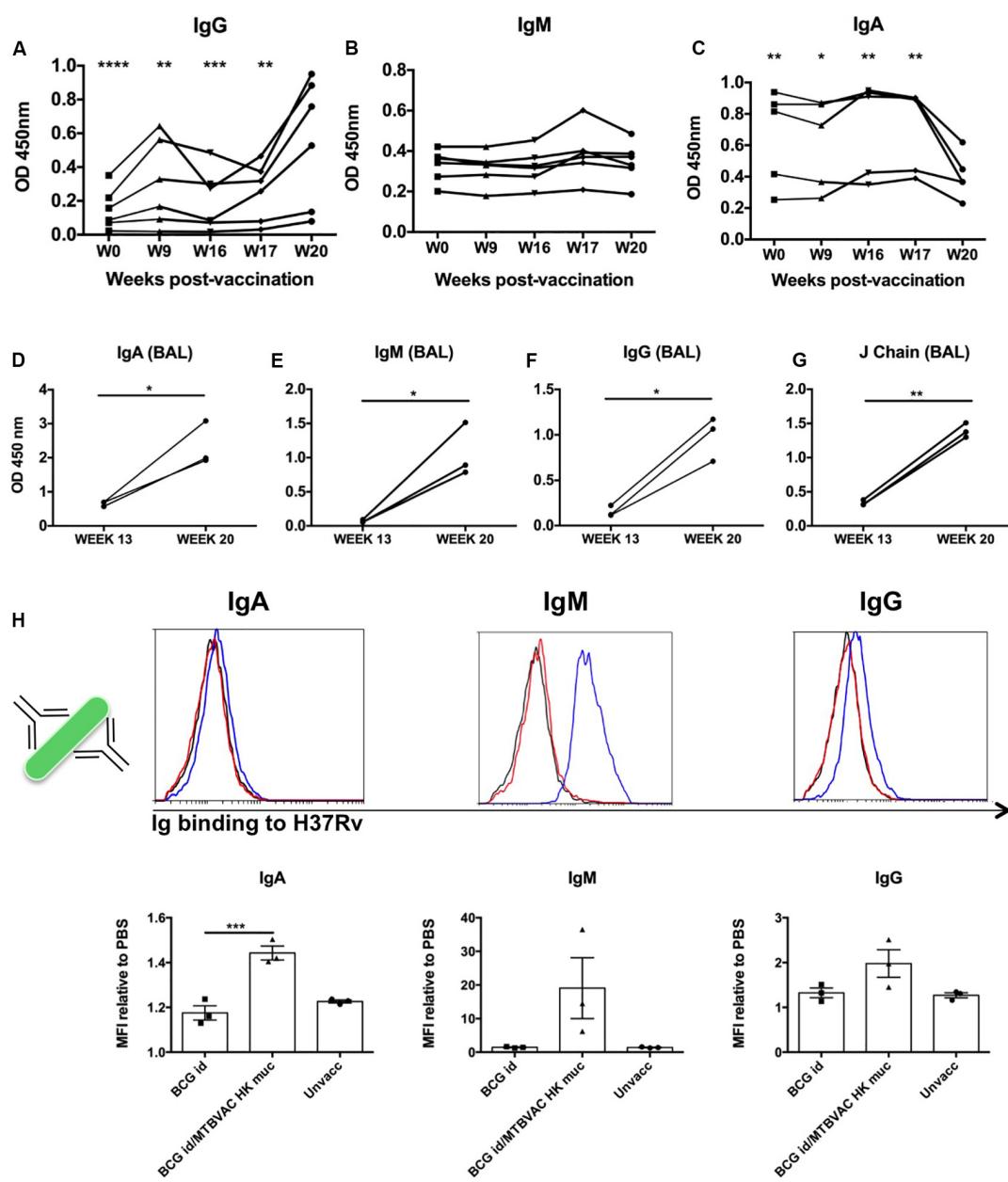


FIGURE 5 | Intrabronchial MTBVAC HK induces mucosal immunoglobulins in respiratory airways from non-human primates. **(A–C)** PPD-specific IgG, IgM, and IgA were measured in sera samples from the different individuals throughout the vaccination phase (until week 20). **(D–G)** PPD-specific IgA, IgM, IgG, as well as J chain were analyzed in BAL samples harvested at week 13 (before MTBVAC HK) and week 20 (after MTBVAC HK) from three individuals from the MTBVAC HK group. Data in the graphs show values for each individual. **(H)** Direct binding of IgA, IgM, and IgG to Mtb surface. H37Rv bacteria were incubated with BAL samples and immunoglobulin binding measured by flow cytometry using specific secondary antibodies. Representative overlay histograms are shown. Black line: unvaccinated; Red line: BCG vaccinated; Blue line: BCG/MTBVAC HK vaccinated. Data in the graphs show the mean fluorescence intensity (MFI) obtained with each BAL compared to the measured when bacteria are incubated with PBS. **(A–G)** Data show individual from one experiment. **(F)** Data are shown as mean \pm SEM and are representative of two independent experiments. **(A–C, H)** * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ by one-way ANOVA and Bonferroni post-test. **(A–C)** Comparisons of the different timepoints with week 20 are shown. **(D–G)** * $p < 0.05$; ** $p < 0.01$ by paired t-student test.

line with what was observed for the different immunoglobulin subtypes previously, we detected a higher fluorescence peak of C3b binding with the BAL from week 20 compared to week 13 (**Figure 6C**). Interestingly, preincubation of week 20 BAL from two different individuals with anti-C3b partially prevented

Mtb phagocytosis, suggesting a functional role of this opsonin in enhanced uptake (**Figure 6D**). These results do not rule out that other opsonins released during complement activation play a role in the enhanced uptake of Mtb after MTBVAC HK booster vaccination.

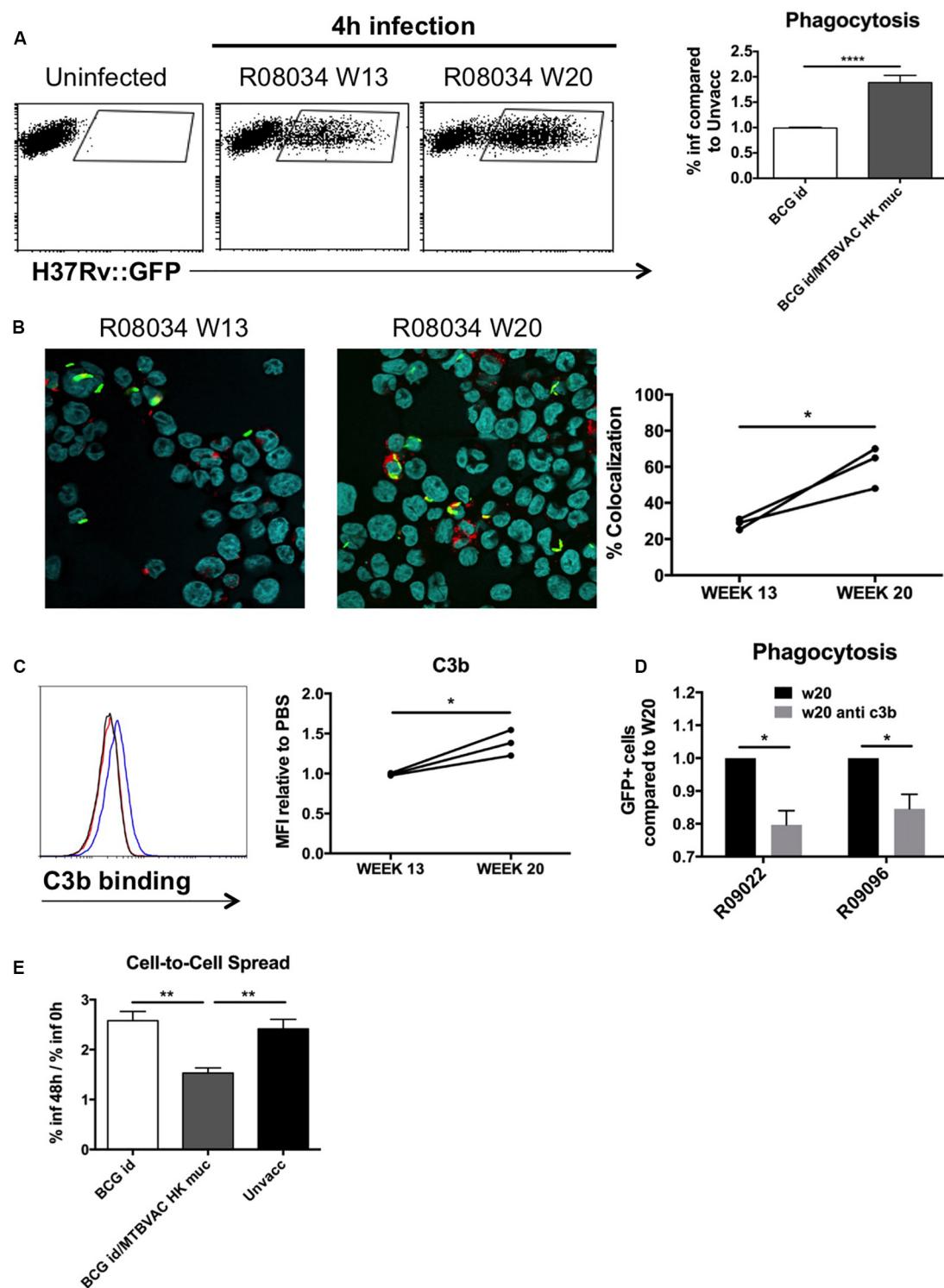


FIGURE 6 | Mucosal immunoglobulins induced by MTBVAC HK opsonize Mtb *in vitro*. GFP-expressing H37Rv coated with BAL samples were added to THP-1 human monocytes. **(A)** Percentage of infected cells was determined by flow cytometry 4 h post-infection. Representative dot-plots from one individual are shown in the left panel. Data in the graph is represented as the fold-change of the percentage obtained with the BAL from BCG-only or BCG/MTBVAC HK compared with the BAL from unvaccinated NHP. **(B)** Representative images of infected cells stained with lysotracker and Hoechst reagents. Data in the graph show colocalization values for each individual from MTBVAC HK group comparing colocalization obtained with BAL from week 13 and 20. **(C)** C3b binding to H37Rv surface was analyzed by flow cytometry using an antibody against C3b. A representative overlay histogram is shown. Black line: unvaccinated; Red line: BCG vaccinated; Blue line: BCG/MTBVAC HK. **(D)** Phagocytosis was measured by flow cytometry using GFP+ cells compared to W20. **(E)** Cell-to-cell spread was measured by flow cytometry using the ratio of GFP+ cells at 48 h to 0 h post-infection.

(Continued)

FIGURE 6 | Continued

line: BCG/MTBVAC HK vaccinated. Data in the graph show the MFI fold-change obtained following incubation with BAL from week 13 and 20 compared to the measured with the value when bacteria are incubated with PBS. **(D)** THP1 cells were infected with H37Rv previously incubated with BAL from week 20 in the presence or absence of a neutralizing antibody antiC3b. **(E)** Fold-change comparison of infected- THP1 cells at 48 h versus 0 h for each experimental condition. **(A,E)** Data in the graphs are mean \pm SEM from a pool of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.0001$ by one-way ANOVA and Bonferroni post-test. **(B)** Data show mean values of at least six images for each experimental condition from one experiment. **(C)** Data show individual from one experiment. **(B,C)** $^*p < 0.05$ by paired *t*-student test. **(D)** Data in the graphs are mean \pm SEM from a pool of two independent experiments. $^*p < 0.05$ by two-way ANOVA and Sidak's multiple comparison test.

Finally, we analyzed the capacity of internalized bacteria to spread from cell to cell. Our data indicate that percentage of infected cells at 48 h of infection was about 2.5-fold higher when compared to initial infection rate in cases where H37Rv had been incubated with control BAL fluid (unvaccinated or BCG only), whereas this ratio dropped to 1.5 with the BAL from MTBVAC HK boosted animals (**Figure 6E**). This result suggests that the ability of *Mtb* to spread to bystander cells was impaired when bacteria were opsonized.

DISCUSSION

During the last years there is rising interest to elucidate the role of immunoglobulins in tuberculosis infection. Different studies using animal models have shown that pulmonary delivery of monoclonal antibodies targeting certain *Mtb* surface proteins leads to a reduction of bacterial load following challenge (Achkar and Casadevall, 2013). In addition, results from clinical vaccine trials suggest a correlation between *Mtb*-specific IgG in sera and lower risk of TB disease (Fletcher et al., 2016). An elegant study recently demonstrated differences in glycosylation of immunoglobulin Fc fractions between latent and active TB individuals, and the importance of these signatures for antibody interaction with different cellular subsets, suggesting a role of antibodies in natural prevention of TB reactivation (Lu et al., 2016). Altogether, these studies provide substantial support for the potential contribution of antibodies to natural or vaccine-induced protection against tuberculosis.

Nevertheless, there is a poor understanding about the processes that drive to generation of protective antibodies during tuberculosis infection, and vaccine strategies that specifically exploit humoral responses are underrepresented in the tuberculosis vaccine pipeline. In the present work, we identify a novel vaccine approach that induces mucosal protective antibodies in the lungs. We describe in mice how a single intranasal immunization with an inactivated whole-cell *M. tuberculosis* vaccine triggers lung protective immunity when given as boost over previously BCG immunized animals. Data from NHP studies also showed a tendency in the MTBVAC HK-immunized group to improve protection in certain disease-associated parameters, even though differences were not statistically significant. Remarkably, results in NHP showing MTBVAC HK-induced inhibition of PPD- and ESAT6/CFP10-specific responses following *Mtb* challenge suggest a protective role of MTBVAC HK in this animal model. However, high dose challenge experimental conditions used in this

study may be obscuring the full protective potential of MTBVAC HK boosting.

We show that mucosal administration of MTBVAC HK induces strong cellular and humoral responses at both systemic and local lung level, which correlates with an improved protection compared to animals immunized only with BCG by parenteral route. Our data evidence the importance of the mucosal route of administration to trigger mucosal immunoglobulins in the lungs. In this regard, we demonstrate that intradermal BCG, the current vaccine regimen used in the clinic, does not generate antibodies in the lung lumen, which is in agreement with our previous observations (Aguilo et al., 2016).

Remarkably, MTBVAC HK fails to protect in the absence of pIgR, a transporter protein that mediates release of secretory Ig (sIg) to respiratory airways, suggesting a crucial role of mucosal antibodies in the protective mechanism triggered by this vaccination strategy. sIg comprise polymeric IgA (dimer) or IgM (pentamer) molecules, covalently linked to J chain, and the secretory component (SC), which corresponds to a pIgR extracellular domain that is cleaved following translocation. This complex conformation provides more stability to the immunoglobulin, making it less susceptible to proteolytic digestion and more mucophilic (Rojas and Apodaca, 2002). Our results in mice suggest no contribution of IgGs to MTBVAC HK-mediated protection, despite their presence in BAL following immunization. Monomeric immunoglobulins have lower capacity to neutralize pathogens in mucosal tissues compared to polymeric conformations (Renegar et al., 1998). In this regard, our results suggest a strong capacity of sIgA and sIgM to impair *M. tuberculosis* infection.

Even though our data do not demonstrate greater susceptibility of IgA-/ and pIgR-/ mice to *Mtb* infection *per se* and therefore, no apparent role of sIg in natural TB protection, the scenario changes when *Mtb*-specific sIg are present in the respiratory airways prior to pathogen encounter. Our *in vitro* functional assays using BAL fluid samples from MTBVAC HK-vaccinated NHP indicate the capacity of vaccine-induced sIg to bind directly to *Mtb* surface and to modulate pathogen interaction with macrophages. *Mtb* incubation with BAL from MTBVAC HK-immunized monkeys led to an increase of the phagocytosis rate, a higher colocalization of intracellular *Mtb* with acidic compartment tracer, and an impaired capacity of bacteria to spread from cell to cell. Thus, our data suggest antibody-mediated opsonization as a plausible protective mechanism of MTBVAC HK vaccination.

Our results indicate similar MTBVAC HK-induced protection in IgA-/ mice (where IgM is the only secretory immunoglobulin available) compared to wild-type animals,

suggesting a primordial role of sIgM in vaccine-induced protective efficacy. However, our results are not sufficient to determine that IgA have not contribution to vaccine-induced protective response. A good approach to elucidate this point could be the use of a mouse strain unable to produce secretory IgM. This experiment should be performed in the future to clarify this question. Unlike IgG and IgA, it is not well defined up-to-date which receptor for IgM could drive IgM-specific opsonization. However, IgM, including secretory IgM (Michaelsen et al., 2017), is a strong complement activator, and IgM-dependent opsonization by complement-derived opsonins is well reported (Guidry et al., 1993). In addition, our results also revealed an important binding of IgG to *Mtb* surface. Since IgG is a strong complement activator, these antibodies might also play a role in the bacterial opsonization events described in this study. One of the major opsonins activated during this process is C3b, released as a consequence of the C3 proteolysis by the enzyme C3-convertase. C3b presents strong avidity of binding to bacterial surfaces directly, and it mediates opsonization following recognition by CD35, a surface receptor on macrophages, including the THP1 cells used in the present study (Baqui et al., 1999). Our results suggest that, at least partially, C3b generated following MTBVAC HK vaccination has a role in *Mtb* opsonization. Although complement proteins are mostly generated in the liver, lungs can be a local source of complement proteins. In particular, alveolar epithelial cells, as well as alveolar macrophages can secrete complement proteins (Strunk et al., 1988; Huber-Lang et al., 2002).

Despite the lower immunogenicity and poorer protective efficacy conferred by inactivated whole-cell tuberculosis vaccines in comparison to their live counterparts, there is an interest in the development of this type of vaccination strategies. As inactivation is associated with an improved safety profile, such vaccines are considered in particular for target populations to which live vaccines might be harmful, such as immunosuppressed individuals. Limited immunogenicity of inactivated vaccines is usually overcome by other strategies, such as the formulation of inactivated vaccines with adjuvants (Haile et al., 2004) or repeated administration by multiple immunizations (Opie and Freund, 1937; Von Reyn et al., 2017). In the case of intranasal MTBVAC HK, our data indicate that it is not protective by itself, but it needs of a previous BCG prime to trigger a response in the lungs and confer protection. Even though we have not fully studied the mechanisms behind this result, it could be interesting to address the cross-talk between mucosal and systemic immune compartments in the future. For now, we speculate that the mucosal delivery of MTBVAC HK might be pulling the systemic cellular response elicited by subcutaneous BCG toward the lungs. Secondary targeting of the lung compartment with inactivated bacilli, would be a relatively safe way of boosting the cellular response and inducing specific antibodies locally against whole-cell bacteria.

Our results do not clarify whether the present vaccination approach is effective only when we use an *M. tuberculosis*-based vaccine as MTBVAC, or whether it can be translated to other whole-cell vaccines like inactivated BCG. In this regard, a previous study reported no additional protection in the lungs

conferred by intranasal heat-killed BCG given as booster of subcutaneous live BCG immunization (Haile et al., 2005). This failure of boosting with inactivated BCG could highlight the importance of the differential antigens expressed by MTBVAC relative to BCG (Marinova et al., 2017), which are shared with the *M. tuberculosis* pathogen. Indeed, contribution of differential antigens to MTBVAC-conferred protection has been previously reported in the case of live MTBVAC (Aguilo et al., 2017). Nevertheless, comparison between inactivated MTBVAC and BCG should be done head-to-head in order to confirm this difference under the same experimental conditions.

The results from the present study indicate that a plausible mechanism of protection of mucosal MTBVAC HK is mediated by the generation of specific antibodies in the respiratory airways, with capacity to opsonize live *Mtb* bacteria. We do not discard that another inflammatory events mediated by the vaccine as production of certain cytokines could also contribute to protection. To our knowledge, this is the first tuberculosis vaccine approach based on whole-cell bacteria that targets to elicit protective mucosal antibodies.

MATERIALS AND METHODS

Bacteria

BCG Danish (Statens Serum Institute) and H37Rv (Institut Pasteur Paris) strains were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with ADC 10% (Difco) and 0.05% (v/v) Tween-80 (Sigma), or on solid Middlebrook 7H11 (Difco) supplemented with ADC 10%. Bacterial suspensions for vaccination or infection were prepared in PBS from glycerol stocks previously quantified by plating serial dilutions. MTBVAC HK vaccines were prepared by heating a log-phase MTBVAC culture at 100°C during 30 min. In the case of formalin inactivation, MTBVAC culture was incubated with 0.5% (vol/vol) formalin during 48 h. Culture viability was checked for each vaccine lot. Bacterial density was determined by plating prior to heat treatment, and was used to calculate MTBVAC HK dose for *in vivo* studies.

Cell Lines

THP-1 cells were purchased to European Collection of Cell Cultures (ECACC) (Lot Number 10/016). All the experiments were done using cells with less than five passages from its thawing. Mycoplasma absence in the cultured cells was confirmed after finalizing the experiments using the MycoAlert PLUS Mycoplasma Detection Kit (Lonza).

Animal Studies Approval

Mouse studies were conducted in agreement with European and national directives for protection of experimental animals and with approval from the Ethics Committee from University of Zaragoza (approved protocols PI14/14, PI50/14, and PI33/15).

Ethical approval for NHP study was obtained from the Dutch authorities prior to start, under dossier registration number DEC726subB.

Mouse Study

All mice were kept under controlled conditions and observed for any sign of disease.

Protective Efficacy

Female, 8–10 weeks-old C57BL/6 (Janvier Biolabs), pIgR-/-(a kind gift from Gerard Eberl, Institut Pasteur Paris), IgA-/-(MMRRC repository), or DBA/2J (Janvier Biolabs) mice were vaccinated subcutaneously with 5×10^5 CFU BCG vaccine in 100 μl PBS. Four weeks post-vaccination, mice were intranasally vaccinated with 10^7 MTBVAC HK bacteria in 40 μl of PBS. Four weeks later, mice were challenged intranasally with 150 CFUs H37Rv. Bacterial burden was assessed four weeks post-challenge by plating homogenized lungs and spleen on solid medium. A group of infected mice was sacrificed 1 day after challenge in order to determine the initial bacterial load in lungs, which resulted in approximately 20 CFU in all experiments. In the experiments involving newborn animals, mice were vaccinated subcutaneously with 2.5×10^5 BCG CFU in 50 μl PBS in the first three days after birth. Unvaccinated controls were inoculated with 50 μl of PBS. 10^7 MTBVAC HK was administered intranasally eight weeks later (or 10^4 when indicated). Four weeks later, mice were challenged with 150 CFUs of H37Rv for bacterial burden determination, or 10^4 CFUs for survival evaluation. In this latter case, disease-associated symptoms (including weight, aspect and individual/social behavior) were monitored weekly, and mice were humanely euthanized according to pre-established endpoint criteria.

Immunogenicity

Four weeks after MTBVAC HK vaccination, mice were euthanized and splenocytes and lung cells collected. 10^6 cells per experimental timepoint were stimulated with Purified Protein Derivative (PPD) (Statens Serum Institute, SSI) 5 $\mu\text{g}/\text{ml}$ during 48 h for supernatants collection and cytokine detection by ELISA. IL17A or IFNy concentrations were determined with specific ELISA commercial kits (MabTECH). For bronchoalveolar lavage (BAL) collection, trachea was cannulated and BAL was performed with 0.8 ml of ice-cold PBS. Supernatant was separated from cells by centrifugation and frozen at -80°C for further protein detection analysis. For antibodies or J chain determination in BAL, maxisorp ELISA plates (NUNC) were coated with 10 $\mu\text{g}/\text{ml}$ of PPD and incubated overnight at 4°C . After a washing step with PBS-Tween20 0.05% (v/v) buffer, plate was blocked with Bovine Serum Albumin 1% (w/v) in washing buffer for 1 h at 37°C . Then, plates were incubated with 100 μl of BAL during 90 min at 37°C . Following washing, plates were incubated for 1 h at 37°C with the corresponding anti IgA, IgG, or IgM antibodies conjugated with Horseradish Peroxidase (HRP) (Sigma Aldrich) at a 1:10000 dilution. Finally, enzyme-substrate reaction was developed using 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma Aldrich) as substrate, and reaction was stopped with H_2SO_4 0.1N.

NHP Study

Bacteria, Vaccination and Infectious Challenge

For primary vaccination of NHPs, clinical grade BCG Danish 1331 (SSI, Copenhagen, Denmark) was reconstituted

according to manufacturer's protocol and applied at a single, standard human dose (of 5×10^5 CFU on average) by intradermal injection.

Inactivated MTBVAC-HK booster vaccination in NHP was established by endobronchial instillation of 1.95×10^8 CFU-equivalent/10 mL/dose, targeting the lower left lung lobe. To this end, one mL of MTBVAC-HK suspension (provided by Biofabri) was added to 9 mL of sterile, isotonic saline solution (Eurovet Animal Health B.V., Bladel, Netherlands) immediately prior to administration.

M. tuberculosis (Mtb) strain Erdman K01 was prepared at 50 colony forming units (CFU) per 3 mL sterile, isotonic saline per dose, for infectious challenge of NHPs by endobronchial instillation targeting the lower left lung lobe. NHPs were challenged in random order in a single session within 3 h from the preparation of the inoculum, using a pool of 3 vials of Mtb Erdman from -80°C frozen stock.

Study Design, Handling and Sampling

Purpose-bred, adult, healthy, Indian-genotype rhesus macaques (*Macaca mulatta*) were selected from BPRC's breeding colonies and stratified into treatment groups. Males and females were represented at a 2:1 ratio per group (unless indicated otherwise; see **Supplementary Table 1**). Animals, per gender, were socially housed throughout the experiment in BPRC's experimental animal facilities at biosafety level three. Enrichment was provided in the form of food and non-food items and welfare was monitored by daily observation.

A schematic diagram of the study design is provided in **Figure 3A**. After a brief period of acclimatization and baseline measurements, animals were primed with BCG – or left untreated as non-vaccinated controls – and BCG vaccinees were either or not boosted with MTBVAC-HK 16 weeks later. Another 9 weeks later – that is 25 weeks post-priming – all animals received an infectious challenge with 50 CFU of Mtb Erdman into the lower left lung lobe. Twelve weeks post-infection, animals were sedated and euthanized by intravenous pentobarbital injection, and subsequently submitted to full post-mortem evaluation and assessment of pathological and bacteriological parameters of infection and disease. Two animals of the non-vaccinated control group reached a premature, humane endpoint due to acute progressive disease development and were euthanized for full post-mortem evaluation 6 and 11 weeks post-infection, respectively (see **Supplementary Table 1**).

All animal handling was performed under ketamine sedation (10 mg/kg, by intra-muscular injection). For pulmonary endoscopic installation and additional relaxation, intramuscular ketamine (5 mg/kg) was supplemented with medetomidine (0.04 mg/kg) and an analgesic was sprayed into the larynx.

Peripheral blood was sampled by venepuncture at various time points, as indicated in the Results section. For immunological assays, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by standard Ficoll®-based gradient centrifugation using Lymphoprep™ (Axis-Shield, United Kingdom). Serum tubes were spun for 10 min at 1000 g to harvest and store cell-free serum at -80°C for further

analysis at later time point. Serum samples were filter-sterilized using 0.2 µm PVDF-membrane plates (Fisher Scientific) before immune response analysis.

Standard hematological parameters were measured in fresh EDTA blood on a Sysmex2000i system (Siemens). Standard clinical chemistry values were determined in fresh serum samples on a CobasTM Integra 400+ platform (Roche Diagnostics).

Bronchoalveolar lavage (BAL) was collected by three times consecutive instillation-and-harvesting of 20 mL prewarmed, sterile 0.9% saline, targeting the lower left lung lobe with an endoscope. BAL was collected 3 weeks before and 4 weeks after MTBVAC-HK boosting from $N = 3$ pre-assigned animals of the MTBVAC-HK booster group, and 3 weeks before boosting also from $N = 3$ pre-assigned animals of the non-vaccinated controls (see also **Supplementary Table 1** and **Figure 3A**). After passage over a 100 µm filter and centrifugation for 10 min at 400 g, supernatant was decanted and stored as BAL fluid at -80°C pending further analysis. BAL cell pellet was resuspended in fetal calf serum-, glutamax- and penicillin/streptomycin-supplemented RPMI culture medium for immediate use in immune assays. BAL fluid samples were filter-sterilized using 0.2 µm PVDF-membrane plates (Fisher Scientific) before immune response analysis.

NHP Post-mortem Evaluation

At study endpoint, gross pathology was arbitrarily scored utilizing a predefined algorithm, adapted from Lin et al. (2009). Lung lobes were slabbed into approximately 0.5 cm slices for ample inspection and lesions were scored by size, frequency and appearance. Extrathoracic organs (typically spleen, liver and kidneys) were scored similarly, and lymph node involvement was graded by size and appearance.

After gross pathological examination, lung lobes were minced and spleen and lung-draining lymph nodes were passed over a 100 µm cell strainer (Greiner Bio One International), prior to further homogenization using GentleMACS M tubes (Miltenyi Biotec). Homogenates were frozen stored at -80°C and later thawed and serially diluted onto Middlebrook 7H10 plates for enumeration of bacterial tissue burden.

NHP Immune Analyses

Immunogenicity of vaccination was measured by a lymphocyte stimulation test using a specific NHP IFNg ELISPOT kit (U-CyTech) for the readout of the frequency of IFNg producing cells. In brief, 2×10^5 PBMC in supplemented RPMI culture medium were incubated in triplicate in microtiter flat-bottom plates for 24 h at 37°C and 5% CO₂, either or not in the presence of specific antigen. Mtb-derived protein purified derivative (PPD, Statens Serum Institute, Denmark) or Mtb-specific, recombinant ESAT6-CFP10 fusion protein (E6C10, for short; a gift from K. Franken, LUMC, Leiden) were used for *in vitro* recall stimulation as indicated, both at a final concentration of 5 µg/ml. After overnight incubation cells were transferred to specific anti-IFNg-coated filter plates (PVDF, Millipore) for an additional overnight incubation. Spots were developed using tetra-methylbenzidine substrate (MAbTech) and quantified on an AELVIS automated reader.

Flow cytometry was used to measure intracellular IFNg cytokine levels in CD4+ and CD8+ T lymphocytes from BAL. In brief, BAL cells (at least 1×10^6 cells) were either or not stimulated with PPD at a final concentration of 5 µg/ml in supplemented RPMI for 3 h in the presence of costimulatory antibodies against CD28 (PE-Cy7 conjugated) and CD49d. Subsequently, GolgiPlug (BD Biosciences) was added and cells were incubated for another 15 h at 37°C and 5% CO₂. Cells were then stained by standard procedures using Cytofix/Cytoperm and PermWash buffer (all from BD Bioscience), using the following antibody conjugates: anti-CD20-V450, anti-CD3-V500, anti-CD4-PerCP-Cy5.5, anti-CD8a-APC-Cy7, and anti-IFNg-AF700. T lymphocytes were gated as singlets, CD20-negative, CD3-positive cells. Within this population CD4+ and CD8+ cells were interrogated for cytokine production (**Supplementary Figure S6**). Cells were measured in a LSR-II flowcytometer (BD Biosciences); data were analyzed using FlowJo software.

PPD-specific IgA, IgM and IgG in sera and BAL samples, as well as J chain in BAL, were determined following a protocol similar to the one described above for mice. For J chain determination, plates were incubated with a primary monoclonal antibody anti J chain (Santa Cruz Biotechnology, sc-271967) at a 1:1000 dilution, followed by incubation with secondary anti mouse IgG. Serum samples were diluted 1:500 in all cases, whereas BAL fluid was used undiluted. Anti human IgA, IgG or IgM antibodies were all conjugated with HRP (Sigma Aldrich) and diluted 1:10000. Primary anti J chain antibody was the same as described above for mice.

Functional Studies With BAL Samples

To evaluate direct Ig binding to Mtb, 10^7 GFP-expressing H37Rv in 50 µl of PBS were added to 50 µl of BAL (or PBS as control) and incubated for 1 h at room temperature. Then, anti human IgA, IgG or IgM antibodies, all biotin-conjugated (Mabtech), were added in 50 µl at a final dilution of 1:200 (30 min at room temperature). Finally, streptavidin conjugated with APC (Miltenyi Biotech) was added in 50 µl at a final dilution of 1 µg/ml (30 min at room temperature). Bacteria were fixed adding 300 additional µl of paraformaldehyde (PFA) (final concentration 4%) and acquired with a flow cytometry Gallios (Beckman Coulter). Use of GFP-expressing H37Rv allowed discerning bacteria from BAL-derived debris. To assess C3b binding, we used an anti C3b as primary antibody (Millipore, clone 3E7) diluted at 1:25. Biotin-conjugated anti mouse IgG (Bethyl), diluted 1:200, and APC-conjugated streptavidin were used to detect fluorescence by flow cytometry.

THP-1 cells (Sigma-Aldrich) were cultured at 37°C and 5% CO₂ in DMEM medium (Invitrogen) supplemented with 10% inactivated foetal bovine serum (Biological industries) and 2 mM glutamine (Biological industries). 5×10^5 cells were seeded in 24-plate wells and incubated overnight with PMA 10 ng/ml. GFP-expressing H37Rv were incubated with the different BAL samples (or PBS as control) as described above. When indicated, anti C3b at 300 µg/ml was added to BAL suspensions. Then, bacterial suspensions were prepared in DMEM complete medium and put in contact with cells for 4 h at a MOI or five bacteria per cell. Cells were washed three times with PBS and detached

with trypsin to evaluate internalization by flow cytometry. In some of the experiments, fresh media was added and GFP-positive cells analyzed 48 h later. To assess bacterial colocalization with acid compartments, cells seeded over microscopy slides were incubated with 1 μ M lysotracker red (Invitrogen). Then, they were fixed with PFA and nuclei stained with Hoechst 33342 10 μ g/ml. Images were acquired with an Olympus FV10-i confocal microscopy. Colocalization was quantified using FV10-ASW 3.0 software (Olympus), and was calculated as the proportion of pixels that were both green and red compared to those that were green. Percentage of colocalization was determined analysing at least six images randomly taken for each experimental condition.

Statistical Analysis

Results from this study were not blinded for analysis. No randomization specific methodology was applied to this study. No statistical method was used to calculate sample size in animal experiments. GraphPad Prism six software was used for statistical analysis. Statistical tests used for each experiment are indicated in the figure legends. All statistical tests were two-tailed. Outlier values were determined applying the Grubbs's test to all data sets, and were discarded from the final statistical analysis. Differences were considered significant at $p < 0.05$.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comisión Ética de Experimentación Animal de la Universidad de Zaragoza.

AUTHOR CONTRIBUTIONS

NA, EP, ER, FV, and CM designed the experiments and directed the study. NA, SU, EM, RT, DoM, AG, IO, RV, and CS performed the experiments. MM and JB provided material and facilities

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crucial for the execution of the experiments. NA, DeM, EP, FV, and CM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.01339/full#supplementary-material>

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Conflict of Interest: NA, SU, DeM, EP, ER, and CM are co-inventors of the patent “inactivated tuberculosis vaccine” filed by the University of Zaragoza and Biofabri.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors CM.

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PUBLICACIÓN 4:

Therapeutic efficacy of pulmonary live tuberculosis vaccines against established asthma by subverting local immune environment.



Research paper

Therapeutic efficacy of pulmonary live tuberculosis vaccines against established asthma by subverting local immune environment



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ABSTRACT

Background: Substantial recent advances in the comprehension of the molecular and cellular mechanisms behind asthma have evidenced the importance of the lung immune environment for disease outcome, making modulation of local immune responses an attractive therapeutic target against this pathology. Live attenuated mycobacteria, such as the tuberculosis vaccine BCG, have been classically linked with a type 1 response, and proposed as possible modulators of the type 2 response usually associated with asthma.

Methods: In this study we used different acute and chronic murine models of asthma to investigate the therapeutic efficacy of intranasal delivery of the live tuberculosis vaccines BCG and MTBVAC by regulating the lung immune environment associated with airway hyperresponsiveness (AHR).

Findings: Intranasal administration of BCG, or the novel tuberculosis vaccine candidate MTBVAC, abrogated AHR-associated hallmarks, including eosinophilia and lung remodeling. This correlated with the re-polarization of allergen-induced M2 macrophages towards an M1 phenotype, as well as with the induction of a strong allergen-specific Th1 response. Importantly, vaccine treatment was effective in a scenario of established chronic asthma where a strong eosinophil infiltration was already present prior to immunization. We finally compared the nebulization efficiency of clinical formulations of MTBVAC and BCG using a standard commercial nebulizer for potential aerosol application.

Interpretation: Our results demonstrate that pulmonary live tuberculosis vaccines efficiently revert established asthma in mice. These data support the further exploration of this approach as potential therapy against asthma.

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1. Introduction

Asthma has reached pandemic levels, with more than 300 million individuals affected all around the world. Asthma is especially prevalent in developed countries compared to low- and middle-income scenarios. One of the most accepted explanations for these differences comes from the named "Hygiene hypothesis", which suggests that asthma development is favoured by the lower exposure of children to determined environmental factors [1]. In this regard, exposure to certain microorganisms and mites (as those present in farms)

during early life stages might contribute to educating the immune system, leading to the acquisition of higher tolerance to allergens [2,3].

Asthma is a heterogeneous disease characterized by chronic airway inflammation and remodeling. Even though asthma can be associated with different types of inflammatory response, type 2 inflammation is present in more than 80% of asthma cases in children. T helper (Th) lymphocytes with a Th2 profile are present in most of the patients, producing cytokines as IL-4, IL-5 or IL-13, which are responsible for some of the characteristic clinical symptomatology. IL-5 plays a central role in the survival and recruitment of eosinophils, one of the main players in asthma, and whose presence in sputum represents one of the most accepted biomarkers for the

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Research in context

Evidence before this study

The current tuberculosis (TB) vaccine, BCG, is the most administered vaccine in history. Since BCG, a live-attenuated vaccine, is classically considered a Th1 response-promoter, the benefits of intradermal BCG vaccination for asthma have been widely assessed, although epidemiological evidences are controversial, with no clear benefits demonstrated. Conversely, different studies suggest a relationship between higher tuberculosis infection rates and lower prevalence of asthma. Indeed, we recently demonstrated this correlation in TB-infected mice subjected to an experimental model of allergen-induced asthma. Since tuberculosis infection is mostly acquired by the respiratory route, whereas BCG is given intradermally, evidences from human and animal studies suggest that the beneficial effects of live mycobacteria against asthma might rest on that bacteria physically reach the lungs to induce an asthma antagonistic response at a local level.

Added value of this study

In the present study, in an attempt to mimic the natural route of tuberculosis infection, we investigated the therapeutic efficacy of intranasal live-attenuated mycobacterial tuberculosis vaccines in different acute and chronic murine models of airway hyperresponsiveness (AHR). Our results showed that intranasal vaccine administration reverted AHR-associated response in all scenarios assayed, both in short-term and long-term acute models, and also in an established asthma scenario induced by chronic allergen exposure, where a strong airway eosinophilia was already present prior to vaccine administration. Our results indicate that vaccine treatment subverted asthma-associated type 2 response, including repolarization of allergen-specific Th2 lymphocytes into Th1. In addition, we described for the first time the use of the live-attenuated mycobacterial TB vaccine MTBVAC against asthma. MTBVAC is a vaccine candidate currently under clinical evaluation, and therefore alternative applications of this vaccine are relevant from a translational point of view.

Implications of all the available evidences

From regulatory point of view, aerosol (but not intranasal) delivery might be the only accepted route of administration for live tuberculosis vaccines into the lung compartment. In this study, we have evaluated the efficacy of nebulization *in vitro* of two clinical formulations of MTBVAC and BCG in a standard commercial nebulizer, demonstrating the feasibility of reaching therapeutic bacterial doses using standard nebulization devices. Altogether, our data support the further exploration of pulmonary application of live-attenuated mycobacteria as potential therapy against asthma.

found in asthma animal models [6,7] as well as in samples from patients [8]. M2 macrophages adopt regulatory skills and trigger an immune modulatory environment that impairs Th1 response and favours expansion of Th2 cells [9]. M2 is a simplified terminology that encloses different subsets of macrophages with regulatory skills. Thus, three different types of M2 macrophages have been defined, M2a, M2b and M2c, each with its own peculiarities. In the particular case of M2a macrophages, their presence has been linked with an induction of Th2 adaptive response [10]. With regard to allergic asthma, M2a macrophages can contribute to triggering the allergen-specific T cell response by at least two different ways, including presentation of allergen-derived peptides to T lymphocytes through MHC-II molecules, and by secretion of cytokines such as IL-4, which drive T cell response towards a Th2 profile [11]. Therapies targeting M2 macrophages have been shown to alleviate allergic responsiveness [12]. Thus, exacerbated M2 macrophage activation represents a highly attractive opportunity to design novel immunomodulatory treatments targeting this misbalance in lung macrophage populations [13].

The current tuberculosis vaccine BCG is the most administered vaccine in history. As live-attenuated *Mycobacterium bovis*, BCG vaccine is classically considered a Th1 response-promoting stimulus and as such, the benefits of intradermal BCG vaccination for asthma have been widely discussed [14]. Different observational studies suggest that vaccination with BCG could provide protection in the development of certain allergies, including asthma [15]. Remarkably, an interventional study conducted in South Korea evidenced that the group of asthma patients treated with BCG presented an improvement of lung function in the next days following vaccination, in comparison with the placebo group [16].

At a preclinical level, whole-cell BCG, either live or inactivated, as well as different mycobacterial components, have been extensively proven to be efficient against asthma in different animal models [17-19]. However, most of these results have been obtained with BCG delivered prior to or concurrently with allergen sensitization. As a result, the therapeutic capacity of BCG to revert established asthma has not been elucidated. In addition, previous studies have focused mainly on the Th1/Th2 response balance without considering other components of the immune system, such as pulmonary macrophages, which seem to play major role during asthma development.

In the present study, we mimicked the natural route of tuberculosis infection by intranasal delivery of live tuberculosis vaccines, to evaluate the therapeutic efficacy of this approach in different models of airway hyperresponsiveness (AHR). We hypothesized that direct interplay between vaccines and the lung compartment might modulate the immune environment associated with asthma. Our results revealed that BCG was able to re-educate M2 macrophages induced by allergen administration towards an M1 phenotype, as well as to convert allergen-specific Th2 lymphocytes to Th1. In addition, we assessed for the first time the efficacy of a live-attenuated *Mycobacterium tuberculosis* vaccine, called MTBVAC, against asthma. MTBVAC is currently under clinical evaluation, and to date it remains the first and only live vaccine based on attenuated *M. tuberculosis* that has reached the clinic [20-22]. Importantly, our data showed strong therapeutic efficacy of both BCG and MTBVAC in allergen-challenged mice in a scenario of established disease, demonstrating the potential of live attenuated tuberculosis vaccines as therapy for asthma.

2. Materials and methods

2.1. Ethics

Experimental work was conducted in agreement with the Spanish Policy for Animal Protection RD53/2013 and the European Union Directive 2010/63 for the protection of animals used for experimental and other scientific purposes. Experimental procedures were

diagnosis of the disease. In addition, IL-4 and IL-13 trigger airway remodeling by inducing proliferation of airway epithelial cells as well as exacerbated mucus production [4].

In addition to adaptive response, over the last years different studies have evidenced the crucial importance of lung innate populations for asthma triggering. Indeed, allergen presentation through MHC-II molecules from antigen-presenting cells (APC) results essential for the induction of allergen-specific T cells [5]. Asthma has been linked with a pathological macrophage polarization towards an M2 phenotype, as exacerbated levels of M2 macrophages have been

approved by the Ethics Committee for Animal Experiments of University of Zaragoza. (protocol PI22/15).

2.2. Bacteria

BCG Danish SSI (Pfizer), BCG Pasteur (strain 1173P2, Institut Pasteur Paris, France), MTBVAC(20) (University of Zaragoza) and MTBVACΔ^{erp} [23] (University of Zaragoza) strains were grown at 37 °C in Middlebrook 7H9 broth (Difco) supplemented with ADC 10% (Difco) and 0.05% (v/v) Tween-80 (Sigma), Sauton's medium or on solid Middlebrook 7H10 (Difco) agar medium supplemented with ADC 10%. BCG Pasteur and MTBVAC were transformed with the replicative pJKD6 plasmid encoding green fluorescent protein (GFP) (a kind gift from Luciana Leite, Butantan Institute, Brazil). MTBVAC heat-killed was obtained by boiling an MTBVAC culture at 100 °C for 15 min. MTBVAC produced under GMP conditions was provided by Biofabri (Spain). OncoTICE® was purchased from MSD. Bacterial suspensions for vaccination were prepared in PBS from glycerol stocks previously quantified by plating serial dilutions.

2.3. Animal studies

All mice were kept under controlled conditions and observed for any sign of disease.

For induction of OVA-specific AHR, 8 to 10 weeks old C57BL/6 (Janvier Biolabs) female mice were sensitized by two intraperitoneal injections of 50 µg chicken egg ovalbumin (lyophilized powder, ≥98% (Sigma)) with 2 mg aluminum hydroxide (Sigma, St. Louis, MO) one week apart. One week after second sensitization, mice were given a single intranasal administration of BCG, MTBVAC or MTBVAC-derived vaccine at the dose indicated in the figure legends, resuspended in 40 µl of PBS. In the acute model, four weeks after vaccine administration, animals were intranasally challenged with 100 µg OVA in sterile PBS for 3 consecutive days and one day after the last challenge, mice were humanely sacrificed. In some of the experiments, OVA challenge was delayed until 4 months after BCG vaccination. In the chronic OVA model, three weeks after immunization, mice were intranasally challenged with 10 µg OVA twice per week during eight weeks. In this case, vaccines were administered at week 9 of the procedure, in the middle of the challenge phase. For HDM-induced chronic AHR, mice were intranasally challenged twice a week for three consecutive weeks with 10 µg HDM. Vaccines were delivered at week 4 of the experiment (i.e., one week after primary HDM challenge), and one month later, mice were intranasally challenged with 10 µg HDM for three consecutive days. One day after last HDM administration animals were humanely sacrificed. Dexamethasone treatment was given according to [24]. Briefly, OVA-sensitized mice were treated intraperitoneally with 5 mg/kg dexamethasone (DEX) (Dexamethasone-Water Soluble, Sigma-Aldrich) the day prior to initiating the challenge phase, and then three additional DEX inoculations were given 1 hour before each OVA intranasal administration.

For bronchoalveolar lavage (BAL) collection, trachea was cannulated and BAL performed with 0.8 ml of ice-cold PBS. Supernatant was separated from cells by centrifugation for 5 min at 4500 xg.

Lungs were removed aseptically. For obtaining cellular suspensions, they were added to HEPES buffer (HEPES 10 mM; NaCl 0.15 M; KCl 5 mM; MgCl₂ 1 mM; CaCl₂ 1.8 mM pH 7.4) containing collagenase D 100 mg/ml (Roche) and DNaseI 400 IU (AppliChem), incubated at 37 °C for 30 min, and homogenised using GentleMACS (Miltenyi Biotech) dissociator with the lung specific program according to manufacturer instructions. Afterwards, residual red blood cells were lysed using Red Blood Cells Lysing Buffer (Sigma) and the homogenized filtered to eliminate tissue debris. For bacterial burden determination, lungs were homogenized with the GentleMACS, using the RNA protocol, and then plated onto agar medium 7H10

supplemented with ADC. In the case of histological analysis, lungs were fixed with 4% formaldehyde solution for 24 h prior to the staining procedure.

2.4. Flow cytometry analysis

10⁶ lung or BAL cells were incubated for 15 min at 4 °C with Fc receptor blocking reagent (Miltenyi Biotech). Then, eosinophil, neutrophil and macrophage presence was determined by extracellular staining with the following antibodies: CD45-FITC (RRID: AB_2658216), SiglecF-APC (RRID:AB_2653441), Ly-6G-Vioblu (RRID:AB_2751964), CD11c-PE (RRID:AB_2654707), CD11b-PerCP/Cy5.5 (RRID:AB_2751174) from Miltenyi Biotech. Eosinophils were defined as SSC^{high}CD45⁺CD11b⁺SiglecF⁺CD11c⁻; neutrophils as CD45⁺Ly6G⁺CD11b⁺CD11c⁻; and Alveolar Macrophages as CD45⁺SiglecF⁺CD11c⁺CD11b^{dim} cells.

For intracellular staining (ICS), after labeling membrane proteins with the above-mentioned antibodies, in addition to MHCII-Vioblu (RRID:AB_2652908) and CD86-PE (RRID:AB_2660746) (Miltenyi), and CD206-APC (RRID:AB_2739133) (BD Biosciences), cells were fixed and permeabilized with the FoxP3 staining set (Miltenyi Biotech), according to manufacturer instructions. As intracellular antibodies, we used iNOS-APC (RRID:AB_2727527) and iNOS-PE (RRID: AB_2727486) (Miltenyi), and Arg1-APC (RRID:AB_2734835) (eBiosciences). Cells were acquired using a Gallios flow cytometer (Beckman Coulter) and analyzed with Weasel software.

2.5. Cytokine analysis

Quantification of IL-5, IL-4, IL-13 and IFN-γ was performed using specific commercial ELISA kits following manufacturer instructions (Mabtech Biotech). Cytokine determination in the lungs was done from organ explants. These were prepared by cutting the lung into small pieces and incubating them overnight at 37 °C in 0.5 ml of culture medium.

To analyze OVA specific response, mediastinal lymph nodes were removed aseptically and mechanically disrupted for cell collection. 2 × 10⁶ cells were incubated with or without OVA at 1 mg/ml for 96 h. Then, supernatant was collected to determine cytokine concentration. OVA-specific response for each cytokine was calculated as the difference between cytokine concentration obtained following OVA stimulation minus the unstimulated control. For ICS, cells were incubated with 1 mg/ml OVA or 1 µg/ml of anti CD3/CD28 (RRID: AB_394590; RRID:AB_394763, respectively) (BD Biosciences) for 24 h, and 10 µg/ml Brefeldin A (Sigma) was added during the last six hours. For surface staining, cells were labelled with anti-CD4-FITC (RRID:AB_394582) (BD Biosciences) and anti-CD3-PerCPVi0700 (RRID:AB_2752207) (Miltenyi Biotech) in culture medium with 10% FCS. Then, cells were fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) following manufacturer instructions, and stained with anti-IFNγ-APC (RRID: AB_2784369) and anti-IL5-PE (RRID:AB_2660015) (Miltenyi Biotech).

2.6. qRT-PCR

For RNA extraction, lungs were immersed into TRIzol reagent (Invitrogen) just upon harvesting, and frozen immediately on dry ice. Once thawed, lungs were homogenised with the GentleMACS, using the RNA 0.2 protocol. 200 µl of chloroform were added per ml of TRIzol and after vigorous vortexing, tubes were centrifuged at 18,000 x g during one hour at 4 °C. Aqueous upper phase containing eukaryotic RNA was recovered, added to 700 µl of isopropanol and centrifuged at 18,000 x g during 10 min at 4 °C. The resulting pellet was washed with 70% EtOH and stored at -20 °C. Residual DNA was eliminated by DNase treatment, RNA was purified with an extraction based on phenol-acid-chloroform and precipitated ON at -20 °C with isopropanol

and sodium acetate. cDNA libraries were constructed for gene expression analysis by RT-qPCR. Primer pairs used in the present study were the following:

Gene	5' sequence	3' sequence
Actin	5'-ACCAAGTCGCATGGATGAC	5'-TGCGGGAGCCGTGTC
18S	5'-TTCTGATTGCCGCCCTAGA	5'-CTTCGCTCTGGCTCGTCTT
Gata3	5'-GACCGAAACCGGAAGATGT	5'-GCGCGCATGCACCTTT
Il11a	5'-ACCGCAGCACTTCAGAACATCAC	5'-CACCAAGCATGCCCTTGCTCA
Il11b	5'-TGGACACTCCCCATTCCT	5'-TGCCTGGATTCGAACAA
Ifng	5'-TTGGCTTGTGAGCTCTTCT	5'-TGACTGTGCCGTGGCAGTA
Il5	5'-TTGACAAGCAATGAGACGATGAG	5'-TCCAATGCATAGCTGGTGTGATT
Il4	5'-GGAGATGGATGTGCCAAACG	5'-CGAGCTCACTCTCTGGTGT
Il13	5'-TTGAGGAGCTGAGCAACATCAC	5'-CCATGCTGCCGTGCA
Stat1	5'-CTCTGAATGATGGGTGCA	5'-TTGAGCAGAGCGGCTTC
Stat4	5'-CATTGCAACCCAAGGAGATG	5'-TGGCAGGCCCTGTTCC
Stat6	5'-AACTGCAACGGCTATGTGA	5'-AGCCAGTCAGCCAGGAGATG
Tnf	5'-CAGCCGATGGTTGTACCT	5'-GGCACCCCTGTCCTTGA
T Bet	5'-ACCTGTTGTTCCAAGTCAA	5'-GCCGCTCTGCTTGTGATGA
Ifnb1	5'-CCCTATGGAGATGACGGAGAAG	5'-GAGCATCTCTGGATGCCAAA
Ccl11	5'-GACCAGTTGGCCAAGAGA	5'-GGCATCTGCCAGGACCATCT
Ym1	5'-CTCTGCCCTGGACATG	5'-AGAGGAAATCTCTGGTGCAC
Il11b	5'-AGTTGACGGACCCAAAAGA	5'-GGACAGCCCAGGTCAAAGG
Retnla	5'-CAGCTGATGGTCCAGTGA	5'-TCCCTTGACCTATCTCCACGAT
Nos2	5'-GGATCTCCAGGCAACCA	5'-TCCACAACTCGCTCAAAGATT
Arg1	5'-GCTCAAGCCAAGTCTTAGA	5'-CCTGAGGCTGTCCTTGA

2.7. Nebulization studies

MTBVAC® and OncoTICE® GMP vials were resuspended with 1 ml/vial of eluent indicated by manufacturers, concentrations normalized at 10^7 CFU/ml, and 2 ml of each vaccine preparation placed in the reservoir of the clinical nebulizer U100 (OMRON). Nebulizer was connected with a plastic tube to a gas washing flask with 5 ml of sterile water, coupled with a vacuum pump to recover the nebulized fraction. Bacteria were nebulized for 5 min and both nebulized and reservoir fractions were plated on solid agar 7H10 medium supplemented with ADC. Nebulization efficacy index for each vaccine strain was calculated as the percentage of bacteria in the nebulized fraction compared to that in the reservoir.

2.8. Statistics

Mice were randomly distributed in groups of 6 animals per cage prior to start experimental procedures. Results were not blinded for analysis. No statistical method was used to calculate sample size in animal experiments. GraphPrism software was used for statistical analysis. Statistical tests used for each experiment are indicated in the figure legends. All statistical tests used were two-tailed. Outlier values were determined applying the Grubb's test to all data sets, and discarded from the final statistical analysis. Differences were considered significant at $p < 0.05$.

2.9. Role of funding source

The funders had no role in study design, data collection, interpretation and analysis, decision to publish or preparation of the manuscript.

3. Results

3.1. Intranasal BCG and MTBVAC prevent AHR in allergen-sensitized mice

Initially, we tested vaccine efficacy in an acute model of AHR driven by ovalbumin (OVA) administration. Appearance of asthma symptoms in humans comes preceded by an asymptomatic phase of allergen sensitization, which is usual to occur at an early life stage during childhood [25]. Thus, to make our conclusions more

representative of the real situation, we delivered vaccines over previously sensitized animals (Fig. 1a). With the objective to reach the primary organ (lungs) affected during asthma episodes, we inoculated the vaccines by the intranasal route. Our long experience with this route of administration in mouse models indicate that a substantial proportion of bacteria reaches the lungs under the experimental settings used. The dose of BCG initially administered was 10^6 CFUs. As primary marker of asthmatic responsiveness we measured eosinophil infiltration in lungs and respiratory airways the day after last challenge (Fig. 1b, 1c, S1), relevant from a clinical point of view. Our data revealed a strong ability of intranasal BCG to abrogate eosinophilia both in BAL and lungs (Fig. 1c-e). In addition, we found no changes in other myeloid populations, as neutrophils and alveolar macrophages, suggesting that BCG did not trigger an uncontrolled cellular infiltration into the respiratory airways and indeed, the number of total leukocytes (CD45+ cells) were significantly lower in the BCG OVA group compared to OVA (Fig. 1e). Another typical asthma hallmark is the remodeling of the airway epithelium, which is characterized by a thickening of the alveolar walls and the proliferation of goblet cells, responsible for mucus production and secretion. These features were observed in the lungs from OVA group after staining with Periodic Acid Schiff (PAS) technique, where different layers of epithelial cells could be distinguished in the alveolar walls, with an important proportion of PAS-positive cells (goblet cells). In addition, presence of mucus substances was found in the airways. Remarkably, BCG treatment prevented this phenotype. Goblet cells were hardly visible and no mucus secretion was observed in alveolar spaces, whereas airway epithelium was restricted to one layer (Fig. 1f).

We also assessed intranasal BCG in a long-term AHR model, in which OVA challenge was performed four months after BCG immunization. BCG also reduced eosinophilia in this model (Fig. 1g). Interestingly, bacterial counting in lungs at the time of sacrifice indicated that BCG persistence was dramatically reduced compared to the one-month model (Fig. S2a), indicating that intranasal BCG could modulate asthma-associated immune environment even after almost complete bacterial clearance, which might suggest a contribution of memory response to the vaccine-protective effect.

MTBVAC is a novel live tuberculosis vaccine, attenuated from a clinical isolate of *Mycobacterium tuberculosis*, the pathogen causative of TB in humans, whereas BCG is a derivative vaccine from *Mycobacterium bovis* [20]. MTBVAC has demonstrated higher efficacy and immunogenicity than BCG against tuberculosis in different preclinical models [26], and is currently under clinical evaluation in newborn [22] (ClinicalTrials.gov Identifier NCT03536117) and adult populations [21] (NCT02933281) in TB-endemic countries in Africa, showing acceptable safety and immunogenicity profile to date. In the present study, we evaluated intranasal MTBVAC for the first time in a model of asthma. Using the OVA-driven acute AHR model described above, we observed a strong capacity of intranasal MTBVAC to abrogate eosinophil infiltration into airways, comparable to that observed with BCG (Fig. 1h).

To address whether vaccine persistence could affect protection against asthma, we compared MTBVAC with two other MTBVAC-derived vaccines: MTBVACΔerp strain, which contains an additional deletion of the gene *erp* resulting in a hyperattenuated phenotype with lower persistence as previously described [23] and demonstrated in this study (Fig. S2b); and MTBVAC killed by heat (MTBVAC HK). MTBVAC HK did not reduce eosinophils following OVA challenge, whereas MTBVACΔerp showed significant reduction as compared to the OVA group but lower than that observed with MTBVAC (Fig. 1i). These results suggested an influence of bacterial persistence on protection. To study this in more detail, we conducted a dose-response experiment with intranasal BCG and MTBVAC. We observed robust correlation of the dose with a stronger eosinophilia reduction, both in BAL (Fig. 1j) and lungs (Fig. 1k). In the case of the lungs, the highest dose groups (10^7 MTBVAC and 10^6 BCG) showed a level of

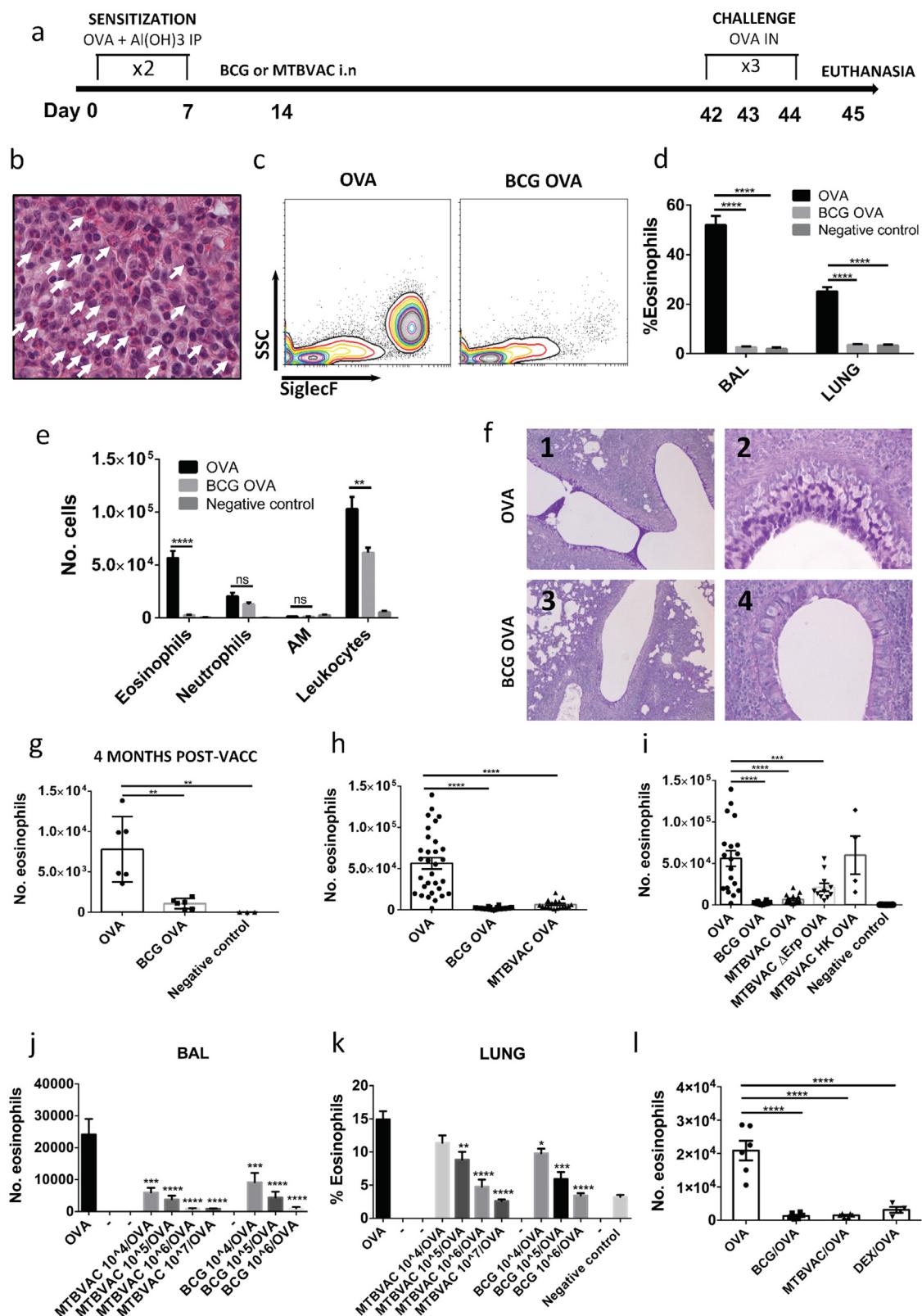


Fig. 1. Intranasal BCG and MTBVAC prevent allergic airway responsiveness in allergen-sensitized mice. (a) Eosinophils were determined by flow cytometry in an OVA-driven acute AHR model, with mice treated intranasally with 10⁶ BCC CFUs one week after second sensitization. (b) A representative hematoxylin-eosin lung image of OVA-challenged mice. Presence of eosinophils is highlighted with arrows. (c) Eosinophils were quantified by flow cytometry, corresponding to a population SSC^{high}SiglecF+CD11b⁺CD11c⁻. A representative diagram is shown in the figure. (d) Percentage of eosinophils in BAL and lungs with respect to CD45+ cells. (e) Total number of leukocytes, eosinophils, neutrophils and alveolar macrophages (AMs) in BAL determined by flow cytometry. (f) Representative images of PAS-stained fixed lungs from OVA-challenged mice untreated (1,2) or BCG-treated (3,4). (g) Eosinophils in BAL using long-term AHR model, in which OVA challenge was performed 4 months after BCG immunization. (h) Eosinophils in BAL following intranasal treatment with 10⁶ MTBVAC or BCG CFU. (i) Total number of eosinophils in BAL following intranasal treatment with 10⁶ MTBVAC, or the derivative strains MTBVACΔerp and MTBVAC Heat-Killed (HK). (j, k) Eosinophils in BAL (j) and lungs (k) following intranasal treatment with increasing doses of MTBVAC or BCG. (l) BAL eosinophils comparison following BCG, MTBVAC and DEX/OVA.

eosinophils similar to the negative control, and therefore we used these doses for the subsequent experiments. Finally, we assessed eosinophilia following intranasal MTBVAC or BCG in comparison with systemic administration of dexamethasone (DEX), which is a standard drug used in asthmatic patients. Our results showed comparable eosinophil reduction induced by intranasal MTBVAC and BCG and systemic DEX administration (Fig. 11).

3.2. Intranasal vaccination subverts asthma-associated lung immune environment

We hypothesized that the beneficial effects against AHR observed upon intranasal vaccination could be related with the ability of the vaccine to locally interact with resident phagocytic cells in the lungs. To assess *in vivo* infected cell populations following intranasal immunization, we delivered a BCG strain that expressed the green fluorescent protein (GFP). Our data demonstrated that level of BCG infection was efficient enough to discern infected cells by flow cytometry (Fig. 2a). Using a panel of antibodies to detect myeloid cell markers, we identified that the majority of BCG-infected cells corresponded to alveolar macrophages (AMs). Noteworthy, an important proportion of infected AMs expressed high levels of iNOS and CD86, two well-known markers of classical macrophage activation (or M1 phenotype) (Fig. 2a). We next compared macrophage polarization of total lung macrophages in OVA-challenged mice treated or not with BCG. Percentage of iNOS- and CD86-positive macrophages was substantially higher in the vaccinated group, whereas no difference was found in the case of MHC-II, with most of the macrophages positive for this marker in both groups (Fig. 2b). Importantly, expression of CD206, a classical marker associated with M2 polarization, was greater in macrophages from OVA group compared to those of the vaccinated group, which expressed similar levels of CD206 as naïve cells (Fig. 2c). To complement these data, we performed another experiment in which we isolated lung RNA from untreated or BCG-treated OVA-challenged mice. Expression analysis of genes linked with macrophage polarization clearly demonstrated that BCG immunization led to an upregulation of the M1 profile-associated genes *Nos2* and *Il1b*, whereas it inhibited expression of the M2 activation markers *Ym1*, *Arg1* and *Retnla* (Fig. 2d). Interestingly, in a dose-response experiment with MTBVAC and BCG, iNOS expression was found highest in the groups treated with the most protective vaccine doses (10^7 MTBVAC and 10^6 BCG) (Fig. 2e), suggesting a correlation between M1 phenotype and protection against asthma. Altogether, our data demonstrated the enrichment of M2-like macrophages during asthmatic responsiveness, and suggested that the therapeutic efficacy of live attenuated mycobacteria could rest on the reversion of this response by inducing classical activation of lung macrophages.

We next studied pulmonary Th1 and Th2 responses, whose misbalance towards the latter one has been classically connected with the immune pathogenesis of asthma. Gene expression analysis of lung RNA revealed that BCG vaccination led to induction of Th1-associated genes as *Ifng*, *Il12a*, or the transcription factor *Tbet*. Conversely, genes codifying for typical Th2 cytokines and chemokines, as IL-5, IL-4, IL-13 or CCL-1, were down modulated by BCG (Fig. 3a) compared to untreated mice. In consonance with these results, IL-5 and IL-4 cytokines were found elevated in BAL and lungs respectively in the OVA control group, whereas IFN γ was increased when treated with BCG (Fig. 3b, c). In addition to total cytokine levels, we also studied allergen-specific T cells after *ex vivo* OVA stimulation of harvested lymphocytes from mediastinal lymph nodes. Data showed a higher IL-4, IL-5 and IL-13, and lower IFN γ OVA-specific production in the OVA group compared to BCG-vaccinated mice (Fig. 3d-g). Intranasal MTBVAC showed a similar ability as BCG to shift CD4+ cells towards Th1 profile

(Fig. S3). We also found a correlation between vaccine-induced Th1 profile and bacterial dose, indicating the importance of bacterial persistence for the shift of Th2 response toward Th1 (Fig S4).

Importantly, using intracellular staining and flow cytometry we directly visualized cytokine-producing CD4+ T cells expressing the memory marker CD44. Both following stimulation with CD3/CD28 or with OVA, we observed an opposite T cell response polarization between OVA controls and BCG-treated mice. BCG treatment abrogated OVA-specific IL-5-producing cells, whereas it triggered allergen-specific Th1 IFN γ -producing CD4+ lymphocytes (Fig. 3h, i). This suggests that T cell-driven IFN γ production in BCG-treated mice is not only generated by expanded lymphocytes that recognize mycobacterial antigens, but also by allergen-specific T cells, that might be re-shaped from a Th2 towards a Th1 profile due to vaccine-associated inflammatory environment.

3.3. BCG and MTBVAC inhibit eosinophilia and lung remodeling in a scenario of established asthma

With the aim to elucidate the therapeutic potential of live attenuated vaccines against established asthma, we conducted experiments using a chronic AHR model with multiple OVA challenges, where vaccines were delivered halfway through the challenge phase (Fig. 4a). BAL eosinophils were determined in an additional group sacrificed the day prior to vaccination, at week 8, confirming eosinophil infiltration at the time of vaccine administration (Fig. 4b). Data at the end of the procedure, at week 13, revealed that eosinophils were reduced in the treated group, indicating the capacity of BCG to overcome established allergen-induced eosinophilia (Fig. 4c).

In another experiment, we also observed that MTBVAC was able to revert eosinophilia under similar experimental settings as BCG (Fig. 4d). In this experiment we additionally assessed lung remodeling by PAS staining and histological evaluation. Our data revealed an important disorganization of the alveolar epithelium surface in the OVA control group, as well as a substantial presence of goblet cells. This phenotype was not observed following MTBVAC treatment (Fig. 4e). We also analyzed by flow cytometry expression of the M2 and M1 polarization markers Arg-1 and iNOS, respectively. A significant proportion of Arg-1-positive macrophages was found in the OVA group but not in the MTBVAC-treated mice. Conversely, iNOS-positive macrophages were detected in the MTBVAC group, suggesting the ability of the vaccine to re-polarize asthma-associated M2 macrophages towards M1 profile (Fig. 4f, g).

We next assessed the therapeutic efficacy of intranasal BCG and MTBVAC in a model of established AHR induced by house dust mites (HDM), a relevant allergen in clinical asthma. Allergen-induced airway responsiveness was triggered by successive intranasal HDM challenges (Fig. 5a), which elicited a strong eosinophil infiltration in the BAL (Fig. 5b). BCG and MTBVAC administration resulted in abolishment of BAL eosinophilia, as measured in frequency (Fig. 5b) and absolute number of cells (Fig. 5c). PAS-stained lungs showed that HDM exposure in untreated mice strongly induced proliferation of the alveolar epithelium and appearance of goblet cells, a phenotype that was not observed in the vaccine-treated groups (Fig. 5d).

Finally, we observed that vaccine administration diminished production of Th2 cytokines and substantially increased IFN γ in BAL (Fig. 5e, g) and lymph node cells following stimulation with HDM (Fig. 5f, h).

3.4. MTBVAC and BCG aerosolization feasibility with a clinical nebulizer

There are strong regulatory safety concerns about intranasal delivery of vaccines in humans [27]. However, aerosol administration

dexamethasone (DEX) treatment. Data in the graphs are pooled means \pm SEM from three independent experiments (d, e, h), two (i), or one (g, j, k, l). A minimum of 6 mice were used per group and experiment. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$, by two-way ANOVA (a, e) or by one-way ANOVA (g, h, i, j, k, l), with Bonferroni post-test.

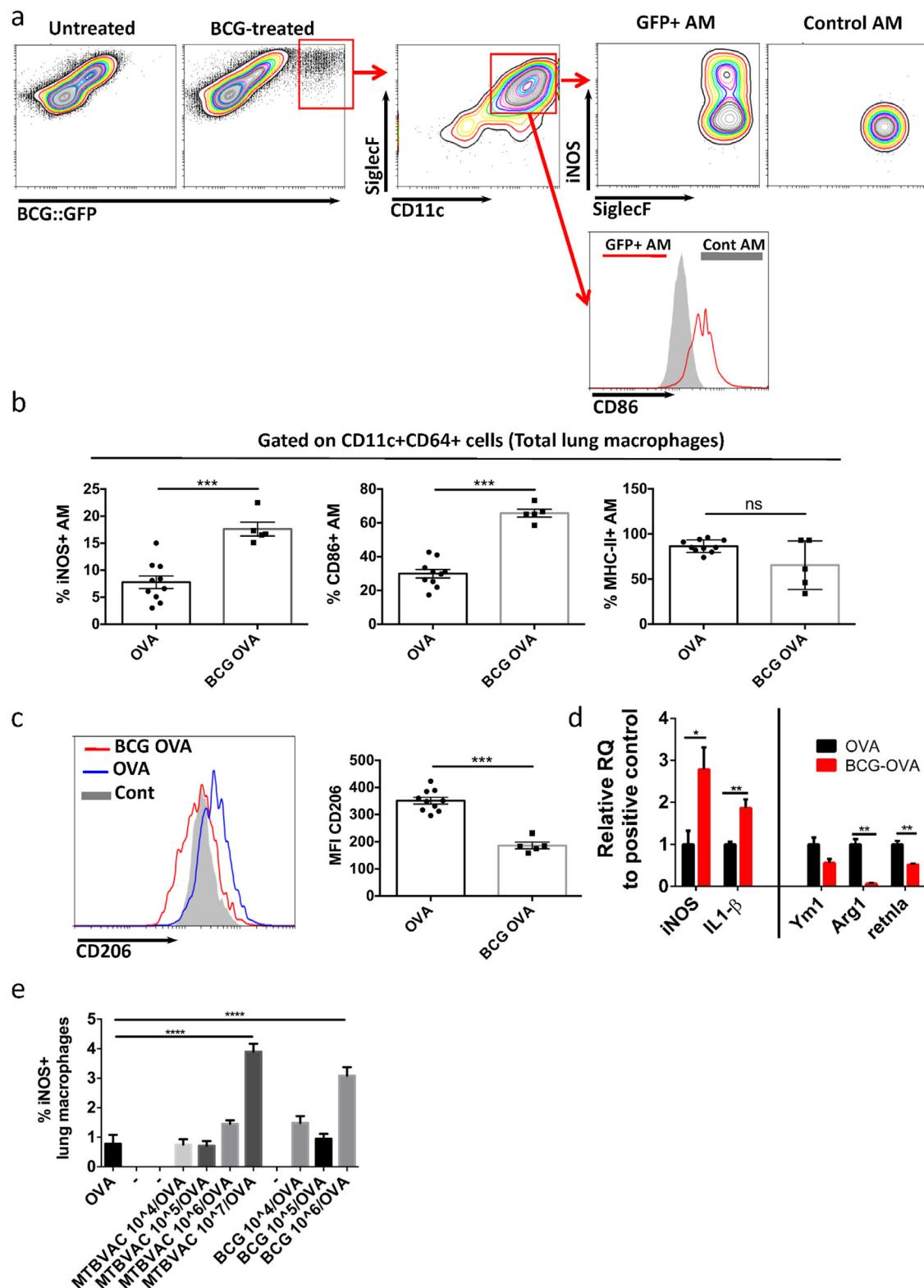


Fig. 2. BCG intranasal infects lung resident macrophages and induces classical activation. (a) Groups of mice were immunized with 10⁶ CFU of GFP-expressing BCG. One month later, infected cells were monitored and characterized by flow cytometry. Expression of M1-polarization markers iNOS and CD86 was analyzed. Representative diagrams are shown in the figure. (b) Percentage of iNOS-, CD86- and MHC- positive lung macrophages in OVA-challenged untreated or BCG-treated. (c) Surface expression of the M2-activation marker CD206. Representative overlay histogram showing CD206 surface expression of indicated experimental groups. Graph shows comparison of Mean Fluorescence Intensity (MFI) corresponding to CD206 level of expression. (d) M1 and M2 activation markers measured by qRT-PCR in lungs from OVA-challenged mice, untreated or BCG-treated. (e) Percentage of iNOS-positive cells in lung macrophages following intranasal treatment with increasing doses of MTBVAC or BCG. Data in the graphs are representative mean±SEM from two independent experiments (b, c) or one (d, e). A minimum of 6 mice was used per group and experiment. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; by unpaired single (b, c) or multiple (d) t-student test; or by one-way ANOVA (e) with Bonferroni post-test.

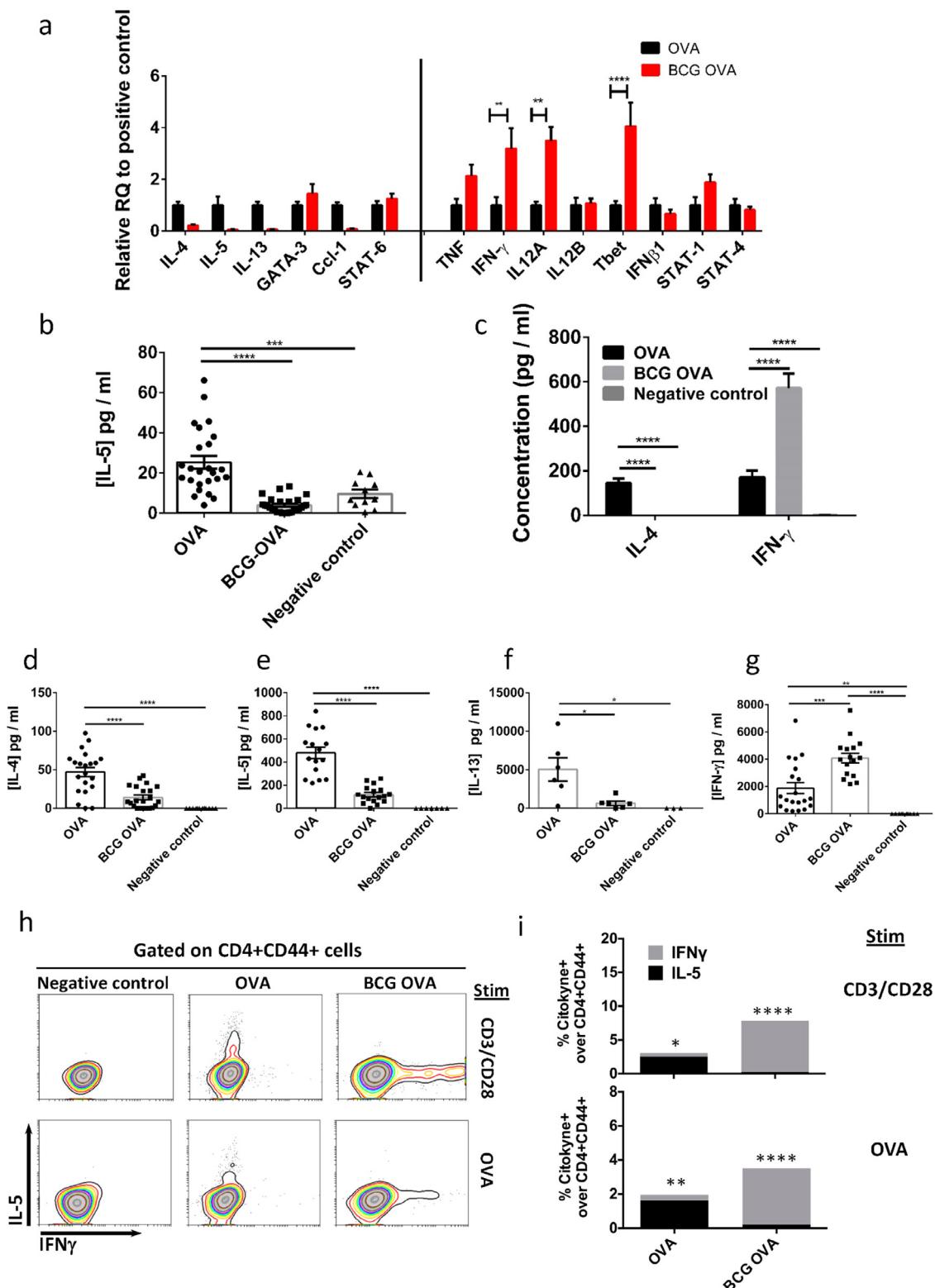


Fig. 3. BCG intranasal reshapes allergen-specific response into a Th1 profile. (a) Th1 and Th2 activation markers measured by qRT-PCR in lungs from OVA-challenged mice, untreated or treated with BCG one week after sensitization. (b) IL-5 determination in BAL (c) IL-4 and IFN γ determination in lung explants (d-g) Allergen-specific IL-4, IL-5, IL-13 and IFN γ produced by mediastinal lymph node cells, following ex vivo stimulation with OVA. Data are represented following subtraction of the value obtained in the absence of allergen. (h, i) IL-5- and IFN γ -producing cells visualized by intracellular staining and flow cytometry following ex vivo stimulation with anti CD3/CD28 or with OVA. Representative diagrams are shown. Data in the graphs are pooled means \pm SEM from three independent experiments (b-e, g) or one (a, i). A minimum of 6 mice was used per group and experiment. * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001, by multiple t-student test (a), one-way ANOVA with Bonferroni post-test (b, d-g), and two-way ANOVA with Bonferroni post-test (c, i).

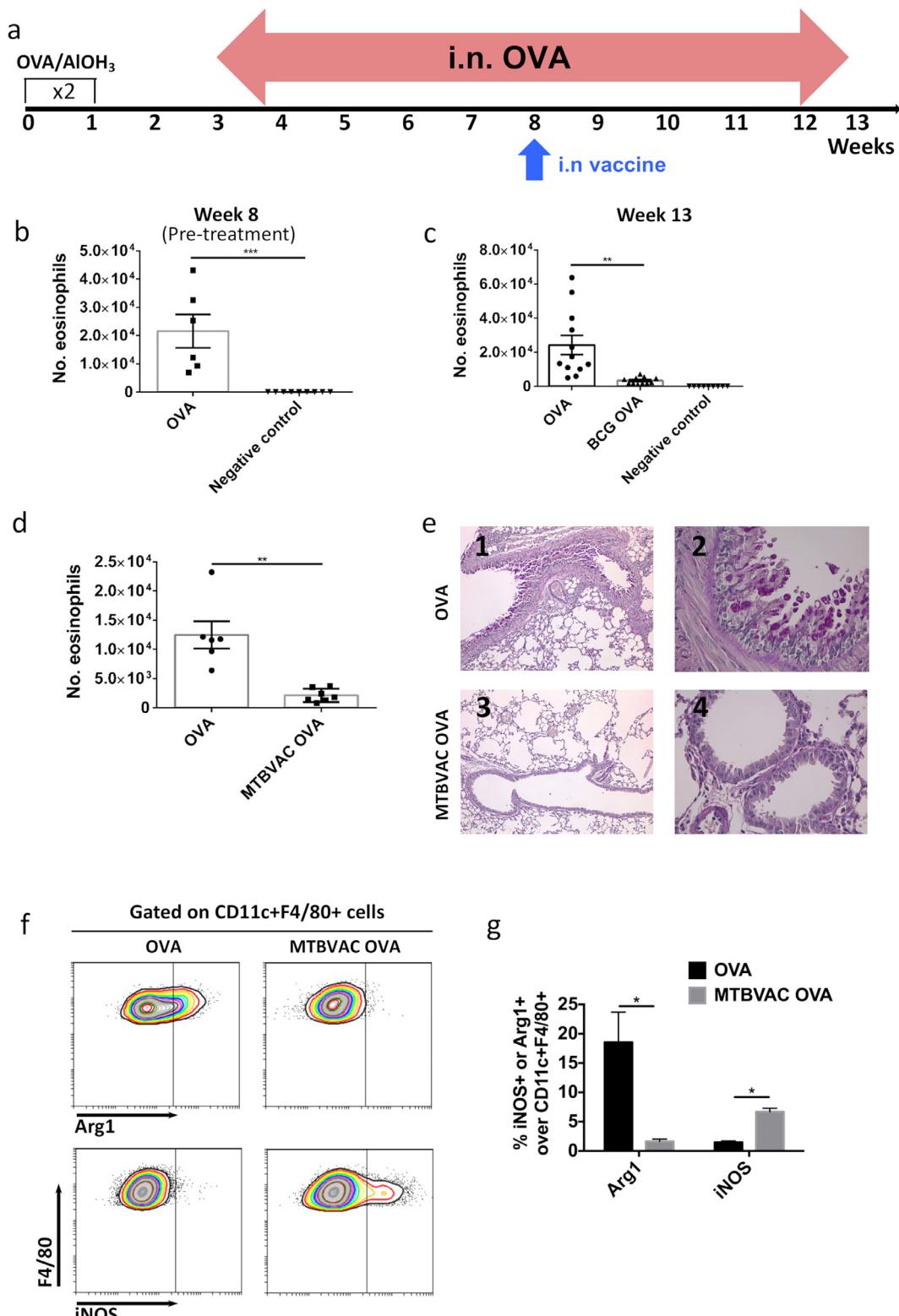


Fig. 4. BCG and MTBVAC intranasal revert established allergic airway responsiveness in an OVA-driven chronic model. (a) Scheme of the experiment based on an OVA-induced chronic model. Sensitized mice were challenged from week 3 to 13 with two intranasal inoculations weekly of OVA 10 µg. Vaccines were delivered at week 9, in the half of the challenge phase. (b) Total number of eosinophils in BAL at week 8, the week before vaccine immunization. (c) Total number of eosinophils in BAL at week 13, four weeks after BCG treatment. (d) Total number of eosinophils in BAL at week 13, four weeks after treatment with 10⁷ CFU of MTBVAC. (e) Representative images of PAS-stained fixed lungs from OVA-challenged mice untreated or MTBVAC-treated. (f, g) Intracellular expression of iNOS and Arg-1 in lung macrophages at week 13. Representative dot-plot diagrams are shown. Data in the graphs are pooled means±SEM from two independent experiments (b, c), or one (d, g). A minimum of 6 mice was used per group and experiment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, by t-student test (b, e), one-way ANOVA with Bonferroni post-test (b, c), and multiple t-student test (g).

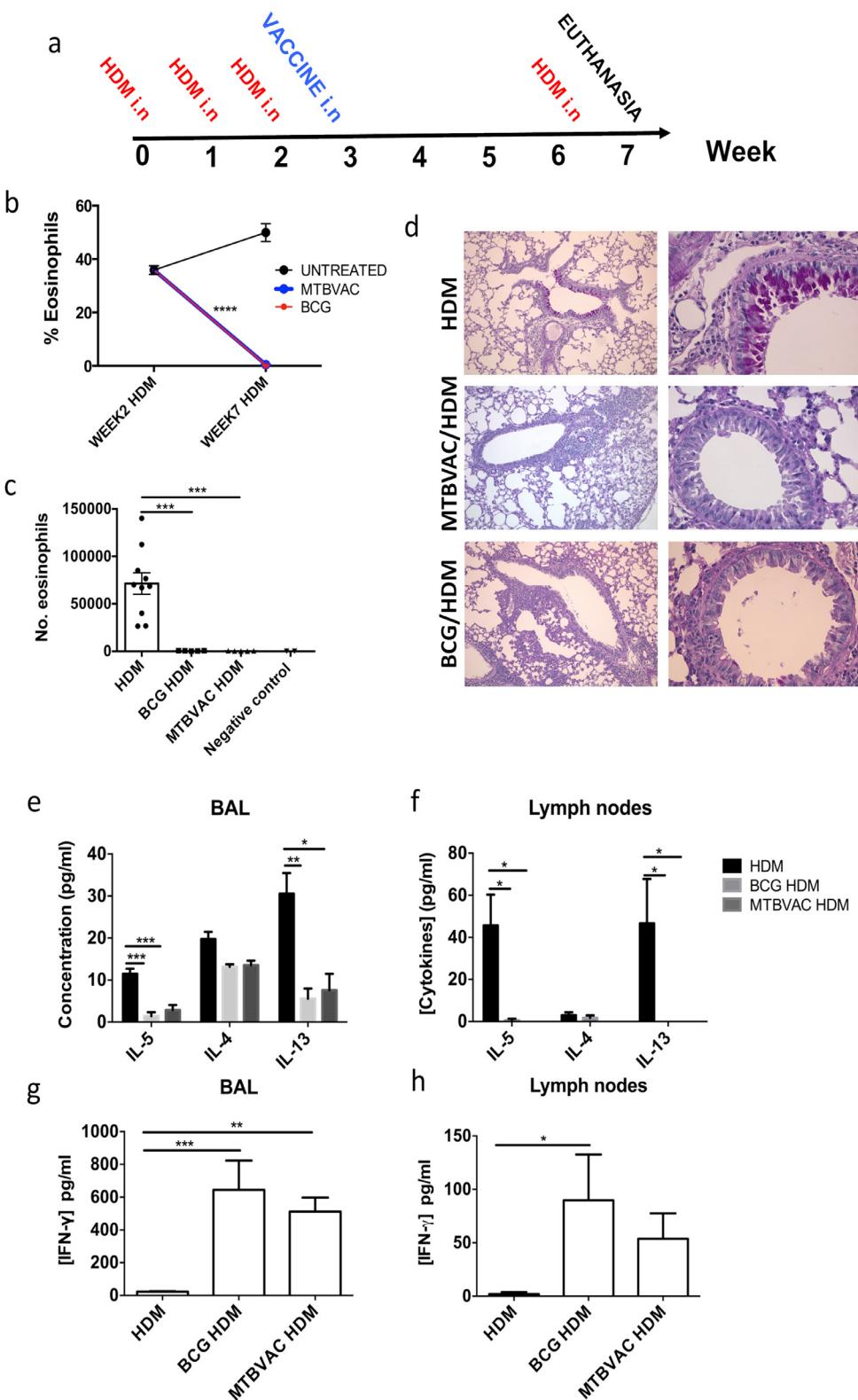


Fig. 5. BCG and MTBVAC intranasal vaccination reverts established allergic airway responsiveness induced by the relevant allergen HDM. (a) Scheme of the experiment. Mice were challenged twice a week during 3 consecutive weeks with intranasal HDM 10 µg. Vaccines were delivered the week after. (b) BAL percentage of eosinophils (with respect to CD45+ cells) comparison between week 2 (prior to treatment) and week 7 (end of the procedure), following treatment with MTBVAC or BCG. (c) Total number of eosinophils in BAL at the end of the procedure. (d) Representative images of PAS-stained fixed lungs from HDM-challenged mice untreated or BCG- or MTBVAC-treated. 10x left panels and 40x right panels (e, f) Th2 cytokines IL-5, IL-4 and IL-13 were analyzed in BAL, and in lymph nodes following ex vivo stimulation with HDM. (g, h) Th1 cytokine IFN- γ - was analyzed in BAL, and in lymph nodes following ex vivo stimulation with HDM. Data in the graphs are mean \pm SEM from one experiment ($n=6$ mice per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ by one-way ANOVA with Bonferroni post-test (a, c, g, h), and two-way ANOVA with Bonferroni post-test (e, f).

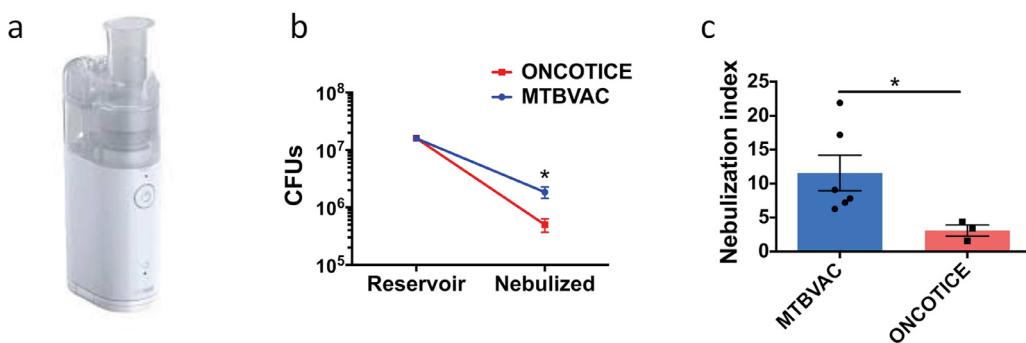


Fig. 6. MTBVAC and BCG are feasibly nebulized with a clinical device. (a) OMRON U100 nebulizer used in the study. (b) MTBVAC and BCG OncoTICE® CFUs determined in nebulized and reservoir fractions. (c) Nebulization efficacy index calculated as the percentage of bacteria recovered in the nebulized fraction compared to the contained in the reservoir. Each dot corresponds to the result obtained with a different filter. Data in the graph represents mean \pm SEM from six independent nebulizations with MTBVAC, and three with BCG OncoTICE®. * $p < 0.05$, by t-student test.

is an accepted way to reach the pulmonary compartment in clinic, and multiple commercial nebulizers authorized for human use are available. From a practical perspective, an important step to bring pulmonary administration of live mycobacteria to clinical development is the feasibility to deliver therapeutic vaccine doses through the aerosol route. To evaluate this, we measured the nebulization efficacy of GMP formulations of MTBVAC and BCG (OncoTICE®) adapted for human use, through the clinical nebulizer OMRON U100 (Fig 6a). Bacterial concentration loaded into the reservoir was similar for both vaccines, around 1.5×10^7 CFUs/ml. After 1 min of nebulization, the mean amount of bacteria recovered in the nebulized fraction was 1.85×10^6 CFUs for MTBVAC and 5×10^5 for BCG (Fig 6b). The mean efficacy of nebulization with respect to the initial bacterial load in the reservoir was 11.7% for MTBVAC and 3.1% for OncoTICE® (Fig 6c).

Studying different characteristics of BCG and MTBVAC vaccines we noticed a different appearance between MTBVAC and BCG clinical formulations upon reconstitution (Fig S5a), with BCG showing a cloudy suspension, whereas MTBVAC suspension was more clear. It is important to remark that the amount of viable bacteria in both formulations was comparable, and therefore the more turbid aspect of BCG could be a consequence of a higher proportion of dead bacteria produced during the manufacturing process. In addition, this could be related with the higher tendency of BCG to clump during bacterial growth. As we have observed under laboratory conditions, in the absence of detergent, MTBVAC tended to grow in suspension, whereas BCG suspension showed more clumps (Fig. S5b). We also visualized both individual bacilli of MTBVAC and BCG by electronic microscopy. Images revealed lower bacterial length of MTBVAC ($< 1 \mu\text{m}$) in comparison to BCG ($> 2 \mu\text{m}$) (Fig. S5c).

4. Discussion

Data available in the literature evidence a primary role of lung innate cells in the development of asthma. Concretely, alternatively activated or type M2 macrophages are elevated in lungs from asthmatic individuals, both in animal models and humans. Reasons behind this pathological macrophage polarization are not clear. A plausible explanation is that allergens can directly cause damage in the alveolar epithelium, and macrophages might be alternatively activated as reaction to the injury to induce a wound-healing response [28].

In the present study, we evaluated the therapeutic potential of live attenuated tuberculosis vaccines BCG and MTBVAC delivered by the intranasal route in different preclinical models of asthma. Our results are highly robust. Intranasal BCG and MTBVAC revert AHR-associated response in all scenarios assayed, both in short-term and long-term acute models, and also in established asthma induced by

OVA or HDM, where a strong airway eosinophilia was already present prior to vaccine administration. Our results showed that airway eosinophilia reduction induced following single intranasal delivery of BCG or MTBVAC was comparable to the observed following dexamethasone (DEX) treatment achieved after four drug systemic inoculations. This reflects a qualitative difference between treatment with vaccines or anti-inflammatory standard drugs. Whereas in the former case, vaccines are administered once and they provided therapeutic efficacy in the long-term by subverting the asthma-associated immune response; in the relative to standard treatments their target are the symptoms instead of the causes of the disease, and therefore they carry out the beneficial effect only in the short-term, with the consequence that the patient is usually subjected to a chronic treatment during the whole life.

BCG and MTBVAC are efficiently internalized by alveolar macrophages upon immunization, leading to the expression of M1 markers like iNOS or CD86. These proinflammatory macrophages might trigger an inflammatory response antagonist to the generated by alternatively activated macrophages usually associated with asthma. Importantly, our analysis with GFP-expressing BCG bacteria indicates that not only infected macrophages adopt an M1 phenotype, but also uninfected cells. This suggests that macrophages initially infected by vaccine bacteria generate a chain of signals that would lead to activation of bystander neighbours, favouring the expansion of a response that globally counterbalances asthma-associated lung environment.

BCG-associated benefits for asthma patients have been extensively studied in the clinic, both in observational studies comparing asthma prevalence among BCG vaccinated populations [15], and in interventional controlled trials with two arms vaccinated either with BCG or placebo [16]. Data are controversial, with studies showing opposite findings. Reasons to explain this controversy can be attributed to different aspects, such as age-group (children or adults), different doses of vaccine administered, previous exposure to mycobacteria, or even use of different BCG substrains. For instance, in an interventional study conducted in 2002 in South Korea, authors found an improvement of lung function in the BCG group with respect to placebo. However, study subjects were mostly BCG-vaccinated, since this vaccine is usually administered during childhood in the country, and importantly, the intradermal BCG dose was extremely high (58.2×10^7 CFUs) (standard intradermal dose is 5×10^5) [16]. These experimental settings are different from other studies performed in countries without a history of BCG vaccination, where a standard dose of BCG would be used. This is the case of another study from 2008 conducted in the Netherlands, where no significant benefit was found in the BCG group [29]. Indeed, at least two meta-analysis reports comparing different published studies suggested poor association between intradermal BCG vaccination and low risk of developing allergies [14,30].

Our data convey that the beneficial effect of BCG might be organ dependent, and therefore BCG needs to be physically present in the lungs to induce an efficient antagonist response against asthma. Indeed, subcutaneous BCG is not protective against asthma in mice [18], a result that we have confirmed with MTBVAC (data not shown). This agrees with our observation that live mycobacteria must interact with resident lung macrophages to activate them in a classical way. Data from the literature regarding asthma incidence and TB infection and disease could endorse this hypothesis. A significant correlation between lower prevalence of asthma and TB notification cases has been reported [31]. A study conducted in individuals with latent TB infection (LTBI) showed a strong association between tuberculosis skin test (TST) positivity and lower prevalence of different types of allergy [32], including asthma. We recently demonstrated this result using an experimental tuberculosis infection mouse model [33]. Interestingly, Obihara et al. found a substantial reduction of asthma incidence in the group with the highest TST value, suggesting a correlation between magnitude of the TB-specific immune response and degree of unspecific protection [32]. TST assay rate of false LTBI positives is high, mainly due to the interference of the test with BCG vaccine or environmental mycobacteria. However, the authors found the lower asthma prevalence in the group with higher TST positivity (>20 mm), which makes more likely that this unspecific benefit could be due to latent tuberculosis infection [34,35].

Finally, reports of individual cases have described lower rates of exacerbations in asthmatic patients who suddenly undergo active TB [36]. Since *M. tuberculosis* infection is mostly acquired by the respiratory route (whereas BCG is given intradermally), we speculate that our results might be reflecting a natural unspecific protection against asthma that already occurs in nature.

Allergen-specific CD4+ T cells are thought to play a central role in asthma pathogenesis. Use of allergen-MHC-II tetramers has allowed characterization of allergen-specific CD4+ T cells in asthmatic individuals, finding tetramer-positive T cell clones that express central memory markers [37]. These long-lived T cells are ultimately responsible of the perpetuation of asthma throughout lifetime. Upon allergen exposure, specific memory CD4+ T cells migrate to lungs where they efficiently recognize allergen-derived peptides presented by APCs, and respond quickly by secreting Th2-associated cytokines leading to asthma inflammatory response. Thus, a therapy that pursues asthma abrogation should focus on hampering allergen-specific Th2 memory cells. For instance, immunotherapy based on low-level allergen epitope exposure works by inducing anergy on allergen-specific Th2 T cells [38].

Our results analysing OVA-specific T cells in draining lymph nodes indicate that intranasal vaccines have a strong impact in OVA-specific Th2 cells. Intracellular staining and flow cytometry, which allows direct visualization of cytokine-producing cells, showed a population of IL-5-producing T cells in the OVA group that is diminished upon BCG treatment, as long as a population of OVA-specific IFN γ -producing cells arises. This suggests that live mycobacteria could reshape the phenotype of T cells involved in asthma responsiveness. Interestingly, BCG-induced protection against AHR was also observed in long-term studies (where OVA challenge was delayed until 4 months post immunization), though bacteria were almost totally cleared at the time of analysis, suggesting that vaccines might be triggering allergen-specific memory T cells with a Th1 profile that persisted even after bacterial clearance, and which could provide a long-lasting protection against allergen exposure.

A question that remains unanswered in the present study is whether the T cells that adopt a Th1 profile after immunization are generated *de novo* from naïve T cells, or they are the same Th2 clones induced by allergen exposure, which are re-polarized to Th1 by the effect of the vaccine. Supporting the second hypothesis, T cells present high plasticity and Th2 cells have been shown to become Th1 in

the presence of an appropriate environment enriched with interferon and IL-12 [39].

Our results demonstrate in mice the efficacy of intranasal BCG and MTBVAC subverting AHR. Nevertheless, it is unlikely that intranasal route is authorized for live tuberculosis vaccine delivery in clinic. Together with the anatomical limitations associated with this route [40], there are safety concerns with intranasal administration. A flu vaccine intranasally delivered caused transient face palsy episodes in clinic [27]. In addition, proximity of the nose cavity to the brain would be likely a cause of reluctance for regulatory authorities. Conversely, the aerosol route results more plausible [41]. Recent studies in non-human primates (NHP) have demonstrated that BCG reaches the lungs following aerosolization with clinical nebulizers [42,43]. With regard to human studies, BCG has been already administered in the past by the aerosol route without major toxicity issues [44,45], and at present there are at least two on-going clinical trials to assess safety of aerosol BCG (NCT03912207, NCT02709278). Interestingly, our results showed the feasibility of nebulizing GMP formulations of MTBVAC and BCG through a clinical nebulizer, reaching bacterial loads proven to be effective in models of established asthma. Of note, our data indicated that MTBVAC was more efficiently nebulized than BCG. As mentioned above, two distinct phenotypic characteristics between MTBVAC and BCG could account for the different nebulization efficiency of the vaccines. Considering that the mean particle size released by clinical nebulizers is around 5 μm , it is realistic to speculate that clumping and bacterial size could crucially affect the efficacy of vaccine nebulization. In this regard, MTBVAC presents a reduced clumping capacity during growth and smaller bacterial size compared to BCG. This could be due to the loss of most of the extractable surface lipids in MTBVAC as a result of the deletions in the virulence genes *phoP* and *fadD26* [20]. Indeed, *phoP* mutant strains show a reduced cording factor [46], which is highly related to clumping in wild-type strains. Intradermal MTBVAC is in advanced clinical development in TB-endemic countries today with demonstrated safety, at least comparable to BCG, and superior immunogenicity characterized by antigen-specific Th1 responses in both adult and newborn populations [21,22].

Although BCG was initially conceived as a prophylactic vaccine against TB, other different clinical applications have been subsequently proposed. The most remarkable one has been the intravesical application of BCG as bladder cancer therapy, successfully used for the last four decades. However, no previous therapeutic application of pulmonary BCG has been reported up-to-date. From a safety perspective, a key step to move this type of treatment into clinic should be to evaluate the reactogenicity of the vaccine in individuals pre-exposed to mycobacteria, including BCG vaccination and/or *M. tuberculosis* infection. In this regard, a recent study conducted in South Africa described the safety of a pulmonary BCG administered by the intratracheal route [47]. Interestingly, the authors inoculated BCG in adult populations with different mycobacterial sensitization status, including BCG-vaccinated and latent (pulmonary) TB infected, finding no major adverse events associated with intratracheal administration of BCG. In the case of intradermal MTBVAC, a phase IIa dose-escalation study for safety and immunogenicity is currently being performed in *M. tuberculosis* infected and uninfected adults (BCG-vaccinated at birth) in South Africa (NCT02933281).

Over the last years, novel experimental immunotherapies against asthma have emerged, mainly based on the monoclonal antibody-mediated blockade of specific molecules that contribute to asthma-induced inflammation, such as IL-4, IL-5, IL-13 or IgE, showing in many cases positive results. However, these studies also evidence that efficacy achieved by inhibition of a single pathway is in general partial. For instance, IL-5-specific therapy substantially inhibits eosinophilia, whereas outcomes for other measures, such as lung function, are less favourable [48]. Since asthma is a highly complex disease, we speculate that an approach as the proposed in the present study,

with a capacity to interact with both innate and adaptive immune components, could be more efficient dealing with the different aspects of the pathology.

Contributors

C.M. and N.A. designed the experiments and directed the study. R.T., E.M., S.U., A.B.G. and I.O. performed the experiments. R.T., D.M., C.M. and N.A. wrote the manuscript. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interests

Raquel Tarancón, Elena Mata, Santiago Uranga, Dessislava Marinova, Carlos Martín and Nacho Aguiló are co-inventors of the patent “Therapeutic efficacy by pulmonary delivery of live attenuated mycobacteria” held by the University of Zaragoza. Santiago Uranga, Dessislava Marinova, Carlos Martín and Nacho Aguiló are co-inventors of the patent “Compositions for use as a prophylactic agent to those at risk of infection of tuberculosis, or as secondary agents for treating infected tuberculosis patients” held by the University of Zaragoza and Biofabri. Carlos Martín is inventor of the patent “Tuberculosis vaccine” held by the University of Zaragoza. There are no other conflicts of interest.

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Data Sharing Statement

The authors declare that the data supporting the findings of this study are available from the corresponding author upon request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103186.

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DISCUSIÓN Y CONCLUSIONES

DISCUSIÓN

Las vacunas vivas de TB son agentes inmunogénicos únicos. A lo largo de los 100 años de historia de BCG se han evidenciado por un lado sus efectos específicos frente a TB, pero también efectos no específicos frente a patologías diversas como cáncer, alergias, diabetes u otros procesos infecciosos.

En el desarrollo de los estudios preclínicos de MTBVAC hemos realizado, durante esta última década, toda una sucesión de distintos experimentos en modelo murino, estudios que han caracterizado *in vivo* el comportamiento de la candidata a vacuna, y que han culminado con la publicación de una veintena de artículos científicos. La selección de los 4 artículos para esta tesis proviene de la última fase de la progresión de la investigación en estos modelos animales.

Dadas las características de seguridad e inmunogenicidad de MTBVAC en estudios clínicos en humanos, así como su eficacia en la de protección contra la enfermedad causada por *M. tuberculosis* en diferentes modelos animales, nuestro grupo de investigación de Genética de Micobacterias se propuso también estudiar interacciones y posibles aplicaciones profilácticas y terapéuticas en otras enfermedades, como también ocurre con BCG.

Para avanzar en nuevas aplicaciones de MTBVAC primero ponemos en marcha un modelo preclínico sencillo, de prueba de concepto, posteriormente, si la prueba de concepto es validada, desarrollamos un paquete preclínico robusto en el que se evalúa la seguridad y eficacia de MTBVAC para cada aplicación concreta, consistente en la optimización de los modelos experimentales de cada patología, evaluando MTBVAC en comparación con BCG y utilizando distintas rutas de administración.

En los 4 trabajos de esta tesis la progresión de la dificultad de aplicación de las rutas utilizadas ha sido variable. Las inoculaciones más simples son la vía subcutánea e intraperitoneal, aplicadas en la 2^a publicación, en el estudio de variantes de MTBVAC para distintos linajes de patógeno de TB, estas rutas han sido ampliamente utilizadas para los estudios previos de seguridad y eficacia de MTBVAC en ratones. La ruta intranasal, de dificultad intermedia, ha sido aplicada en la 3^a y 4^a publicación, para el novedoso estudio de la aplicación de MTBVAC inactivada como refuerzo de vacuna

imitando la vía natural de la infección, y también para el estudio de la interacción entre las vacunas BCG o MTBVAC y el asma alérgica. La ruta de administración más complicada es la vía intravesical, aplicada en la 1^a publicación, utilizada tanto para la inducción tumoral como para los posteriores tratamientos por instilados con las soluciones de vacuna.

Los resultados avalan que se han optimizado los diferentes modelos animales y explorado las nuevas rutas de vacunación de forma reproducible y consistente obteniendo resultados significativos. Los mecanismos de acción mediante los cuales las vacunas, por un lado protegen de la TB, por otro lado revierten los tumores vesicales; y por otro permiten aplicar terapias a enfermedades como el asma alérgica, dichos mecanismos están ampliamente descritos y discutidos tras los resultados de cada publicación.

A continuación se describen los fundamentos que justifican los experimentos aplicados y se discuten los resultados.

-En el primer lugar se ha optimizado el modelo de protección de BCG para el tratamiento del cáncer de vejiga, que depende en gran medida del tiempo transcurrido entre la administración intravesical de las células tumorales MB49 y el tratamiento con las vacunas atenuadas, de manera que hemos observado que BCG únicamente protege cuando se administra al día siguiente de la implantación de las células tumorales. A tiempos más tardíos, se pierde la protección, probablemente debido a que el tumor ya ha crecido lo suficiente para hacer ineffectivo el tratamiento. Este hecho permite establecer condiciones en las que este modelo de cáncer de vejiga es resistente a BCG, tal y como se hizo en la publicación aquí presentada, lo que nos permite establecer un modelo en el que MTBVAC protege en condiciones en las que BCG no lo hace. Así, nuestro modelo es relevante en términos de desarrollo de nuevos tratamientos clínicos para el cáncer de vejiga no musculo invasivo, sobre todo si tenemos en cuenta el problema que supone la resistencia a BCG en estos pacientes, para los cuales la alternativa es la quimioterapia, de baja eficacia y considerables efectos secundarios, o la alternativa quirúrgica por cistectomía, que implica un importante deterioro en la calidad de vida.

-En el segundo lugar se compara la seguridad y de eficacia de diferentes candidatos de vacunas vivas atenuadas de diferentes linajes de *M. tuberculosis*.

Dentro de los planes de nuevas estrategias para erradicar la TB, (vacunas eficaces, mejoras en el diagnóstico y nuevos antibióticos más efectivos), las vacunas vivas atenuadas siguen teniendo su espacio como candidatas, a pesar de que BCG no haya conseguido erradicar la enfermedad tras cien años de uso. Las nuevas candidatas atenuadas están, o basadas en variantes de BCG, potenciadas con alguna inserción dirigida, o basada en variantes *M. tuberculosis*, atenuada por delecciones como MTBVAC. Lo que se espera de estas nuevas candidatas es que confieran una respuesta inmunitaria tipo linfocitos T más eficaz que la actual BCG, ya que estos nuevos candidatos mantienen antígenos que faltan en BCG, que se perdieron en gran medida en su atenuación desde *M. bovis*.

Ya en su primer estudio clínico (de seguridad) se ha descrito que MTBVAC induce una mayor producción células T que BCG, siendo además una respuesta más duradera. Este hallazgo podría estar relacionado con el hecho de que MTBVAC contiene aproximadamente un 50% más epítopos para el estímulo de células T en comparación con BCG. La eficacia de MTBVAC se ha evaluado en modelos animales (ratón y PNH) frente a las cepas patógenas *M. tuberculosis* H37Rv y *M. tuberculosis* Erdman, ambas pertenecientes al linaje L4. Sin embargo, no se ha estudiado la protección de MTBVAC frente a otros linajes que son también de amplia distribución como son los linajes modernos L2 y L3. Para evaluar la protección dependiente del linaje, hemos construido dos nuevas vacunas basadas en idénticas delecciones a las realizadas en la construcción de MTBVAC pero en este caso en los linajes 2 y 3 de *M. tuberculosis* (MTBVAC-L2 y MTBVAC-L3). De este modo hemos obtenido un conjunto de vacunas MTBVAC frente a los tres linajes modernos de *M. tuberculosis*, que posteriormente se han caracterizado molecularmente, revelando que la mayoría de los fenotipos dependientes de PhoP y FadD26 se mantienen igual entre los tres linajes, aunque con algunas diferencias. Al igual que MTBVAC, los dos nuevos derivados construidos aquí reproducen de forma robusta la presencia de CFP-10 y la ausencia de ESAT-6 en la fracción de proteína secretada.

La aportación de esta segunda publicación a la tesis doctoral ha sido la optimización del modelo de ratón (ratones inmunocompetentes C3H/HeNRj) para determinar *in vivo* la seguridad y la eficacia de las tres construcciones. Los

experimentos de seguridad en ratones inmunodeficientes SCID revelaron que aunque los tres derivados de MTBVAC estaban atenuados existen diferencias entre los linajes. MTBVAC-L2 es la más virulenta, MTBVAC-L3 tiene una atenuación inferior pero cercana a BCG, y solo MTBVAC construido a partir del linaje 4 está significativamente más atenuado que BCG Pasteur. La virulencia de MTBVAC-L2 podría estar en relación con su procedencia de la cepa Beijing que a diferencia de los linajes L3 y L4, presenta glicolípidos fenólicos o la ausencia de proteínas secretadas PE_GRS, y que ambas se asocian a mayor virulencia en experimentos con ratones.

Al contrario que con el estudio de la atenuación, no se detectaron diferencias importantes respecto de la eficacia de las tres vacunas, lo cual nos sugiere que no hay protección dependiente del linaje en ratones. Los resultados con los tres derivados de MTBVAC muestran que diferentes niveles de atenuación en ratones SCID no necesariamente predicen la eficacia de la vacuna en ratones inmunocompetentes, ya que no se encontró una asociación clara entre atenuación y protección. Sorprendentemente, MTBVAC y MTBVAC-L2 confieren una mayor protección en comparación con BCG Pasteur frente a la infección con la cepa W4-Beijing, esta diferencia podría justificar la ineficiencia de la vacunación de BCG que está permitiendo la aparición de recientes brotes de TB producidos por las cepas Beijing y que para más preocupación se asocian a fenotipos hipervirulentos y con resistencia a antibióticos. Para tener una idea más precisa de la protección diferencial entre las tres variantes de MTBVAC y BCG se requieren experimentos de protección adicionales que utilicen diferentes pautas de vacunación y otros modelos animales que permitan contrastar nuestros resultados. Esta tesis ha permitido describir por primera vez que la vacuna MTBVAC es también capaz de proteger en ratones contra diferentes representantes de los linajes modernos de *M. tuberculosis*. Este hallazgo es muy relevante para la evaluación clínica de MTBVAC.

-En el tercer lugar se aplica una pauta de inmunización con vacuna inactivada utilizando la novedosa ruta respiratoria.

Durante los últimos años se ha renovado el interés en las vacunas de TB, pero también en explorar nuevas vías de administración como las vías intravenosa y respiratoria. En este último caso, la eficacia superior de la vía respiratoria a la administración intradermal ha sido contrastada en ratones y macacos, y existen en la

actualidad varios ensayos clínicos para evaluar su seguridad por vía aerosol en humanos.

Recientemente ha aumentado el interés por dilucidar el papel de las inmunoglobulinas en la TB. En diferentes estudios que utilizaron modelos animales se demostró que la administración pulmonar de anticuerpos monoclonales dirigidos contra proteínas de superficie de *M. tuberculosis* provoca una reducción de la carga bacteriana tras la infección experimental. Además, los resultados obtenidos en los ensayos clínicos con las vacunas sugieren una correlación entre la IgG específica de *M. tuberculosis* en el suero y un menor riesgo de desarrollar TB. Estos estudios apoyan la contribución de los anticuerpos en la protección natural o inducida por vacunas contra la TB. En el presente trabajo, aplicamos un enfoque de vacunación novedoso, que induce anticuerpos protectores de la mucosa en los pulmones. Se demostró en ratones que una sola inmunización pulmonar con una vacuna de *M. tuberculosis* de células enteras inactivadas desencadena la inmunidad protectora de los pulmones cuando se administra como refuerzo sobre animales previamente inmunizados con BCG.

La administración pulmonar de MTBVAC HK indujo respuesta sistémica y local de tipo celular y humoral, proporcionando una protección mejorada respecto de los animales inmunizados solo con BCG por vía parenteral. La vía de administración pulmonar es adecuada para desencadenar la producción de inmunoglobulinas en mucosa pulmonar, mientras que la pauta actual de vacunación con BCG, que utiliza la vía intradérmica, no genera la secreción de anticuerpos a la luz pulmonar.

A pesar de la menor inmunogenicidad y protección que confieren las vacunas de células enteras inactivadas en comparación con sus homólogas vivas, existe un gran interés en su desarrollo. Dado que la inactivación se asocia con un perfil de seguridad mejorado, estas vacunas pueden emplearse en poblaciones concretas para las que las vacunas vivas pueden ser perjudiciales, como los individuos inmunodeprimidos. La inmunogenicidad limitada de las vacunas inactivadas se puede mejorar con otras estrategias, como su formulación con adyuvantes añadidos, o por la administración repetida mediante inmunizaciones múltiples. En el caso de MTBVAC HK intranasal, nuestros datos indican que no es protectora por sí misma; necesita una vacunación previa con BCG para desencadenar una respuesta en los pulmones y conferir la protección deseada. En la presente tesis no se ha profundizado en este hecho, que deberá ser abordado en el futuro para esclarecer la interrelación de las respuestas inmunológicas sistémica y de mucosas. Por el momento solo podemos especular que la

administración pulmonar de MTBVAC HK podría estar activando o atrayendo hacia los pulmones la respuesta celular sistémica previamente provocada por la administración de BCG subcutánea. La redirección de los bacilos inactivados hacia el compartimento pulmonar en una segunda fase de vacunación sería una forma relativamente segura de impulsar la respuesta celular e inducir anticuerpos específicos locales contra esas bacterias completas.

Los resultados del tercer estudio indican que el mecanismo de protección más probable de MTBAVC HK pulmonar está mediado por la generación de anticuerpos específicos en las vías respiratorias, con capacidad para opsonizar bacterias de *M. tuberculosis* vivas. Hasta donde alcanza nuestro conocimiento, este es el primer enfoque de vacuna contra la TB basado en bacterias inactivadas que consigue la secreción de anticuerpos protectores en la mucosa pulmonar. Promover la secreción de anticuerpos en la mucosa del árbol respiratorio mediante vacunación mejora la protección contra TB, lo que representa un nuevo enfoque preventivo.

-En el último estudio incluido en esta tesis, se evaluó el potencial terapéutico de las vacunas vivas atenuadas BCG y MTBVAC, administradas por la vía intranasal frente al asma alérgica en modelo de ratón. Los resultados obtenidos son evidentes; la vacunación provoca una reducción de los niveles de IgE y de la eosinofilia pulmonar, disminuyendo también la producción de moco bronquial. Ambas vacunas, BCG y MTBVAC, revierten la respuesta asociada a la hiperreactividad de las vías respiratorias en todos los escenarios ensayados, tanto a nivel preventivo como en asma ya establecida. Los experimentos se han realizado en modelo de asma aguda y en asma crónica, y la reducción de la eosinofilia se produce tras una única administración intranasal, tanto con BCG como con MTBVAC. Sorprendentemente esta reducción es comparable a la que se produce tras el tratamiento estándar de dexametasona, con la ventaja añadida de que la eficacia tras la vacunación se mantiene a largo plazo. Esta reversión supone un nuevo paradigma en la terapia de esta patología que aún debe ser corroborada en humanos.

Entre nuestros resultados en modelo animal y los resultados de diversos estudios realizados en poblaciones humanas respecto a la protección de BCG frente al asma hay diferencias. Hay estudios poblacionales de prevalencia de asma basados en meta-análisis, entre poblaciones vacunadas y no vacunadas, y también estudios en

ensayos específicos con seguimiento de grupos vacunados con BCG y de grupo placebo. Los resultados son controvertidos ya que muestran hallazgos opuestos. Las razones para explicar esta ambigüedad se pueden atribuir principalmente a diferencias de aspectos muestrales que no se han caracterizado, como el grupo de edad (niños o adultos), diferentes dosis de vacuna administradas, exposición previa a micobacterias ambientales, el uso de diferentes subcepas de BCG...etc. Estos son estudios de difícil comparación por la heterogeneidad de datos agrupados. La comparación entre los estudios entre humanos y los estudios en ratones tiene además otros aspectos a considerar: por un lado, los grupos de experimentación en ratones son muy homogéneos pero hay diferencias en la dosificación; en relación con el peso corporal la cantidad de BCG administrada a los seres humanos es de varios órdenes de magnitud inferior a la dosis en ratones, y por otro lado por la vía de vacunación utilizada, la vía intranasal o por aerosol es de acción mucho más potente a nivel pulmonar que la vía intradérmica administrada en el hombre. Estos aspectos son cuestiones que se deberán tener en cuenta en futuras investigaciones para que se puedan aplicar en situaciones reales y contribuir a mejorar la clínica de los pacientes con asma alérgica.

CONCLUSIONES

- 1.- Hemos optimizado un modelo de cáncer de vejiga en ratones basado en la administración ortotópica de células MB49. Este modelo permite evaluar la eficacia de nuevos tratamientos y estudiar los mecanismos inmunológicos para el control del tumor.
- 2.- La vacuna MTBVAC es significativamente más eficaz que BCG en el tratamiento de un modelo preclínico de cáncer de vejiga, con lo que podría ser una buena alternativa para el tratamiento inmunoterápico contra este tipo de cáncer.
- 3.- Hemos optimizado el procedimiento para administrar vacunas de tuberculosis de manera subcutánea, tanto en ratones adultos como neonatos. Esto permite el estudio de vacunas vivas de tuberculosis, como MTBVAC o BCG, por una ruta muy próxima y comparable a la vía intradérmica usada en clínica.
- 4.- Los estudios de seguridad y protección de diferentes versiones de MTBVAC, basadas en diferentes linajes de *M. tuberculosis*, indican que MTBVAC es segura y protege contra la tuberculosis independientemente del linaje filogenético utilizado como cepa molde para su construcción.
- 5.- Se ha optimizado en ratones la ruta de vacunación intranasal, que es la vía natural de la infección de tuberculosis. Los resultados indican que aumenta la presencia de la vacuna en pulmón respecto a la ruta intradérmica, provocando una respuesta inmunitaria local más eficaz y mejorando la protección frente a la enfermedad.
- 6.- La revacunación con MTBVAC inactivada por calor e inoculada por vía respiratoria induce una respuesta inmune en los pulmones más eficaz que la simple vacunación intradérmica frente a un desafío posterior con tuberculosis.
- 7.- Hemos optimizado diferentes modelos de asma aguda y crónica en ratones, inducidos por diferentes alérgenos, lo que permite el estudio de nuevos tratamientos frente al asma.

8.- Frente a situaciones de asma aguda o crónica en ratones, la administración intranasal de las vacunas BCG y MTBVAC revierte la respuesta de hiperreactividad en las vías respiratorias, produciendo descenso de la inflamación eosinofílica y de la secreción de IgE.

APÉNDICE

FACTOR DE IMPACTO Y CLASIFICACIÓN DE LAS PUBLICACIONES.

Publicación 1:

Alvarez-Arguedas, S.; Uranga, S.; Martín, M.; Elizalde, J.; Gomez, A.B.; Julián, E.; Nardelli-Haefliger, D.; Martín, C.; Aguiló, N. **Therapeutic efficacy of the live-attenuated Mycobacterium tuberculosis vaccine, MTBVAC, in a preclinical model of bladder cancer.** TRANSLATIONAL RESEARCH. 197, pp. 32 - 42. 2018. ISSN 1931-5244 DOI: 10.1016/j.trsl.2018.03.004

Tipo de producción: Artículo científico

Fuente de impacto: WOS (JCR) Categoría: Science Edition - MEDICAL LABORATORY TECHNOLOGY

Índice de impacto: 4.915 Revista dentro del 25%: Si

Posición de publicación: 2 Num. revistas en cat.: 29

Fuente de impacto: WOS (JCR) Categoría: Science Edition - MEDICINE, GENERAL & INTERNAL

Índice de impacto: 4.915 Revista dentro del 25%: Si

Posición de publicación: 19 Num. revistas en cat.: 159

Fuente de impacto: WOS (JCR) Categoría: Science Edition - MEDICINE, RESEARCH & EXPERIMENTAL

Índice de impacto: 4.915 Revista dentro del 25%: Si

Posición de publicación: 22 Num. revistas en cat.: 135

Publicación 2:

Pérez, Irene; Uranga, Santiago; Sayes, Fadel; Frigui, Wafa; Samper, Sofía; Arbués, Ainhoa; Aguiló, Nacho; Brosch, Roland; Martín, Carlos; Gonzalo-Asensio, Jesús. **Live attenuated TB vaccines representing the three modern Mycobacterium tuberculosis lineages reveal that the Euro-American genetic background confers optimal vaccine potential.** EBIOMEDICINE. 55, pp. 102761 1 - 10. 2020. ISSN 2352-3964 DOI: 10.1016/j.ebiom.2020.102761

Tipo de producción: Artículo científico

Fuente de impacto: WOS (JCR) Categoría: Science Edition - MEDICINE, RESEARCH & EXPERIMENTAL

Índice de impacto: 8.143 Revista dentro del 25%: Si

Posición de publicación: 17 Num. revistas en cat.: 140

Publicación 3:

Aguilo, N.; **Uranga, S.**; Mata, E.; Tarancon, R.; Gómez, A.B.; Marinova, D.; Otal, I.; Monzón, M.; Badiola, J.; Montenegro, D.; Puentes, E.; Rodríguez, E.; Vervenne, R.A.W.; Sombroek, C.C.; Verreck, F.A.W.; Martín, C. **Respiratory Immunization With a Whole Cell Inactivated Vaccine Induces Functional Mucosal Immunoglobulins Against Tuberculosis in Mice and Non-human Primates.** FRONTIERS IN MICROBIOLOGY. 11, pp. 1339 [15 pp.]. 2020. ISSN 1664-302X
DOI: 10.3389/fmicb.2020.01339

Tipo de producción: Artículo científico

Fuente de impacto: WOS (JCR) Categoría: Science Edition - MICROBIOLOGY

Índice de impacto: 5.640 Revista dentro del 25%: Si

Posición de publicación: 28 Num. revistas en cat.: 136

Publicación 4:

Tarancón, R.; Mata, E.; **Uranga, S.**; Gómez, A.B.; Marinova, D.; Otal, I.; Martín, C.; Aguiló, N. **Therapeutic efficacy of pulmonary live tuberculosis vaccines against established asthma by subverting local immune environment.** EBIOMEDICINE. pp. 103186 [14 pp.]. 2021. ISSN 2352-3964
DOI: 10.1016/j.ebiom.2020.103186

Tipo de producción: Artículo científico

Fuente de impacto: WOS (JCR) Categoría: Science Edition - MEDICINE, RESEARCH & EXPERIMENTAL

Índice de impacto: 11.205 Revista dentro del 25%: Si

Posición de publicación: 14 Num. revistas en cat.: 140

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Autor: Santiago Uranga

Programa de doctorado: Medicina y Sanidad Animal

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2. Live attenuated TB vaccines representing the three modern *Mycobacterium tuberculosis* lineages reveal that the Euro-American genetic background confers optimal vaccine potential. Perez I, Uranga S, Sayes F, Frigui W, Samper S, Arbones A, Aguiló N, Brosch R, Martín C, Gonzalo-Asensio J. EBioMedicine. 2020 May; 55:102761. doi: 10.1016/j.ebiom.2020.102761. Epub 2020 Apr 28.

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Autor:	Santiago Uranga Maiz
Programa de doctorado:	Medicina y Sanidad Animal

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Respiratory Immunization with a whole cell inactivated vaccine induces functional mucosal immunoglobulins against tuberculosis in mice and non-human primates.
Aguilo N, Uranga S, Mata E, Tarancón R, Gómez AB, Marinova D, Otal I, Monzon M, Badiola J, Montenegro D, Puentes E, Rodríguez E, Vervenne RAW, Sombroek CC, Verreck FAW, Martín C. Front Microbiol. 2020 Jun 18; 11:1339. doi: 10.3389/fmicb.2020.01339. eCollection 2020.

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Título: Estudio de rutas de vacunación en modelo murino para el uso profiláctico o terapéutico de vacunas atenuadas de tuberculosis

Autor: Santiago Uranga Maiz

Programa de doctorado: Medicina y Sanidad Animal

3.- Publicaciones que formarán parte de la tesis y de las que el firmante es coautor

Respiratory Immunization with a whole cell inactivated vaccine induces functional mucosal immunoglobulins against tuberculosis in mice and non-human primates.

Aguilo N, Uranga S, Mata E, Tarancón R, Gómez AB, Marinova D, Otal I, Monzon M, Badiola J, Montenegro D, Puentes E, Rodríguez E, Vervenne RAW, Sombroek CC, Verreck FAW, Martín C. Front Microbiol. 2020 Jun 18; 11:1339. doi: 10.3389/fmicb.2020.01339. eCollection 2020.

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