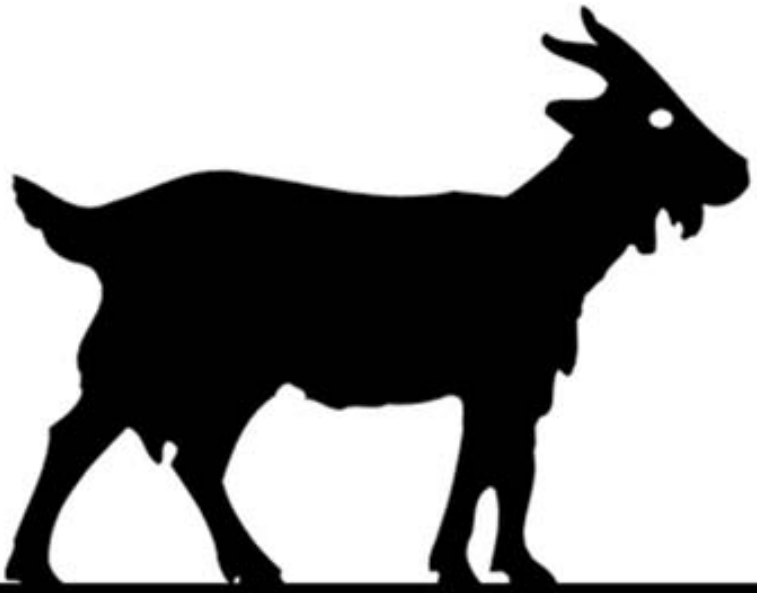
TESI DOCTORAL



Noves estratègies vacunals i diagnòstiques per al control de la tuberculosi. El model caprí

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Juliol de 2013

Universitat Autònoma de Barcelona

Noves estratègies vacunals i diagnòstiques per al control de la tuberculosi. El model caprí

New vaccination and diagnostic strategies for the control of tuberculosis. The goat model

Tesi doctoral presentada per en **BERNAT PÉREZ DE VAL** per optar al grau de Doctor dins del programa de doctorat de Medicina i Sanitat Animals del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Dr. **MARIANO DOMINGO ÁLVAREZ**.

Bellaterra, 2013

CReSA
Centre de Recerca en Sanitat Animal

Disseny de la portada: Enric Vidal, CM & Misc.

El Dr. **MARIANO DOMINGO ÁLVAREZ**, Catedràtic d'Universitat del Departament de Sanitat i Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador adscrit al CReSA,

CERTIFICA:

Que la memòria titulada "**Noves estratègies vacunals i diagnòstiques per al control de la tuberculosi. El model caprí**" presentada per en **BERNAT PÉREZ DE VAL** per a l'obtenció del grau de Doctor, s'ha realitzat sota la seva direcció i, considerant-la acabada, n'autoritza la seva presentació per tal de ser avaluada per la comissió corresponent.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra (Barcelona), a 12 de juliol de 2013.

Dr. Mariano Domingo Álvarez — Director

Bernat Pérez de Val — Doctorand

To Madiba.
We'll persist.

Tot el que facis a la vida serà insignificant,

però és molt important que ho facis.

[Mahatma Gandhi]

TAULA DE CONTINGUTS

RESUM

L'Organització Mundial de la Salut va declarar la tuberculosi (TB) com "una emergència global" ara fa vint anys. En l'actualitat la TB continua sent la principal causa de mortalitat i morbiditat per malaltia infecciosa a nivell mundial. La TB és causada pels bacteris del complex *Mycobacterium tuberculosis*, dels quals *M. bovis* i *M. caprae* en són els patògens zoonòtics més importants, presentant una àmplia varietat d'hostes animals, tant domèstics com silvestres. Per tant, la TB té repercussions en la salut pública i la sanitat animal, així com en l'economia dels sectors públic i ramader. Les cabres poden ser particularment susceptibles a la infecció per TB i el recent augment de casos reportats indiquen que la TB caprina podria ser una malaltia emergent. Així doncs, es necessiten noves eines específiques per al control de la TB en el cabrum.

Les estratègies de control de la TB depenen en gran mesura de la disponibilitat d'una vacuna segura i eficaç. El Bacil Calmette Guerin (BCG), l'única vacuna disponible en l'actualitat, té una eficàcia variable tant en els éssers humans com en animals, i els esforços actuals de la recerca es centren en desenvolupar noves vacunes que reemplacin la BCG, o que n'incrementin l'eficàcia. També són necessaris nous reactius de diagnòstic associats a les vacunes que siguin capaços de distingir els individus vacunats dels infectats. Aquesta tesi pretén abordar aquests reptes mitjançant un nou model animal experimental.

La tesi es divideix en cinc seccions: introducció general, objectius, estudis, discussió general i conclusions:

La introducció descriu una visió global sobre l'epidemiologia, les característiques de l'agent etiològic, la patogènia, la immunitat i el desenvolupament de noves vacunes i estratègies de vacunació, per contextualitzar els objectius que s'enumeren a continuació.

Els quatre estudis contenen cada un dels experiment duts a terme durant el decurs del programa de doctorat. El primer estudi descriu el model experimental de TB en la cabra domèstica, incloent la caracterització dels paràmetres immunològics i *post-mortem* que seran utilitzats en els estudis posteriors. La tomografia computeritzada va ser validada en aquest estudi per al seu ús com a mètode quantitatiu de valoració de lesions tuberculoses. El segon i tercer estudi estan enfocats en l'avaluació de l'eficàcia de la vacuna BCG i de dues noves vacunes adenovirals recombinants de reforç de la pròpia BCG, basades en antígens que són reconeguts pel sistema immune durant la infecció tuberculosa. La BCG va protegir les cabres front a la disseminació extra-pulmonar de la infecció, mentre que quan es reforçava amb les vacunes adenovirals es millorava la protecció en termes de reducció de les lesions tuberculoses i de la càrrega bacteriana. Finalment, el quart estudi va mostrar que les campanyes de vacunació contra la paratuberculosi (PTB) en cabres poden causar interferències en el diagnòstic de la TB quan s'utilitzen les eines de diagnòstic actualment disponibles. La protecció creuada de la vacuna de PTB Silirum® també es va avaluar en aquest estudi. En els quatre experiments es van avaluar nous reactius de diagnòstic dissenyats seguint el principi DIVA (diferenciació d'animals infectats i vacunats) i marcadors immunològics específics associats amb la protecció o la progressió de la infecció.

Els resultats globals i la seva aplicació en els programes de control i eradicació de la TB en animals domèstics, així com en assaigs clínics de vacunes antituberculoses, es discuteixen en la secció de discussió general. Finalment s'enumeren les principals conclusions de la tesi.

ABSTRACT (in English)

The World Health Organization (WHO) declared tuberculosis (TB) "a global public emergency" twenty years ago. Nowadays, TB is still the leading cause of infectious disease mortality and morbidity worldwide. TB is caused by Mycobacterium tuberculosis complex (MTBC) bacteria. M. bovis and M. caprae are the most important zoonotic pathogens among the MTBC, and have a wide range of animal hosts, including both livestock and wildlife. Therefore, TB has repercussions on public and animal health and on the economy of both public and livestock sectors. Goats seem to be particularly susceptible to TB. The recent increase of caprine TB reported cases points to its consideration as an emerging disease. Thus, specific measures to control TB in goat herds need to be implemented.

The TB control strategies depend strongly on the availability of a safe and effective vaccine. The Bacillus Calmette Guerin (BCG), the unique currently available vaccine, has variable efficacy both in humans and animals. Current research efforts are focused on the development of new vaccine candidates to replace BCG, or improve its protective effect. In addition, novel vaccine-associated diagnostic reagents are also needed to distinguish vaccinated from infected individuals. The present thesis addresses these concerns using a new experimental animal model.

The thesis is divided in five sections: General introduction, objectives, studies, general discussion and conclusions:

The introduction describes a global overview of the epidemiology, etiological agent characteristics, pathogenesis, immunity and development of new vaccines and vaccination strategies against TB, to contextualize the thesis objectives.

Each of the four studies contains one of the experiments conducted during the PhD program. The first study addresses a comprehensive description of the experimental model of TB in goats, including the characterization of immunological and post-mortem parameters used in subsequent studies. Computed Tomography was validated for use as quantitative method to evaluate gross TB lesions. The second and third studies are focused on assessing the efficacy of BCG and two BCG-booster recombinant adenoviral vaccines. The latter are based on antigens recognized by the immune system during the TB infection. BCG protected goats against extra-pulmonary dissemination. However, boosting BCG with the adenoviral vaccines showed improved protection by reducing TB gross lesions and bacterial load. Finally, the fourth study showed that vaccination campaigns against paratuberculosis (PTB) in goats may cause interferences on the diagnosis of TB when using the currently available diagnostic tools. Cross-protective effect of the PTB vaccine Silirum® was also evaluated in this study. In addition, throughout the four experiments, new vaccine-associated diagnostic reagents, designed following the DIVA (differentiation of infected and vaccinated animals) principle were assessed, as well as specific immunological markers associated with protection or the progression of the infection.

The global results and their application in the TB control and eradication programs in domestic animals, as well as in TB vaccines clinical trials are discussed in the general discussion section and the main conclusions are subsequently listed.

Aquesta tesi doctoral s'ha realitzat a partir dels treballs duts a terme en el marc dels següents projectes europeus:

Strategies for the eradication of bovine tuberculosis **(TB-STEP)**
FP7-KBBE-2007-1-3-04 (Ref. 212414)

The network of animal disease infectiology research facilities **(NADIR)**
FP7-INFRA-2008-1.1.2 (Ref. 228394)

Els resultats que es presenten en aquesta tesi doctoral han estat publicats o enviats a publicar a revistes científiques indexades:

Pérez de Val, B., López-Soria, S., Nofrarías, M., Martín, M., Vordermeier, H.M., Villarreal-Ramos, B., Romera, N., Escobar, M., Solanes, D., Cardona, P.J., and Domingo, M. 2011. *Experimental model of tuberculosis in the domestic goat after endobronchial infection with Mycobacterium caprae*. *Clinical and Vaccine Immunology* 18:1872-1881.

Pérez de Val, B., Villarreal-Ramos, B., Nofrarías, M., López-Soria, S., Romera, N., Singh, M., Abad, F.X., Xing, Z., Vordermeier, H.M., and Domingo, M. 2012. *Goats primed with Mycobacterium bovis BCG and boosted with a recombinant adenovirus expressing Ag85A show enhanced protection against tuberculosis*. *Clinical and Vaccine Immunology* 19:1339-1347.

Pérez de Val, B., Vidal, E., Villarreal-Ramos, B., Andaluz, A., Moll, X., Martín, M., Nofrarías, M., Vordermeier, H.M., and Domingo, M. 2013. *A multi-antigenic adenoviral-vectored vaccine improves BCG protection of goats against pulmonary tuberculosis infection and prevents disease progression*. Enviat a publicar.

Pérez de Val, B., Nofrarías, M., López-Soria, S., Garrido, J., Vordermeier, H., Villarreal-Ramos, B., Martín, M., Puentes, E., Juste, R., and Domingo, M. 2012. *Effects of vaccination against paratuberculosis on tuberculosis in goats: diagnostic interferences and cross-protection*. *BMC Veterinary Research* 8:191.

INTRODUCCIÓ GENERAL

1. La tuberculosi: passat i present

1.1. Evolució del bacil tuberculós

A finals del segle XIX el metge Robert Koch va descobrir l'etiologia de la tuberculosi (TB) descrivint el bacil que causa la infecció en les persones: *Mycobacterium tuberculosis* (Koch. 1882). Però la TB es remunta molt més endarrere en el temps essent una de les primeres malalties humanes de les que es té constància. Pel cap baix, la TB és tan antiga com la pròpia civilització. Les primeres evidències de tuberculosi òssia en humans daten del Neolític, tot i que recentment s'han descobert a Turquia unes restes fòssils que es remuntarien a uns 500 mil anys enrere, pertanyents a un *Homo erectus* amb lesions cranials que, per la seva forma i localització, serien característiques de leptomeningitis tuberculosa (Kappelman et al., 2008). El primer cas confirmat de TB correspondria a les restes momificades d'un nen d'uns 1300 anys d'antiguitat, descobertes en unes excavacions realitzades a la província de Nazca (Perú). En aquestes restes es van observar lesions amb acumulacions de bacils àcid alcohol resistents, tenyits per mitjà de la tècnica de Ziehl-Neelsen (Allison et al., 1973).

Pel que fa a la TB en animals, i en particular en els bòvids, la malaltia podria ser encara més antiga que en humans. Les anàlisis practicades a les restes d'un bisó extint a Nord Amèrica datades d'uns 17000 anys (molt abans de la domesticació del bestiar vaquí), van permetre detectar ADN d'un ancestre del complex *M. tuberculosis* (MTBC). Aquesta troballa és indicativa que un MTBC ancestral ja era present al Pleistocè i que els bòvids en podien haver estat els primers hostes (Rothschild et al., 2001).

Sigui com sigui, ara fa uns deu mil anys, durant el Neolític, la domesticació de bòvids salvatges a l'Àfrica i el Pròxim Orient hauria propiciat les condicions per a que els bòvids actuessin com a vectors i reservoris de la malaltia (Figura 1). El fet que el *M. tuberculosis* modern sigui un patogen quasi exclusiu de l'ésser humà, mentre que el *M. bovis*, una altra espècie del MTBC, és un patogen capaç de causar la TB a un ampli rang de mamífers, va fer hipotetitzar en un primer moment que l'espècie causant de la TB humana (*M. tuberculosis*) havia evolucionat de l'espècie causant de la infecció en bovins (*M. bovis*) (Stead et al., 1995).

Contràriament a aquesta teoria, estudis filogenètics recents de les espècies integrants del MTBC, han dibuixat un nou escenari d'evolució temporal dels seus diferents llinatges. Concretament, es van analitzar 20 regions variables del genoma, que incloïen les regions de diferència (RD), de 100 soques representatives del MTBC, (Brosch et al., 2002). Els resultats d'aquest estudi han permès especular que les diferents espècies del MTBC haurien evolucionat a partir d'un únic ancestre comú (que filogenèticament era més proper a les soques modernes de *M. tuberculosis*), constituint- se en diferents "ecotips" que s'haurien adaptat a hostes específics. A partir d'aquí, successives mutacions, incloent més delecions de les RD, han donat lloc a la resta d'espècies modernes del MTBC (Brosch et al., 2002; Gutierrez et al., 2005; Smith et al., 2006b).

Figura 1. Reconstrucció d'un fresc de pastors amb el seu ramat, Tassili n'Ajjer, Sàhara algerià (Neolític).

Així doncs, en algun moment de l'evolució, la pressió selectiva va fer saltar la barrera d'espècies produint-se una mutació en un *M. tuberculosis* ancestral que finalment hauria donat lloc al primer *M. bovis*, que presenta una millor adaptació a l'espècie bovina. De la mateixa manera hauria evolucionat *M. caprae*, que el 2003 va ser proposat com a nova espècie separada del MTBC (Aranaz et al., 2003), ja que s'aïlla freqüentment en el bestiar cabrum i presenta diferències bioquímiques i genotípiques respecte a la resta d'integrants del MTBC.

1.2. Epidemiologia de la tuberculosi

La TB és una malaltia de les persones i els animals. L'Organització Mundial de la Salut (OMS) i l'Oficina Internacional d'Epizooties (OIE) recolzen el concepte *One world, one health*, que és un enfocament col·laboratiu i integral de la salut pública i la sanitat animal a escala mundial. La TB animal es considera una malaltia zoonòtica (que pot afectar a les persones), però l'ésser humà també pot transmetre la infecció als animals domèstics. Per aquest motiu, cal considerar també la interfície entre persones i animals a l'hora d'abordar l'epidemiologia, el control i la prevenció de la TB.

1.2.1. La tuberculosi en l'ésser humà

S'estima que actualment hi ha prop de 2000 milions de persones infectades per *M. tuberculosis* a nivell mundial (World Health Organization. 2009). La majoria d'aquestes persones infectades controlen la infecció de forma activa, donant lloc al que es coneix com a infecció tuberculosa latent (en anglès LTBI). Quan, per diferents factors, la immunitat queda compromesa, es produeix una reactivació de la infecció desencadenant coneguda com a TB activa, que és l'evolució de la infecció cap a la malaltia pròpiament

dita. S'ha descrit que el control de la infecció es dóna en un 90% dels casos, mentre que el 10% restant desenvolupa TB activa (Young and Dye, 2006).

L'any 2009 es va reportar la incidència estimada de casos de TB més elevada de la història, arribant fins als 9,4 milions de persones arreu del món (World Health Organization. 2010a). La majoria dels casos es localitzaven a l'Àfrica i l'Àsia Central i Oriental, amb proporcions menors al Pròxim Orient, Europa, Amèrica i Oceania. Un total de 22 països acumulaven el 80% dels casos de TB al món (Figura 2, Lawn and Zumla, 2011). Els països que van presentar un major nombre de nous casos de TB van ser (per aquest ordre): l'Índia, la Xina, Sud Àfrica, Nigèria i Indonèsia (World Health Organization. 2010a).

Figura 2. Distribució geogràfica dels rangs d'incidència estimada de la TB en persones l'any 2009 (Lawn and Zumla, 2011).

Cal tenir present que un 12% dels nous casos de TB al món (més d'un milió) estan associats a casos de VIH (Virus de la Immunodeficiència Humana), dels quals quatre cinquenes parts es donen a l'Àfrica subsahariana (World Health Organization. 2010a).

A més a més, en les darreres dues dècades hi ha hagut una emergència global de les formes MDR (*Multidrug-resistant)* de TB i posteriorment de les formes XDR (*Extensively drug-resistant*). L'any 2007 es va estimar una incidència de 50000 TB-MDR i al voltant de 50000 casos de TB-XDR (World Health Organization. 2009), localitzats principalment a l'Índia, la Xina, Rússia, Sud Àfrica i Bangladesh. Concretament l'Índia i la Xina concentraven més del 50% dels casos (World Health Organization. 2010b).

Amb tot, s'estima que la TB encara avui és la causa de mort d' aproximadament 1,7 milions de persones cada any (World Health Organization. 2009).

1.2.2. La tuberculosi en els animals

S'entén per tuberculosi animal la infecció causada pels diferents micobacteris del MTBC a un ampli rang d'espècies animals, essent *M. bovis* la principal espècie causant de la infecció. Tot i així, encara és força comú el terme "tuberculosi bovina" per referir-se a la infecció en el bestiar vaquí o inclús sovint generalitzada al conjunt d'hostes animals. Amb aquesta denominació apareix a la llista de malalties, infeccions i infestacions de l'OIE de 2013 (www.oie.int, consultat 19/02/2013).

A nivell mundial, la tuberculosi continua sent un problema sanitari important del bestiar i, com a conseqüència, un problema econòmic per al sector ramader, així com un risc per a la salut pública. Al voltant d'un 70% dels països membres de la OIE tenen bestiar infectat per MTBC (www.oie.int, consultat el 19/02/2013) que ocasionaria una disminució del 10% al 20% de la producció de carn o llet (Bennett and Cooke, 2006; Boland et al., 2010). Aquest fet, conjuntament a les restriccions legals de la seva venta i/o exportació en ramats positius, causa pèrdues econòmiques molt importants al sector ramader, a les quals s'hi han d'afegir les despeses públiques en matèria d'indemnitzacions per sacrifici d'animals.

L' estratègia de lluita als països desenvolupats basada en proves diagnòstiques *in vivo*, normalment la prova de la Intradermotuberculinització (IDTB), i el posterior sacrifici dels animals positius (estratègia de prova i sacrifici), ha aconseguit eradicar o reduir molt significativament la tuberculosi en bovins en la majoria dels casos, incloent gran part de l'Europa continental, els EUA, el Canadà, Austràlia, Cuba i alguns països de Sud Amèrica (Cosivi et al., 1998; Ayele et al., 2004; Amanfu. 2006; Smith et al., 2006a; Thoen et al., 2006).

Per altra banda, la TB és endèmica en el bestiar vaquí a gran part de Sud Amèrica (de Kantor and Ritacco, 2006) i, tret d'algunes excepcions, també podria ser endèmica a la resta del món, però es disposa de poques dades i molt fragmentades per a confirmar-ho. Fins a la data s'han realitzat uns pocs estudis de les soques presents a cada país (Cosivi et al., 1998; Thoen et al., 2006; Jeon et al., 2008; Tadayon et al., 2008). Més concretament, es coneix que la TB és present en el bestiar vaquí a l'Àfrica, tot i que en general la realitat de la malaltia en els països d'aquest continent es desconeix (Cosivi et al., 1995; Ayele et al., 2004), amb l'excepció de Sud Àfrica, on també s'ha dut a terme l'estratègia de prova i sacrifici reduint la malaltia fins a nivells mínims (Michel et al., 2008).

A la unió Europea l'estratègia de prova i sacrifici ha tingut resultats desiguals, malgrat els grans esforços econòmics dedicats al control i a l' eradicació de la TB en bovins (71 milions d'euros previstos per al 2013, Decisió d'execució de la Comissió de 30 de novembre de 2012, 2012/761/UE). A estats com Espanya, Portugal o Itàlia, tan sols s'ha aconseguit l'estabilització de la prevalença (Pavlik. 2006), mentre que en altres estats, com el Regne Unit, la incidència s'ha incrementat significativament en les darreres dècades (Smith et al., 2006a). Actualment a la Unió Europea hi ha un total de 14 estats membres que es consideren no lliures de tuberculosi (Irlanda, Regne Unit, Portugal, Espanya, Itàlia, Hongria, Romania, Bulgària, Grècia, Malta, Xipre, Lituània, Letònia i Estònia) si bé cal tenir

en compte que Escòcia i algunes regions d'Itàlia es consideren oficialment lliures (Figura 3). Els estats membres que van presentar una major proporció de ramats positius l'any 2010 van ser el Regne Unit (8,6%) i Irlanda (4,7%) (European Food Safety Authority. 2012).

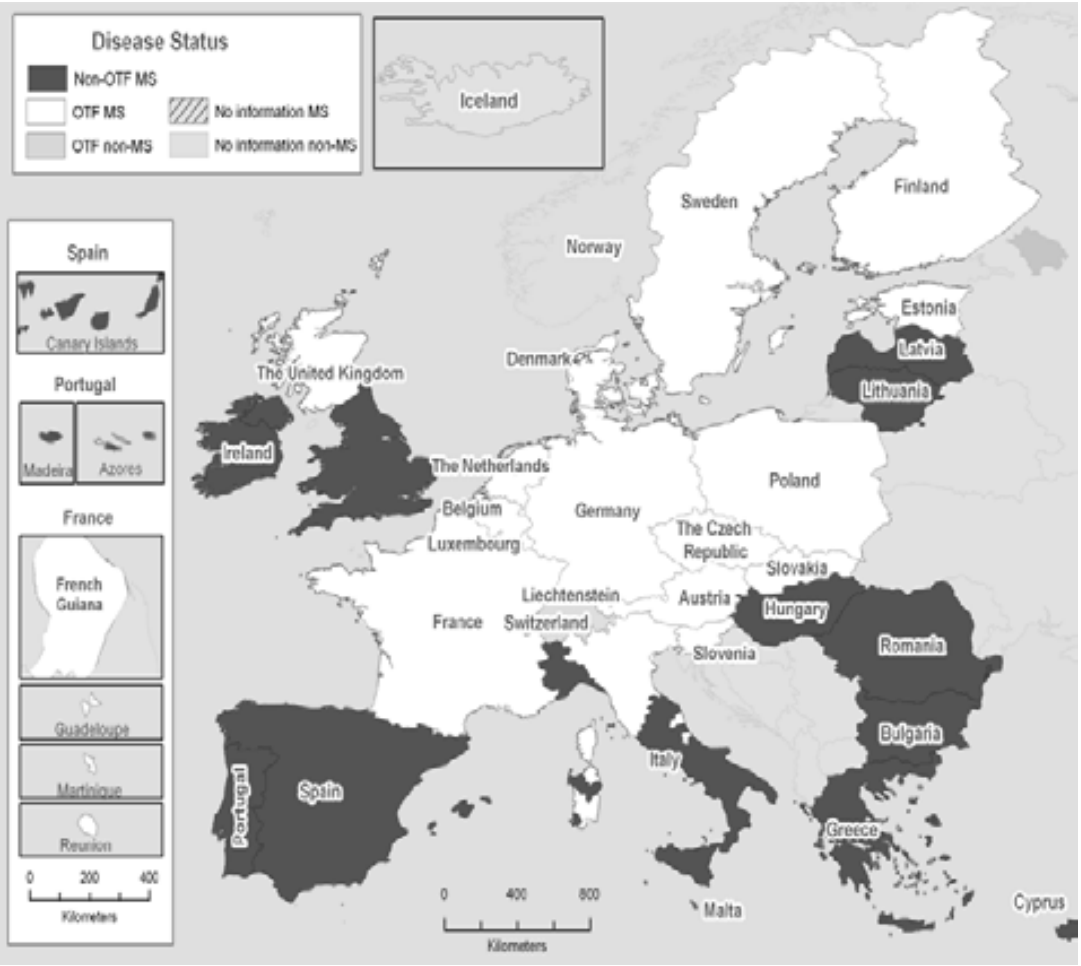

Figura 3. Estatus sanitari de TB dels estats membres (MS) de la UE l'any 2012. OTF, Oficialment lliures de TB (European Food Safety Authority. 2012).

A l'estat espanyol no és fins a l'any 1986 quan s'inicia l'actual programa d'eradicació en el bestiar boví. Aquest programa, basat fonamentalment en l'estratègia de prova i sacrifici, va donar uns resultats altament satisfactoris durant els seus primers anys, reduint la prevalença a nivell de ramat en uns 10 punts, però aquesta s'ha mantingut molt estable entorn al 1,5-2% durant la darrera dècada (*Ministerio de Medio Ambiente Rural i Marino*, 2012). Val a dir que a l'estat espanyol existeixen grans diferències entre àrees geogràfiques. Els majors nivells de prevalença se situen al sud-oest de l'estat. A Catalunya l'evolució de la TB ha estat semblant, si bé a partir de l'any 2007 la prevalença de TB a nivell de ramat s'ha situat entre mig punt i un punt per sota de la mitja de l'estat (Taula 1).

Taula 1. Evolució de la tuberculosi bovina a Catalunya i Espanya (2004-2011).

		Any							
		2004	2005	2006	2007	2008	2009	2010	2011
Prevalença de ramat (%)	Catalunya	1,78	1,7	1,65	1,08	0,85	0,83	0,59	0,81
	Espanya	1,8	1,52	1,76	1,63	1,59	1,65	1,51	1,33

1.2.3. La tuberculosi en el bestiar cabrum

Segons la FAO (*Food and Agriculture Organitzation*), l'any 2010 el cens de bestiar cabrum a nivell mundial s'estimava en uns 910 milions de caps. D'aquests, més del 97%, així com el gruix de la producció caprina mundial, es localitzava en països empobrits o en vies de desenvolupament (Taula 2, FAOSTAT 2010, consultat 18/12/2012), la majoria dels quals ja pateixen una epidèmia greu de TB en humans i/o en el bestiar vaquí. La TB en el cabrum ja s'ha pogut confirmar en alguns d'aquests països com Nigèria o Etiòpia (Cadmus et al., 2009; Hiko and Agga, 2011), per tant, com ja s'ha observat en països desenvolupats, la TB a les explotacions ramaderes de cabrum, pot suposar un problema econòmic i sanitari afegit a tenir en compte (Daniel et al., 2009; Shuralev et al., 2012).

Taula 2. Cens i producció de la ramaderia de bestiar cabrum de països representatius a escala mundial.

Estat	Cens		Producció (carn)		Producció (llet)	
	Caps	Posició	Tones	Posició	Tones	Posició
Índia	154.000.000	1	586.500	2	4.594.000	1
Xina	150.706.554	2	1.872.863	1	277.500	13
Pakistan	59.900.000	3	275.000	4	739.000	4
Nigèria	56.524.100	4	291.300	3	ND	-
Bangladesh	50.000.000	5	191.100	5	2.496.000	2
Etiòpia	22.786.900	8	66.300	10	ND	-
Brasil	9.312.780	17	30.000	28	148.149	20
Grècia	4.850.000	30	53.700	14	470.000	9
Argentina	4.250.000	35	9.900	53	ND	-
EUA	3.038.000	42	ND	-	ND	-
Espanya*	2.933.800	43	9.000	57	602.000	7
Rússia	2.136.640	55	17.944	39	255.175	15
França	1.349.030	70	12.053	50	645.176	6

*Catalunya: 91.023 caps, 781 T de carn i 7.181 L de llet (www.Idescat.cat, 18/12/2012).

Als països desenvolupats l'existència de TB en l'espècie caprina va ser descrita durant la primera meitat del segle XX en casos esporàdics al Regne Unit i els EUA (Bishop et al., 1934; Schwabacher. 1934; Francis. 1958), però en els darrers anys hi ha hagut un increment molt significatiu de publicacions que han reportat brots de TB en granges de cabrum al Regne Unit (Crawshaw et al., 2008; Daniel et al., 2009; Sharpe et al., 2010a), Itàlia (Zanardi et al., 2013), Portugal (Duarte et al., 2008; Quintas et al., 2010; Cunha et al., 2011) o Espanya (Domingo et al., 2009; Rodriguez-Campos et al., 2012; Napp et al., 2013).

No obstant, a data d'avui la tuberculosi caprina encara no apareix al llistat de malalties de notificables de la OIE i normalment no està inclosa als programes d'eradicació de tuberculosi bovina, tret que existeixi una cohabitació amb el bestiar vaquí en granges mixtes o pastures compartides. Aquesta realitat contrasta amb el fet que alguns dels estats membres de la UE no oficialment lliures de TB en el vaquí, com Grècia o Espanya,

són els que tenen censos més importants de bestiar cabrum (Taula 2). Concretament a Espanya alguns estudis apunten que la TB és present amb una prevalença molt significativa en els ramats de cabrum (Aranaz et al., 1999; García Marín. 2010; Rodriguez et al., 2011).

Recentment s'han trobat evidències que els caprins poden actuar com a reservoris domèstics de la TB i representar una font d'infecció tant de *M. bovis* com de *M. caprae* per al bestiar vaquí (Napp et al., 2013; Zanardi et al., 2013). Aquest factor, tot i que no és l'únic, pot ser una de les causes de l'aparició constant de nous brots de TB en el vaquí, impedint la disminució de la prevalença a nivell de ramat i dificultant l'eradicació de la malaltia en aquesta espècie (Humblet et al., 2009).

És interessant destacar que mentre els brots de TB en el cabrum al Regne Unit són causats per *M. bovis*, a Espanya la majoria dels casos són deguts a *M. caprae*. Les lesions causades per ambdues espècies en animals són macroscòpicament indistingibles, tot i que algun estudi recent assenyala que podrien existir algunes diferències histopatològiques (Garcia-Jimenez et al., 2012). En tot cas, l'aïllament d'una o altra espècie pot tenir importància en termes de valor epidemiològic.

1.2.4. La tuberculosi per *Mycobacterium caprae*

A banda de ser un agent causatiu de la TB en el caprí domèstic (Aranaz et al., 1999), *M. caprae* també s'ha aïllat en casos de TB en altres animals domèstics i en fauna silvestre, tant en estats de l'Europa central oficialment lliures de TB bovina (Haddad et al., 2001; Prodinger et al., 2002; Erler et al., 2004; Prodinger et al., 2005; Pate et al., 2006; Domogalla et al., 2013), com en estats del sud d'Europa que no tenen estatus oficialment lliure de TB bovina com Grècia (Ikonomomopoulos et al., 2006), Itàlia (Boniotti et al., March 2009), Espanya (Rodriguez et al., 2011) i Portugal (Duarte et al., 2008). A Espanya *M. caprae* representa un 7,4% de tots els aïllaments de MTBC realitzats a partir d'animals domèstics i silvestres (Rodriguez et al., 2011). Darrerament també s'han reportat nombrosos casos d'aïllaments de *M. caprae* en l'ésser humà a Europa (Kubica et al., 2003; Prodinger et al., 2005; Cvetnic et al., 2007; Rodríguez et al., 2009; Bayraktar et al., 2011; Aimé et al., 2012; Hansen et al., 2012).

2. Agent etiològic i patogènia

2.1. Característiques generals

Tots els bacteris causants de TB pertanyen al MTBC que inclou les següents espècies: *M. tuberculosis* (Koch. 1882), *M. microti* (Wells and Oxon, 1937)*, M. africanum* (Castets et al., 1968), *M. bovis* (Karlson and Lessel, 1970), *M. caprae* (Aranaz et al., 2003), *M. pinipedii* (Cousins et al., 2003) i recentment s'ha identificat una nova espècie: *M. mungi* (Alexander et al., 2010). Tradicionalment també s'ha inclòs *M. canettii* (van Soolingen et al., 1997) dins del MTBC, però anàlisis filogenètics recents el situen com una de les possibles espècies ancestrals del MTBC (Gutierrez et al., 2005; Supply et al., 2013). El grau de similitud de les espècies del MTBC és virtualment idèntic en termes de genòmica comparada amb un nivell d'identitat del 99,95% de les seqüències de nucleòtids (Garnier

et al., 2003; Huard et al., 2006). La seva elevada proximitat filogenètica està d'acord amb la hipòtesi que les espècies del MTBC són ecotips (Taula 3) que han evolucionat d'un ancestre comú sovint anomenat *M. prototuberculosis* (Supply et al., 2013).

Taula 3. Evolució filogenètica dels ecotips del MTBC.

Espècie	Hoste natural	Delecions de RD[a]
M. prototuberculosis[b]	Desconegut	-
M. tuberculosis	Humans	-
M. africanum WA[c]1	Humans	9
M. africanum WA2	Humans	9, 7, 8 i 10
M. mungi	Mangostes	9, 7, 8 i 10
Bacil "Dassie"	Damans	9, 7, 8 i 10
Bacil "Oryx"	Antílops	9, 7, 8 i 10
M. microti	Talpons	9, 7, 8 i 10
M. pinipedii	Foques	9, 7, 8 i 10
M. caprae	Caprins	9, 7, 8 , 10, 2, 12 i 13
M. bovis	Bovins	9, 7, 8 , 10, 2, 12, 13 i 4

[a] RD, regions de diferència del genoma.

[b] Hipotètic ancestre comú del MTBC.

[c] WA, *West-African*.

El MTBC s'integra dins del gènere *Mycobacterium*, que ja agrupa 159 espècies diferents (www.bacterio.net, consultat el 19/02/2013) i és l'únic membre de la família Mycobacteriaceae, que al seu temps està integrada dins de l'ordre Actinomycetales. El gènere *Mycobacterium* inclou altres patògens estrictes per humans, com *M. leprae* (agent causant de la lepra), o per animals, com *M. avium* subespecies *paratuberculosis* (agent causant de la malaltia de *Johne* o paratuberculosi en bovins i petits remugants).

Els micobacteris (tal com es denominen genèricament les espècies del gènere *Mycobacterium*) són bacils filamentosos, Gram-positius, àcid alcohol resistents, immòbils, aerobis estrictes i amb alt contingut de Guanina i Citosina en el seu ADN. En general, el seu estudi ha representat un nucli separat dins de la bacteriologia per les seves elevades singularitats: dificultat de ser cultivats, lent creixement (en la majoria dels casos), elevada patogenicitat o la necessitat de condicions de treball en laboratoris d'alt grau de biocontenció.

Una de les singularitats principals dels micobacteris, que els distingeix de la resta de bacteris gram positius, és la complexitat de l'envolta cel·lular. Aquesta està formada per la membrana plasmàtica, la paret cel·lular i la membrana externa (o càpsula). Al seu temps, la paret cel·lular està formada pel peptidglicà, l'arabinogalactà i els àcids micòlics (Figura 4). Aquests últims són uns àcids grassos de cadena llarga de 60 a 90 àtoms de carboni que s'estenen per tota la regió hidrofòbica i tenen una consistència quasi sòlida a temperatura ambient (Vincent Lévy-Frébault and Portaels, 1992). En conjunt, aquesta composició atorga unes propietats fortament hidrofòbiques a l'envolta cel·lular dels micobacteris que, en el cas dels micobacteris del MTBC, els confereix una major resistència al procés de fagocitosi per part dels macròfags (Rastogi et al., 2001), permetent-los sobreviure i replicar- se intracel·lularment en un ambient hostil (Daffé and Draper, 1998).

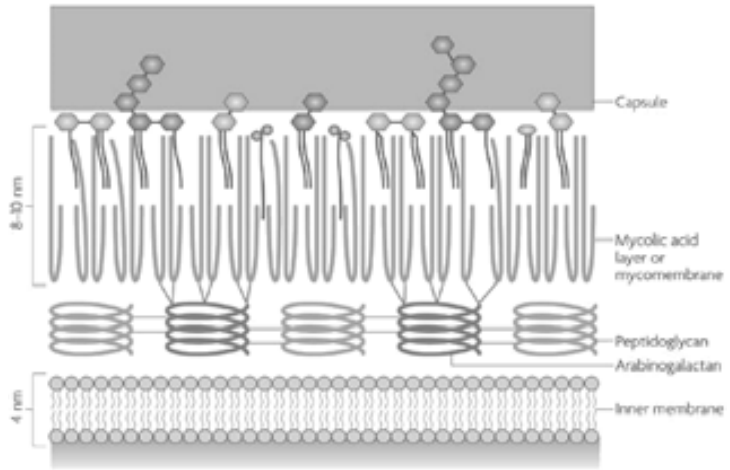

Figura 4. Esquema de l'envolta cel·lular de *M. tuberculosis* (Abdallah et al., 2007). D'interior a exterior: la membrana plasmàtica o membrana interna; la paret cel·lular formada pel peptiglicà, l'arabinogalactà, els àcids micòlics i glicolípids (exterior); i la càpsula formada per polisacàrids.

L'envolta cel·lular dels micobacteris del MTBC també té un especial interès pel seu paper fonamental en les interaccions hoste- patògen, incloent tant factors de virulència com possibles dianes terapèutiques o vacunals (Gordon et al., 2009).

2.2. Patogènesi

Tant en humans com en remugants domèstics la principal via de transmissió de la TB és per mitjà d'aerosols (tos, expectoració) a partir d'individus amb TB pulmonar activa (Pollock and Neill, 2002; Russell. 2007). Les gotícules aerosolitzades que contenen els bacils poden evadir la barrera mucociliar i arribar als alvèols pulmonars, on els bacils seran fagocitats pels macròfags alveolars. A partir d'aquí es pot establir la infecció amb una lesió inicial o focus primari a l'òrgan d'entrada (per ex: el pulmó en el cas de la via d'infecció respiratòria).

Un cop s'ha establert la infecció inicial es produeix un drenatge bacil·lar de l'òrgan d'entrada als seus limfonodes associats, on es pot iniciar una nova lesió. S'anomena complex primari complet al conjunt de lesions a l'òrgan d'entrada i els limfonodes associats (per ex: pulmó i limfonodes pulmonars).

Un cop establert el complex primari es poden obrir diferents escenaris. En la majoria dels casos en humans no es produeix la malaltia, així doncs, o bé hi ha una destrucció ràpida dels bacils per part dels macròfags activats per la resposta immunitària de l'hoste, o bé s'esdevé la LTBI.

En altres casos es pot donar una disseminació post- primària o reinfecció al produir-se un nou focus d'infecció, normalment per disseminació de bacils per via broncoalveolar a partir del focus primari. Aquests bacils infectaran un nou macròfag alveolar inactiu i generaran una nova lesió.

Finalment també es pot produir una disseminació per via limfohematògena que doni lloc a la sembra de bacils a diferents òrgans i un procés de generalització. Sovint aquesta

infecció generalitzada origina lesions granulomatoses multifocals a les membranes seroses donant lloc al que es coneix com a tuberculosi mil·liar.

Pel que fa referència als animals la patogènesi de la TB no està tan extensament estudiada com en el cas del humans, però existeixen suficients indicis per assumir, si més no en remugants domèstics, la preponderància de la via de transmissió aerògena, ja que en la majoria de casos es detecten lesions tuberculoses al pulmó o als limfonodes respiratoris (Cassidy. 2006; Daniel et al., 2009). Tot i que la localització de les lesions no és necessàriament indicativa de la via de transmissió, s'ha pogut comprovar que en bovins infectats experimentalment amb *M. bovis* per via oral, les lesions apareixien fonamentalment en pulmons i el teixit limfoide associat, així com en limfonodes del cap, però no en òrgans o teixit limfoide del sistema digestiu (Palmer et al., 2004).

2.2.1. Formació del granuloma

El granuloma, també denominat "tubercle" per la seva morfologia, és la característica histopatològica fonamental de la lesió tuberculosa. És una conseqüència de la resposta de l'hoste que remodela el lloc d'infecció en un agregat cel·lular disposat en un patró típic que es reconeix com a granuloma (Russell. 2007). L'arquitectura del granuloma permet la contenció de la disseminació dels micobacteris encerclant el nucli central de la lesió amb múltiples barreres concèntriques de cèl·lules inflamatòries (Saunders and Cooper, 2000). Els primers estudis sobre la formació i el rol dels granulomes en la patogènesi de la TB es van realitzar en el model de conill (Dannenberg. 2001). Anteriorment, l'any 1955, el metge i biòleg Georges Canetti havia fet una descripció histopatològica a partir d'un cas de LTBI en un humà. Canetti la va descriure com una "evolució benigna" de la infecció perquè en la necròpsia el subjecte presentava granulomes petits encapsulats per un anell fibrós amb necrosi mineralitzada al centre (Canetti. 1955).

Així doncs, un cop els micobacteris inhalats són fagocitats pels macròfags alveolars, aquests envien senyals a altres cèl·lules inflamatòries del sistema immunitari que són reclutades cap al focus d'infecció a través dels vasos sanguinis propers. El granuloma es va formant a partir d'un nucli de macròfags infectats, vorejat concèntricament per les diferents cèl·lules proinflamatòries reclutades: a) primerament per macròfags escumosos (caracteritzats per un alt contingut de lípids i freqüents en teixits amb estímuls proinflamatoris crònics), cèl·lules gegants multinucleades o cèl·lules de Langhans (macròfags fusionats amb els nuclis en disposició de corona) i altres fagòcits mononuclears; i b) en segon lloc per limfòcits. Posteriorment es forma una beina fibrosa de col·lagen i altres components de la matriu extracel·lular que delinea la perifèria de l'estructura disminuint sensiblement el nombre de vasos sanguinis que penetren a l'estructura (Russell. 2007, Figura 5). Aquesta resposta tissular defineix una primera fase de "contenció" de la infecció.

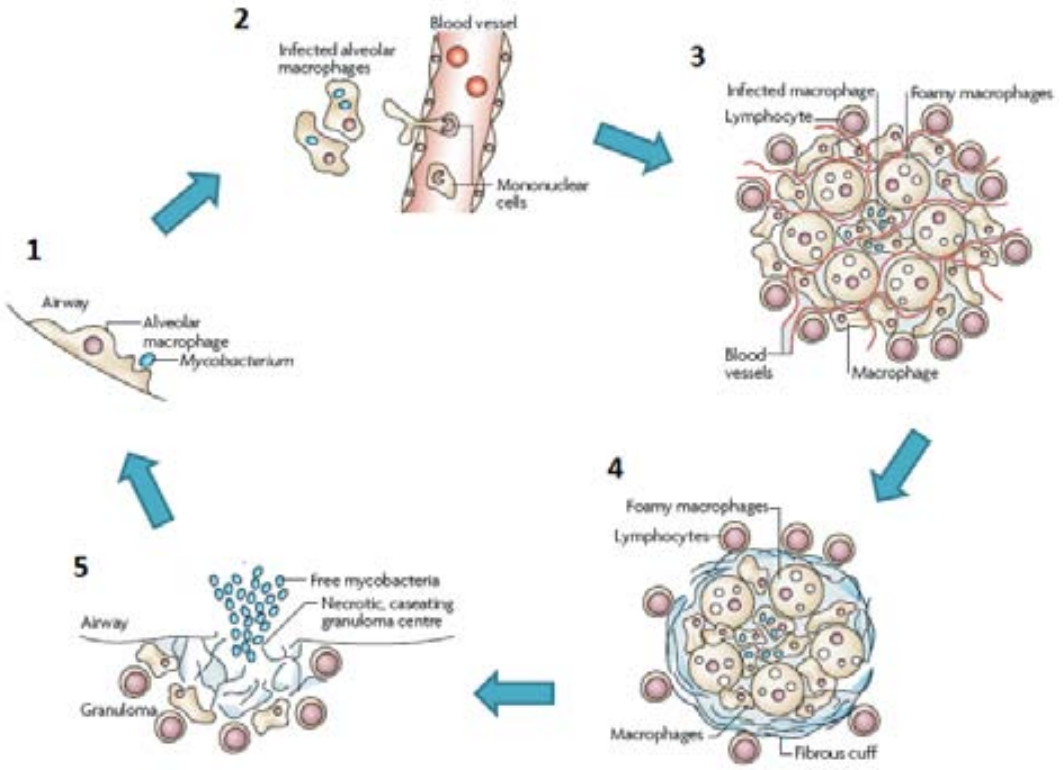

Figura 5. Representació de les etapes de formació i destrucció del granuloma (adaptat de (Russell. 2007)). (1) Infecció de macròfags alveolars. (2) Reclutament de cèl·lules inflamatòries al focus d'infecció. (3) Formació del granuloma inicial (macròfags escumosos, limfòcits, vasos sanguinis). (4) Granuloma consolidat (amb càpsula fibrosa). (5) Liqüefacció del granuloma i obertura de la lesió a les vies aèries.

La regió central d'aquests granulomes és hipòxica i en la majoria de mamífers s'hi acaba desenvolupant necrosi. Aquesta característica clàssicament s'ha atribuït a una reacció tòxica anomenada "fenomen de Koch" (Bothamley and Grange, 1991). Estudis més recents atribueixen la necrosi intragranulomatosa a l'acció de diferents factors que es poden donar de forma conjunta o inclús alternativa: a) infiltrats de cèl·lules T que actuen activant els macròfags que contenen els bacils i posteriorment es destrueixen per mecanismes apoptòtics (Keane et al., 1997); b) una resposta immunitària no efectiva impossibilitant l'activació dels macròfags que són destruïts per la multiplicació dels bacils (Orme. 1998); o c) una resposta immunitària innata exagerada amb un ràpid influx de macròfags i granulòcits al focus d'infecció (Turner et al., 2003).

La generació d'un medi extracel·lular necròtic produeix una evolució de les lesions cap a la necrosi, la caseïficació que finalment pot derivar en la liqüefacció i cavitació dels teixits. La ruptura i desintegració del granuloma provoca un vessament de milers de bacils infecciosos a les vies respiratòries, resultant en el desenvolupament d'una tos productiva que facilita la propagació d'aerosols de bacils infecciosos (Russell. 2007).

En resum, el granuloma és un complex d'interaccions dinàmiques entre macròfags, cèl·lules T, producció de citocines i activitat micobacteriana que determinaran l'evolució de la lesió cap a necrosi, mineralització, liqüefacció o regressió (Cassidy. 2006).

2.2.2. Factors de virulència

Els factors que influeixen en la severitat de la TB en individus infectats són múltiples i depenen de les característiques genètiques de la soca bacteriana, de l'hoste i de la seva interacció (Collins. 2001; Kaufmann. 2008). Les manifestacions clíniques de la TB també estan influenciades per altres factors que transcendeixen les característiques intrínseques de l'hoste i el patogen com són la ruta d'infecció o la dosi infectiva (Pollock and Neill, 2002).

En animals també s'ha assenyalat l'edat de l'individu com un factor a tenir en compte. En general la progressió de la infecció és més ràpida i la severitat de la patologia és major en animals joves (Martin-Hernando et al., 2007). En explotacions ramaderes de producció intensiva també s'ha comprovat que la freqüència i disseminació de la infecció tuberculosa és més elevada degut a la major concentració d'animals característica dels sistemes productius ramaders i al compromís de la immunocompetència derivat de l'estrès que pot ocasionar la vida productiva (Ramirez-Villaescusa et al., 2010).

Els patògens del MTBC presenten factors de virulència propis. La virulència de les soques vindrà determinada per la seva habilitat per superar les defenses de l'hoste. Per exemple, s'ha descrit que els lípids de la paret cel·lular juguen un paper important com a efectors defensius, ofensius o adaptatius de la virulència (Hotter and Collins, 2011).

En diversos estudis de factors de virulència, s'han comparat soques patògenes amb soques atenuades. La soca vacunal atenuada *M. bovis* BCG (Bacil de Calmette-Guerin) ofereix un bon marc per a aquest camp d'estudi. Aquesta soca, a diferència de la resta d'espècies del MTBC, ha patit la deleció de la RD1 del genoma (Mahairas et al., 1996) i conseqüentment, els gens que formen part de la RD1 han estat ampli objecte d'estudi. Concretament el locus Esx-1 (ESAT-6 *secretion complex 1*) és el més estudiat (Gey et al., 2001; Abdallah et al., 2007). El sistema Esx-1 permet la secreció de les proteïnes ESAT-6 (*6-kDa early secretory antigenic target*) i CFP-10 (*10-kDa culture filtrate protein*), que formen un heterodímer estable i són un factor clau en les interaccions hoste- patogen (Renshaw et al., 2005). Concretament s'ha suggerit que estan involucrades en la lisi de la membrana de l'hoste i la disseminació cèl·lula- cèl·lula (de Jonge et al., 2007). Recentment també s'ha demostrat que ESAT-6, després de ser secretat pel micobacteri, s'integra entre els fosfolípids de la membrana del fagosolisosoma dels macròfags i la destrueix permetent als micobacteris accedir al citosol (Houben et al., 2012). La capacitat de translocació d'alguns micobacteris patògens al citosol ha esta descrita en el MTBC, *M. leprae* i *M. marinum* (Stamm et al., 2003; van der Wel et al., 2007) i s'ha demostrat que és un factor clau associat de la patogènesi de les infeccions micobacterianes (Houben et al., 2012).

Finalment, la transcriptòmica és una eina que també ha estat utilitzada darrerament per comparar diferents perfils d'expressió entre soques de *M. bovis* amb la finalitat d'identificar polimorfismes cromosòmics que puguin donar lloc a nous determinants de virulència (Inwald et al., 2002; Blanco et al., 2009).

3. Immunitat i antigenicitat

La patologia causada per MTBC és fonamentalment immunopatològica, ja que és el resultat de l'alteració tissular concurrent provocada per la resposta immune de l'hoste contra la infecció tuberculosa (Ulrichs and Kaufmann, 2006). Tanmateix, el coneixement dels mecanismes immunològics implicats és l'àrea de recerca central per al disseny racional d'estratègies vacunals contra la TB (Kaufmann. 2006).

La LTBI causada per *M. tuberculosis* en l'ésser humà és inicialment controlada de forma activa per la resposta immune i, en determinades circumstàncies, pot progressar cap a TB activa (malaltia). Les característiques qualitatives i quantitatives de la resposta immune seran un dels principals factors que determinaran si un individu infectat emmalalteix (desenvolupa clínica o lesions macroscòpiques) o no (Kaufmann. 2005).

En els grans mamífers, tot i que menys estudiats, els fonaments immunològics i immunopatològics són en general similars als desenvolupats en els humans, salvant certes diferències fisiològiques i patogèniques (Buddle et al., 1994; Caro et al., 2001; Palmer and Waters, 2006; Gil et al., 2010). Concretament els estudis a partir d'infeccions experimentals de bovins amb *M. bovis* han contribuït significativament al coneixement de la immunitat enfront a la TB en aquesta espècie (Pollock et al., 2005; Palmer and Waters, 2006; Pollock et al., 2006).

3.1. Control immunitari de la tuberculosi

Les interaccions entre els micobacteris i les diferents dianes cel·lulars de la mucosa respiratòria de l'hoste determinen el desenllaç de la TB pulmonar. En aquestes interaccions, tant la immunitat innata com l'adaptativa juguen un paper crític.

Tant en animals com en l'ésser humà, el control de MTBC es basa principalment en la immunitat mitjançada per cèl·lules (en anglès CMI) que és el resultat del treball en equip de diferents poblacions de cèl·lules T i macròfags (Pollock et al., 2005; Cooper. 2009). Concretament la resposta que es considera imprescindible per al control de MTBC és la CMI basada en cèl·lules T cooperadores de tipus I (en anglès Th1). La intensitat de la CMI, sobretot els nivells d'interferó-gamma (IFN-γ) produïts per les cèl·lules T, és el paràmetre que s'associa a nivells de protecció front a la TB (Flynn et al., 1993; Andersen et al., 2007).

Per la seva banda la immunitat mitjançada per anticossos o immunitat humoral en general no s'ha considerat rellevant per al control dels micobacteris que, degut a la seva característica de patògens intracel·lulars, pràcticament no estarien exposats a l'acció neutralitzant dels anticossos. No obstant, estudis recents emprant el model murí han demostrat que teràpies basades en anticossos són eficaces en impedir la reactivació de la infecció per *M. tuberculosis* (Guirado et al., 2006; Balu et al., 2011) i el rol dels anticossos en la protecció contra la TB és encara matèria de discussió.

3.1.1. Immunitat innata

Els micobacteris poden ser endocitats pels macròfags i també per les cèl·lules dendrítiques (en anglès DCs) (Russell. 2007). Ambdós tipus cel·lulars, que alhora són

cèl·lules presentadores d'antigen (en anglès APCs), expressen receptors de superfície de dos tipus: 1) receptors endocítics, com els receptors fixadors del complement (en anglès FcRs) o els receptors de manosa, que fixen el micobacteri i faciliten l'inici del procés de fagocitosi; i 2) receptors de senyalització o receptors de reconeixement de patrons (en anglès PRRs), que detecten patrons moleculars associats a patògens (en anglès PAMPs) (Akira et al., 2006). La principal família de PRRs són els receptors de tipus *toll* (en anglès *toll-like receptors*, TLRs) que interactuen amb components de la paret cel·lular dels micobacteris i desencadenen la resposta immune innata.

El macròfag, com a fagòcit professional, és la principal cèl·lula diana i també l'hàbitat de preferència dels micobacteris del MTBC, que hi duen a terme mecanismes sofisticats de supervivència i d'evasió de la resposta immune innata, com el segrest del fagosoma en una etapa immadura, impedint la seva acidificació i prevenint la fusió del fagosoma amb el lisosoma i la formació del fagolisosoma (Figura 6).

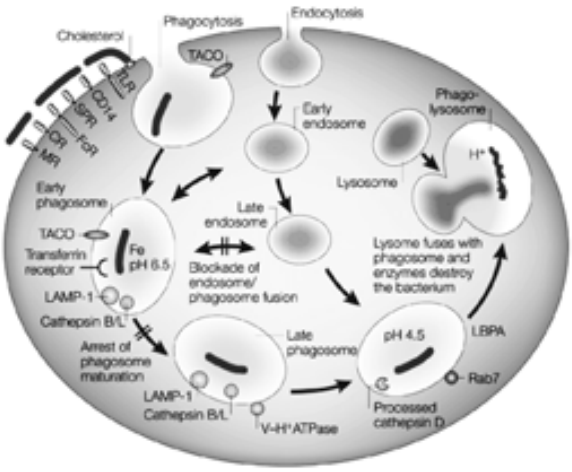

Figura 6. Esquema de la influència de *M. tuberculosis* durant el procés de fagocitosi per part dels macròfags (Kaufmann. 2001). Els micobacteris impedeixen la maduració del fagosoma i sobreviuen dins del macròfag en repòs.

La maduració del fagosoma és un punt crític per a completar la fagocitosi i eliminar els micobacteris. En el fagosoma immadur els micobacteris podran tenir accés al ferro, que resulta essencial per a la seva supervivència intracel·lular, i competir per ell amb la cèl·lula hoste, que el requereix per diferents mecanismes de defensa (Kaufmann. 2001). Els macròfags activats promouen la maduració del fagosoma, particularment després de ser estimulats per l'IFN-γ secretat per cèl·lules T efectores que han migrat al granuloma (Kaufmann. 2001). L'IFN-γ, conjuntament amb el factor de necrosi tumoral alfa (TNFα), són els principals promotors de l'activitat fagocítica i antimicobacteriana dels macròfags. Ambdues citocines estimulen diferents mecanismes efectors fonamentals per a l'eliminació dels micobacteris: maduració i acidificació del fagosoma (que passa de pH 6,5 a pH 4,5), restricció de l'accés al ferro, disminució de la concentració d'oxigen o síntesi d'òxid nítric (Schaible et al., 1998; Endsley et al., 2007; Russell et al., 2009).

La destrucció del micobacteri desencadena una cascada de senyalització que acaba amb l'activació del factor de transcripció NF-κB (factor nuclear de les cadenes lleugeres kappa

de les cèl·lules B) que indueix l'expressió de citocines proinflamatòries com les interleuquines (IL) IL-1β, IL-6 o els interferons de tipus I (IFN-α i IFN-β), així com de quimioquines que són responsables del reclutament d'altres cèl·lules inflamatòries al lloc d'infecció.

3.1.2. Immunitat adaptativa

La immunitat adquirida, fonamentalment la CMI que involucra cèl·lules T, juga un paper central i tanmateix dual davant la infecció tuberculosa, proporcionant immunitat protectora en determinats casos, però també contribuint al desenvolupament de les lesions granulomatoses i la necrosi caseosa que facilita la progressió transmissió de la infecció. Si la resposta és massa lenta, el creixement bacterià pot arribar a un punt en què, malgrat s'expressi una potencial immunitat protectora, l'ambient que s'ha generat al focus d'infecció impedeix que aquesta sigui eficaç (Cooper. 2009).

Els limfonodes regionals de drenatge són el lloc d'iniciació de la immunitat efectora contra MTBC (Wolf et al., 2008). L'activació i expansió limfocitària antigen-específica es correlaciona temporalment amb l'arribada dels bacils disseminats des de l'òrgan d'entrada al limfonode de drenatge (Chackerian et al., 2002). Un cop els micobacteris arriben al limfonode, les cèl·lules T verges són activades a través de la presentació antigènica per part de les APCs (normalment DCs) que migren del interstici alveolar al limfonode regional (Demangel et al., 2002; Bhatt et al., 2004). Les APCs presenten els antígens de MTBC, que han estat prèviament processats a través del complex major d'histocompatibilitat (en anglès MHC), unes molècules que s'expressen a la superfície de les APCs. La presentació a les cèl·lules T CD4+ es realitza a través del MHC de tipus II, mentre que la presentació a les cèl·lules T CD8+ es realitza per mitjà del MHC de tipus I. Per la seva banda, per iniciar l'activació i proliferació de les cèl·lules T verges, aquestes necessiten expressar a la seva superfície el receptor de cèl·lules T (en anglès TCR).

Les cèl·lules T efectores activades, migren del limfonode a través del torrent circulatori i són reclutades al focus primari d'infecció per participar en la resposta inflamatòria. Aquesta migració pot trigar 15-18 dies post-infecció segons s'ha descrit en ratolins (Reiley et al., 2008). Tant les poblacions de cèl·lules T CD4+ com les cèl·lules T CD8+, juguen un paper essencial en la lluita contra la infecció per MTBC:

Les cèl·lules T CD4+ es polaritzen en diferents subpoblacions. La disponibilitat de determinades citocines definirà el fenotip d'aquestes subpoblacions. Les DCs exposades a MTBC, indueixen IL-12 que contribueix a diferenciar les cèl·lules T CD4+ en cèl·lules Th1 (Flynn and Chan, 2001; Flynn. 2004). Les cèl·lules Th1 activen els mecanismes antimicobacterians dels macròfags i per aquest motiu la seva estimulació és un punt crucial per als candidats vacunals (Kaufmann. 2005).

Les cèl·lules Th1 efectores i de memòria produeixen diferents citocines com la IL-2, que participa en l'activació i proliferació de cèl·lules T, o com l'IFN-γ i el TNFα que activen els macròfags (Foulds et al., 2006). Recentment s'han descrit altres subpoblacions, com les cèl·lules productores de IL-17 o Th17, que reforcen les respostes Th1 al focus d'infecció (Khader et al., 2007) i activen els granulòcits polimorfonuclears (en anglès PNG). La IL-23,

secretada per les DCs i els macròfags activats, contribueix a la polarització de les cèl·lules T CD4+ cap a Th17 i és essencial per una resposta òptima de la IL-17 front a la infecció (Khader and Cooper, 2008). De la mateixa manera, la IL-23 també contribueix a polaritzar les cèl·lules T CD4+ a cèl·lules T productores de IL-22 (Th22). La IL-22, produïda per les cèl·lules Th22 i també per les Th17 té una activitat de barrera protectora tissular i promotora de la resposta inflamatòria front a agents infecciosos (Sonnenberg et al., 2011). Recentment s'ha comprovat que els nivells de IL-22 en bovins vacunats contra la TB s'associen amb la protecció (Bhuju et al., 2012).

Les cèl·lules Th2 secreten IL-4 i les cèl·lules T reguladores (Treg) IL-10 i TFGβ. Aquestes citocines contraresten l'activitat de les cèl·lules Th1 (19). Per tant, el balanç Th1/Th2 és important per determinar l'evolució de la infecció (Pollock et al., 2005; Welsh et al., 2005).

Per la seva banda les cèl·lules T CD8+ també participen en l'activació dels macròfags produint IFN-γ i TNFα (Cooper. 2009) i, per una altra banda, també actuen com a limfòcits T citolítics (en anglès CTL). Els CTL secreten perforina i granulisina, lisen la cèl·lula hoste i ataquen directament els micobacteris (Stenger et al., 1998).

L'acumulació de les cèl·lules T CD4+ i T CD8+ a dins o al voltant del granuloma resultarà clau per activar de forma efectiva els macròfags i desencadenar una resposta immunitària efectiva. S'ha observat en ratolins, que els animals que no són capaços d'infiltrar adequadament els limfòcits dins de la lesió granulomatosa, són més susceptibles a morir davant una eventual infecció per *M. tuberculosis* (Orme. 1998). El treball en equip de les diferents subpoblacions de cèl·lules T i dels macròfags determinarà la capacitat de l'hoste per controlar la infecció en granulomes sòlids mantenint una forma latent de TB o d'evolucionar cap a granulomes caseosos i la forma activa de la malaltia (Figura 7).

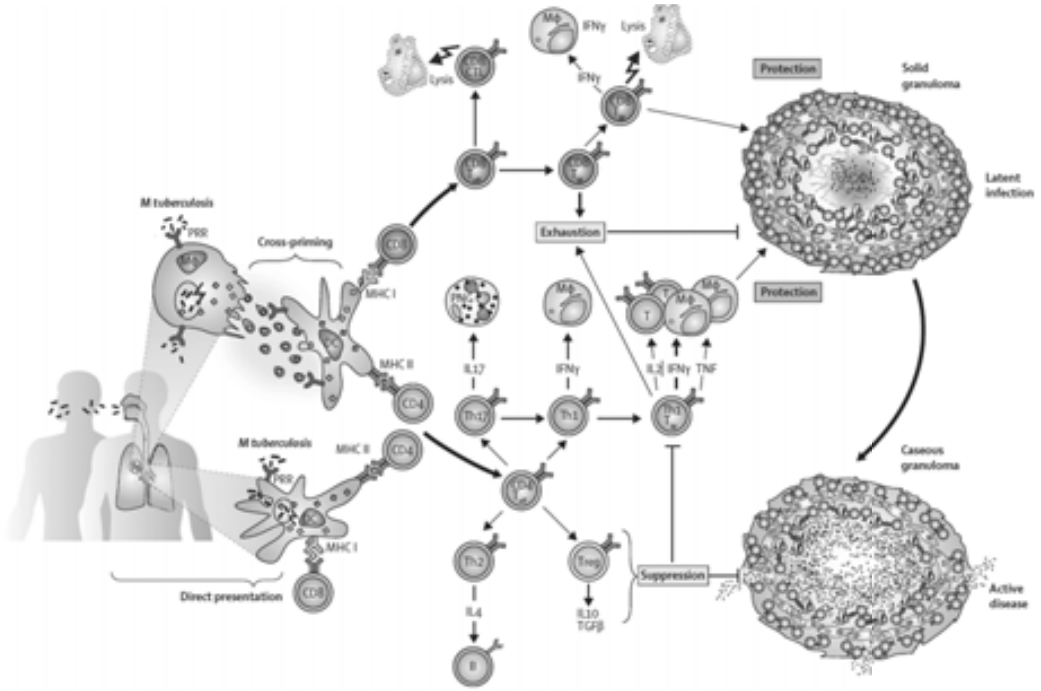

Figura 7. Esquema general de respostes immunològiques en la infecció tuberculosa (Kaufmann et al., 2010). El balanç de la resposta immunològica determinarà si es produeix una contenció activa de la infecció (protecció) en un granuloma sòlid o si s'indueix la seva caseïficació, evolucionant cap a una infecció activa.

A banda de les cèl·lules T CD4+ i T CD8+, una altra subpoblació de cèl·lules T també participa en la resposta immune enfront durant la infecció per MTBC, les cèl·lules T gamma delta (γδ), que són particularment abundants en remugants, especialment en animals joves (Rhodes et al., 2001). Les cèl·lules T γδ s'anomenen així perquè expressen un TCR γδ (format per una cadena γ i una cadena δ), distint al de les cèl·lules que expressen un TCR αβ, com les T CD4+ i T CD8+. El seu mecanisme d'activació i de reconeixement antigènic és encara força desconegut però s'ha descrit que aquestes cèl·lules podrien jugar un paper important en la primera fase de les infeccions micobacterianes i que estarien involucrades tant en la immunitat innata, com en l'adquirida (Blumerman et al., 2007; Plattner et al., 2009). En bovins infectats experimentalment amb *M. bovis*, s'ha observat que la primera subpoblació de cèl·lules T en actuar són les T γδ, seguides de les T CD4+ i les T CD8+ (Pollock et al., 1996). També s'ha descrit que les cèl·lules T γδ poden tenir un efecte supressor sobre l'activitat antigen-específica de les T αβ (Rhodes et al., 2001).

Concretament, l'expressió o no del receptor WC1 (*Workshop cluster* 1) a la superfície de les cèl·lules T γδ és la principal característica per distingir les subpoblacions d'aquestes cèl·lules en remugants (Wijngaard et al., 1992). S'ha observat que les cèl·lules T γδ WC1+ de sang perifèrica de bovins infectats amb *M. bovis* responen significativament front a antígens micobacterians proteics i no proteics (Welsh et al., 2002). A més, s'ha demostrat que cèl·lules T γδ WC1+ d'animals sans i sense sensibilització prèvia, també proliferen i produeixen grans quantitats d'IFN-γ en resposta a estimulacions amb antígens de la paret cel·lular de MTBC (Vesosky et al., 2004).

Tenint en compte que les cèl·lules T γδ constitueixen la major població en sang perifèrica de remugants (sobretot en animals joves), la seva activitat té un impacte important en la resposta de l'hoste contra la infecció tuberculosa i esdevé una àrea de recerca d'especial interès en la tuberculosi animal.

3.2. Hipersensibilitat retardada

La resposta d'hipersensibilitat de tipus IV o retardada (en anglès DTH) enfront a determinats antígens de MTBC és una CMI indicativa d'un encontre previ de l'hoste amb el micobacteri. Es tracta d'una reacció inflamatòria local que es produeix entre 24 i 72h després de la sensibilització.

Durant força temps es va associar la DTH (considerada una forma independent de la CMI) al *fenomen de Koch* i la causa principal de la necrosi caseosa intragranulomatosa (Bothamley and Grange, 1991; Dannenberg Jr. 1991). Posteriorment s'ha observat que la DTH és una resposta induïda per citocines secretades fonamentalment per cèl·lules T CD4+ i macròfags (principalment TNFα i IFN-γ) que estimulen la secreció de quimioquines per part de les cèl·lules tissulars locals que atrauen de forma massiva cèl·lules inflamatòries del torrent sanguini al lloc d'inflamació, ja sigui el focus infecciós o un punt d'inoculació (Orme. 1998).

Aquesta reacció ha tingut (i encara té) una gran transcendència en el camp diagnòstic de la TB. La prova diagnòstica de l'aplicació intradèrmica de la tuberculina o IDTB es basa en

la DTH a nivell local produïda enfront al derivat proteic purificat (en anglès PPD) o tuberculina. El PPD prové de *M. tuberculosis* en la prova de Mantoux en humans, o de *M. bovis* i *M. avium* en la IDTB comparada que es realitza en bovins.

3.3. Antigenicitat

El MTBC conté antígens que estan àmpliament conservats entre els micobacteris, molts del quals es troben també en el PPD. Dins del gènere *Mycobacterium* es poden classificar els antígens en 4 grups en funció de la seva especificitat (Standford. 1983): a) comuns a tots els micobacteris; b) comuns a les espècies de creixement ràpid; c) comuns a les espècies de creixement lent; i d) específics d'una espècie o d'un conjunt d'espècies estretament relacionades. Aquest darrer grup d'antígens acostumen a ser immunodominants a l'hora generar respostes immunològiques enfront als patògens.

La resposta immune front a la infecció tuberculosa és predominantment de base cel·lular, però els antígens del MTBC tenen determinants antigènics (o epítops) específics per cèl·lules T i per cèl·lules B. Alguns d'aquests antígens, per les seves característiques immunogèniques, han estat objecte d'estudi per a ser utilitzats en el diagnòstic o el disseny de nous candidats vacunals (Mustafa et al., 2006; Mustafa et al., 2008; Lindestam Arlehamn et al., 2012).

La identificació d'antígens associats a latència també és un recent camp d'interès. Aquests antígens són aquells que tindran una major expressió durant la fase latent de la infecció i poden ser incorporats en vacunes contra la LTBI (Aagaard et al., 2011; Niu et al., 2011) o com a reactius diagnòstics (Schuck et al., 2009).

A la Taula 4 es mostren els antígens proteics de MTBC més representatius en funció del seu ús recent en el disseny de noves vacunes i reactius de diagnòstic. Els antígens de la Taula 4 es divideixen en 4 grups funcionals:

a) ***Complex Antigen 85 (Ag85)***. Complex que inclou 3 micoliltransferases (Ag85A, B i C) involucrades en la síntesi de la paret cel·lular (Armitige et al., 2000). Recentment s'ha descrit que l'Ag85A també podria estar involucrat en la síntesi de triacilglicerols generant cossos d'emmagatzematge de lípids en el micobacteri que li permetrien persistir dins del macròfag (Elamin et al., 2011).

   Tant l'Ag85A (Wang et al., 2004; McShane et al., 2005; Santosuosso et al., 2006; Hawkridge et al., 2008) com l'Ag85B (Dietrich et al., 2005; Langermans et al., 2005; Dietrich et al., 2006; van Dissel et al., 2010) estan entre els antígens més immunogènics identificats fins ara i són els més utilitzats en les diferents vacunes subunitat (constituïdes per només algun dels components del micobacteri) que actualment es troben en fase d'assajos clínics. Són principalment dianes per a les cèl·lules T però també poden induir alts nivells d'anticossos antigen-específics.

   b) ***Sistema de secreció del complex ESAT-6 (Esx)***. Sistema de secreció de proteïnes. El genoma de MTBC conté 5 locus Esx dels quals l'Esx-1 és el més extensament descrit (Cole et al., 1998; Gey et al., 2001) i és el responsable de la secreció dels antígens

ESAT-6 (EsxA) i CFP-10 (EsxB) (Behr and Sherman, 2007; McLaughlin et al., 2007) (Figura 8). Aquests antígens formen un complex heterodimèric 1:1 estable i, com s'ha comentat amb anterioritat, juguen un paper central en les interaccions hoste-patogen (Renshaw et al., 2005). A banda de la seva rellevància com a factors de virulència, ESAT-6 i CFP-10 són antígens immunodominants i tenen un gran interès en el diagnòstic de la TB per dues raons: 1) perquè estan codificats a la regió RD1, absent al genoma de les diferents soques de *M. bovis* BCG (Mahairas et al., 1996; Ganguly et al., 2008) (Figura 8); i 2) perquè ambdós antígens són potents inductors d'IFN-γ per part de les cèl·lules T durant les primeres fases de la infecció tuberculosa (Pollock and Andersen, 1997). Múltiples estudis han avaluat el seu ús en el diagnòstic de TB basat en l'alliberament d'IFN-γ en humans (Pai et al., 2004) i animals (Vordermeier et al., 2001; Aagaard et al., 2006; Cockle et al., 2006; Bezos et al., 2011). ESAT-6 també s'incorpora en alguns candidats vacunals (Dietrich et al., 2005; Dietrich et al., 2006; Lin et al., 2012).

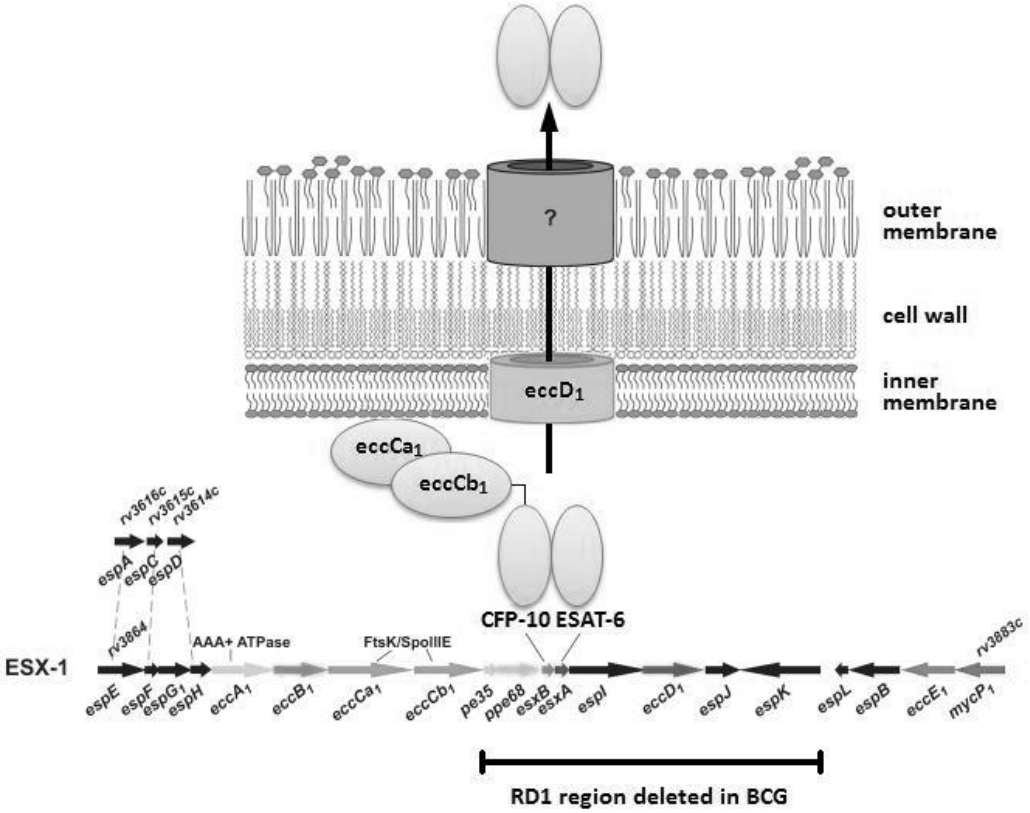

Figura 8. Esquema de l'organització de la regió genòmica que conté els gens del sistema de secreció del complex ESAT-6 1 (ESX-1) i de la participació d'alguns dels seus productes en la formació i posterior secreció de l'heterodímer ESAT-6:CFP-10. La barra inferior indica els gens deleccionats a les soques de *M. bovis* BCG (figura adaptada de Gey et al., 2001 i Behr and Sherman, 2007).

Per altra banda el locus Esx-3 és responsable de la secreció de TB10.4 (EsxH) i TB9.8 (EsxG) (Bitter et al., 2009). Com ESAT-6 i CFP-10, ambdós antígens contenen epítops fortament reconeguts per les cèl·lules T durant la infecció tuberculosa, però també en individus vacunats amb BCG (Skjøt et al., 2002).

TB10.4, en combinació amb altres antígens, també s'ha incorporat en diferents vacunes prototip (Dietrich et al., 2005; Dean et al., 2008; Niu et al., 2011). Per la seva banda TB9.8 també s'ha identificat com un potencial candidat per incorporar en vacunes subunitat, tot i que en una fase preliminar i encara sense estudis d'eficàcia vacunal publicats a data d'avui (Mustafa et al., 2006).

Respecte a TB7.7, no se'n coneix la funcionalitat però sí que també és fortament reconegut per les cèl·lules T durant les primeres etapes de la infecció (Aagaard et al., 2004). Tampoc està present en el genoma de les diferents soques de *M. bovis* BCG. Aquestes característiques el converteixen en un bon candidat per el diagnòstic de la TB i ja s'utilitza en l'actualitat per aquest propòsit en persones (Diel et al., 2008). Malauradament, té restringit el seu ús veterinari degut a que el gen que codifica per TB7.7 es troba a la regió RD11 que no està present ni en *M. bovis,* ni en *M. caprae* (Brosch et al., 2002; Mostowy et al., 2002).

Rv3615c o EspC (*Esx-1 substrate protein C*) és una proteïna codificada fora de la regió RD1 però la seva secreció també depèn del sistema Esx-1 (Millington et al., 2011). Com que el sistema Esx-1 està interromput a BCG (degut a l'absència de la regió RD1), es creu que *M. bovis* BCG no pot secretar el Rv3615c i per això els individus vacunats amb la vacuna BCG no generen respostes cèl·lules T-específiques enfront a Rv3615c, mentre que els infectats responen amb una intensitat similar a la d'ESAT-6 i CFP-10 (Millington et al., 2011). Per aquest motiu també s'ha estudiat el seu ús en el immunodiagnòstic de TB en pacients humans (Millington et al., 2011) i en bovins (Sidders et al., 2008; Whelan et al., 2010; Casal et al., 2012).

c) ***Antígens involucrats en l'adaptació del micobacteri (Adapt)***. Són proteïnes de xoc tèrmic i xaperones moleculars que estan involucrades en l'adaptació del micobacteri a les condicions d'estrès i també són dianes de la resposta immunològica de l'hoste enfront a MTBC (Wilkinson et al., 2005).

Acr (*α-crystalin*), també anomenat HspX o proteïna de 16kDa, és un antigen amb epítops immunodominants tant per cèl·lules T com per cèl·lules B (Wilkinson et al., 1998) i s'associa a la fase de latència de la infecció tuberculosa ja que es requereix per a la persistència del micobacteri en l'ambient d'estrès (privació d'oxigen i exposició a l'òxid nítric) en que es troba dins del macròfag (Yuan et al., 1998). S'ha demostrat que pacients amb LTBI indueixen una resposta de cèl·lules T específica que no s'indueix en individus vacunats amb BCG (Geluk et al., June 2007). Aquestes característiques han convertit a Acr en un bon candidat com a reactiu diagnòstic (Lindestam Arlehamn et al., 2012) i per vacunes subunitat (Geluk et al., June 2007). Recentment s'ha incorporat en vacunes multiestadi (que incorporen antígens secretats en fase aguda i en latència) en combinació amb TB10.4 (Niu et al., 2011).

Per la seva banda, Acr2 (*α-crystalin* 2), també anomenat Hsp20, és una proteïna que conté un 30% d'homologia amb Acr en la seqüència aminoacídica, però, mentre aquesta última és predominant durant la latència, Acr2 genera una resposta immunitària específica de cèl·lules T en les etapes primerenques de la infecció

tuberculosa. Per aquesta característica també s'ha postulat com un bon candidat per vacunes subunitat (Wilkinson et al., 2005).

Recentment s'ha identificat la nova hipotètica proteïna Rv2660c que tindria un patró d'expressió preferent durant la privació de nutrients, característica de la fase latent de la infecció (Aagaard et al., 2011). Aquest antigen també s'ha incorporat amb èxit en vacunes multiestadi conjuntament amb Ag85B i ESAT-6 (Aagaard et al., 2011; Lin et al., 2012).

d) ***Antígens associats a la paret cel·lular (PC)***. Grup d'antígens format per proteïnes de superfície com MPB70, MPB83 i MPB64 (com s'anomenen en *M. bovis*) o MPT70, MPT83 i MPT64 (com s'anomenen en *M. tuberculosis*) que presenten immunodominància per cèl·lules B (Oettinger and Andersen, 1994; Lightbody et al., 1998).

Tant MPB70 com MPB83 són antígens serodominants de *M. bovis* que són reconeguts pel sistema immunològic en les primeres etapes de la infecció (Lyashchenko et al., 2004; Waters et al., 2006). Per aquest motiu, sobretot MPB83 és un antigen altament empleat en el desenvolupament de noves eines de diagnòstic serològic de la TB (Waters et al., 2006; Lyashchenko et al., 2007; Lyashchenko et al., 2008).

Taula 4. Característiques dels principals antígens proteics del complex *M. tuberculosis*.

Antigen	Num. Rv [a]	Grup	PM [b]	Dianes	Ús
Ag85A	Rv3804c	Ag85	37 kDa	Cèl·lules T i B	Vacunes
Ag85B	Rv1886c	Ag85	35 kDa	Cèl·lules T i B	Vacunes
ESAT-6	Rv3875	Esx	6 kDa	Cèl·lules T	Vacunes/Diagnòstic
CFP-10	Rv3874	Esx	10 kDa	Cèl·lules T	Diagnòstic
TB10.4	Rv0288	Esx	10 kDa	Cèl·lules T	Vacunes
TB9.8	Rv0287	Esx	10 kDa	Cèl·lules T	Vacunes
TB7.7 [c]	Rv2654c	-	6 kDa	Cèl·lules T	Diagnòstic
EspC	Rv3615c	Esx [d]	6 KDa	Cèl·lules T	Diagnòstic
Acr (HspX)	Rv2031c	Adapt	16 kDa	Cèl·lules T i B	Diagnòstic
Acr2 (Hsp20)	RV2051c	Adapt	18 kDa	Cèl·lules T	Vacunes
Rv2660c	Rv2660c	Adapt	6 kDa	Cèl·lules T	Vacunes
MPT70/MPB70	Rv2875	PC	19 kDa	Cèl·lules B	Diagnòstic
MPT83/MPB83	Rv2873	PC	22 kDa	Cèl·lules B	Diagnòstic
MPT64/MPB64	Rv1980c	PC	25 kDa	Cèl·lules B	Diagnòstic

[a] Num. Rv: Número que s'atribueix a totes les seqüències codificants de *M. tuberculosis* H37Rv (Cole et al., 1998).
[b] PM: Pes molecular.
[c] Absent a *M. bovis* i *M. caprae* (Brosch et al., 2002; Mostowy et al., 2002)
[d] Pertany a la família proteica Esp que està associada al sistema Esx.

3.4. Diagnòstic immunològic

El coneixement de quins components de la resposta immunològica durant la infecció tuberculosa poden ser utilitzats en cada una de les de la infecció, és crucial per poder diagnosticar la TB.

Els microorganismes del MTBC són patògens intracel·lulars que resideixen principalment dins dels macròfags. Per aquest motiu, des de fa temps està àmpliament reconegut el paper predominant de la CMI (especialment la resposta Th1) durant la infecció. Així doncs, tot i que recentment s'han identificat antígens que generen una resposta d'anticossos en les primeres etapes de la infecció tuberculosa (Lyashchenko et al., 2004; Waters et al., 2006), la principal via per detectar una infecció recent continua sent la mesura de la CMI.

Tant en persones com en animals, la intensitat de la CMI, particularment dels nivells d'IFN-γ contra alguns antígens específics de MTBC, es correlaciona amb la càrrega bacteriana, i per tant, amb la progressió de la infecció (Vordermeier et al., 2002; Andersen et al., 2007).

Durant la LTBI en persones el creixement de *M. tuberculosis* es manté en uns nivells extremadament baixos, dificultant la detecció d'antígens micobacterians per part del sistema immune. De la mateixa manera les respostes d'anticossos són normalment indetectables. Per contra els assajos d'alliberament d'IFN-γ (en anglès IGRAs) poden diagnosticar la LTBI en persones dues setmanes després i durant tot el curs de la infecció, mentre que un pacient amb LTBI pot o no ser positiu a la IDTB. Finalment, si es produeix una progressió cap a TB activa, la infecció podrà ser detectada per proves de detecció antigènica (com la PCR) o per proves de detecció d'anticossos contra *M. tuberculosis* (Andersen et al., 2007, Figura 9).

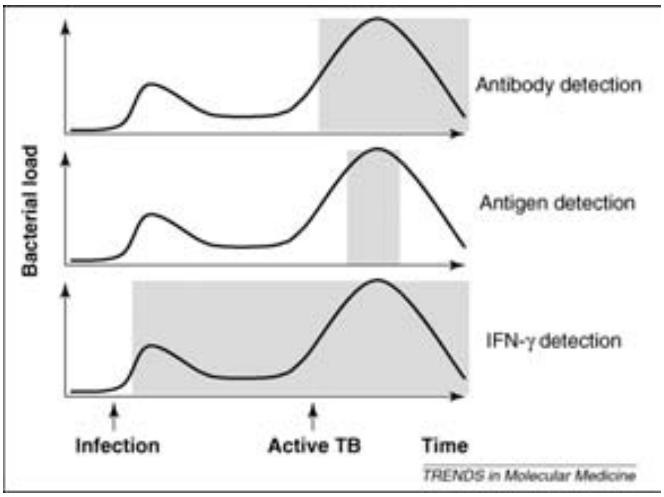

Figura 9. Representació esquemàtica de les diferents possibilitats de detecció de la TB humana en les fases latent i activa (Andersen et al., 2007).

La infecció per *M. bovis* en bovins no reprodueix la LTBI tal i com es dona en les persones infectades per *M. tuberculosis*, però sí que té en comú la predominança de la CMI en les primeres fases de la infecció.

En una primera etapa de "latència" (absència de lesions macroscòpiques i molt baixa càrrega bacteriana) es pot detectar la resposta d'IFN-γ (entre 2 i 4 setmanes post-infecció, Dean et al., 2005) i posteriorment la DTH per mitjà de la IDTB. En fases més avançades de la infecció, on la càrrega bacteriana és més significativa, es pot detectar també la resposta d'anticossos i, finalment, en fases molt cròniques, es pot produir un fenomen d'anèrgia durant el qual no és possible detectar la CMI contra els principals antígens de MTBC.

Aquests animals encara poden ser detectats per proves de detecció de la resposta humoral (de la Rua-Domenech et al., 2006, Figura 10).

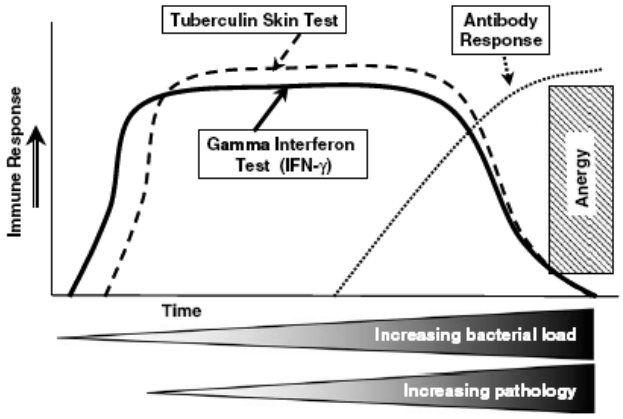

Figura 10. Representació esquemàtica de l'evolució de la infecció tuberculosa en bovins i la capacitat de les proves de diagnòstic immunològic per detectar la infecció (Vordermeier et al., 2004).

3.4.1. Intradermotuberculinització (IDTB).

La prova de la IDTB és el mètode estàndard per detectar la TB tant en persones com en bestiar boví. Aquesta prova es fonamenta en la reacció de DTH, mesurant la induració (en persones) o l'engruiximent del plec cutani (en animals), del punt on prèviament s'han inoculat tuberculines (PPDs) per via intradèrmica.

En persones la IDTB o *Prova de Mantoux* s'interpreta mesurant el diàmetre transversal de la induració. A l'estat espanyol s'utilitza el PPD-RT23 de *M. tuberculosis* (*Statens Serum Institut*, Dinamarca) i normalment es considera que un resultat és positiu quan la induració és major a 5 mm (Taula 5).

En bovins s'utilitza PPD de *M. bovis* o boví (PPD-B) i es mesura l'espessor del plec de pell al punt d'inoculació. A l'estat espanyol s'utilitza la soca *M. bovis* AN-5 per produir el PPD-B (*CZ Veterinaria*, Espanya) i els resultats s'interpreten tenint en compte tant l'engruiximent del plec cutani com el signes clínics associats a la inoculació de la tuberculina (edema difús, exsudat, necrosi dolor o reacció inflamatòria dels limfonodes regionals). La interpretació de la reacció es realitza de la següent manera (*Ministerio de Medio Ambiente, Medio Rual y Marino*, 2008; Taula 5): a) positiva, quan l'engruiximent sigui major o igual a 4 mm i/o hi hagi presència de signes clínics; b) dubtosa, si hi ha absència de signes clínics i un engruiximent major a 2 mm i menor a 4mm; c) negativa, engruiximent menor o igual a 2 mm i absència de signes clínics.

Els PPDs no són específics del MTBC, com a conseqüència, la IDTB pot donar lloc a resultats falsos positius per exposició de l'individu a altres micobacteris no tuberculosos o per efecte de la vacuna BCG. En determinades circumstàncies, s'aconsella realitzar la prova de la IDTB comparada, per evitar la possible interferència en el diagnòstic que pot provocar la sensibilització per micobacteris ambientals o la infecció per *M. avium* subsp. *paratuberculosis*, l'agent causant de la malaltia de *Johne* o paratuberculosi, que és

endèmica en el bestiar vaquí i els petits remugants en diversos països d'Europa (de Juan et al., 2006; Good et al., 2009).

Taula 5. Característiques de la Intradermotuberculinització (IDTB) en les persones i el bestiar vaquí.

	IDTB	
	Persones	Bovins
Tuberculina	PPD-RT23	PPD-B
Soca MTBC	Diverses *M. tuberculosis*	*M. bovis* AN-5
Dosi [a]	2 UT	> 20000 UI
Punt d'inoculació	Avantbraç	Coll
Temps de lectura	48-72h	72h
Interpretació [b]	> 5 mm (+), > 15 mm (++)	≥ 4 mm o sc (+), > 2 < 4 (d)

[a] UT, Unitats de Tuberculina; UI, Unitats Internacionals.
[b] +, positiu; ++, positiu fort; d, dubtós; sc, signes clínics.

La IDTB comparada consisteix en valorar la reacció produïda en resposta a la inoculació de la PPD-B, en relació a la reacció produïda per la inoculació de PPD de *M. avium* o aviar (PPD-A). A l'estat espanyol s'utilitza la PPD-A de la soca *M. avium* subsp. *avium* D4-ER (*CZ Veterinaria*, Galícia, Espanya). La interpretació de les IDTB comparada es defineix de la següent manera (*Ministerio de Medio Ambiente, Medio Rual y Marino*, 2008): a) positiva, quan la reacció a PPD-B sigui positiva i > 4 mm que la reacció a PPD-A i/o hi hagi presència de signes clínics; b) dubtosa, quan la reacció a PPD-B sigui positiva o dubtosa i entre 1 i 4 mm major que la reacció a PPD-A; c) negativa, quan la reacció a la PPD-B sigui negativa o ≤ a la reacció a PPD-A.

En els darrers anys s'està estudiant l'ús de reactius alternatius a la PPD, per ser utilitzats en la prova cutània de valoració de la DTH en bovins i poder diferenciar animals infectats i vacunats o sensibilitzats amb altres micobacteris no tuberculosos (Whelan et al., 2010; Casal et al., 2012).

3.4.2. Assajos d'alliberament d'IFN-γ (IGRAs).

El primer IGRA antigen- específic va ser desenvolupat el 1990 per Rothel i col·laboradors per diagnosticar la TB bovina (Rothel et al., 1990). Diverses proves experimentals i estudis de camp realitzats a Austràlia, van confirmar la seva idoneïtat com a prova diagnòstica *in vitro* i *ex vivo* complementària a la IDTB per incrementar la sensibilitat diagnòstica (Wood and Rothel, 1994). Aquest immunoassaig actualment es comercialitza amb el nom de Bovigam® (Prionics, Suïssa) i a la UE és una prova oficial complementària (Decisió de la Comissió de 8 de juliol de 2012 que corregeix l'anex B de la Directiva Europea 64/432). La prova IGRA actualment s'inclou en programes d'eradicació i control de la TB bovina a la UE, Estats Units, Nova Zelanda i Austràlia. També és reconeguda per la OIE com a prova alternativa per al comerç internacional (Manual d'animals terrestres de la OIE 2009, capítol 2.4.7).

Aquest primer IGRA es duu a terme en dues etapes (veure Figura 11): 1) estimulació de sang perifèrica amb PPD-B i PPD-A durant 16-24h a 37ºC, 2) detecció de l'IFN-γ alliberat al plasma per mitjà d'un assaig immunoenzimàtic (ELISA).

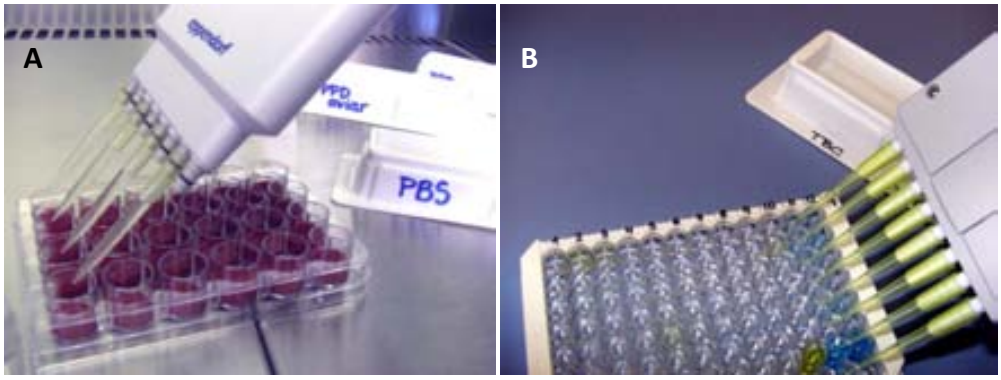

Figura 11. Realització de la prova de l'IFN-γ (Bovigam®). A, estimulació de sangs; B, ELISA de detecció d'IFN-γ alliberat en plasma sobrenedant.

El fonament del mètode es basa en la CMI front a la infecció per MTBC. Si l'animal està infectat, les APCs presentaran els antígens del PPD-B a les cèl·lules T efectores de sang perifèrica que proliferaran i produiran IFN-γ entre altres citocines. Els nivells d'IFN-γ produïts en la mostra estimulada amb PPD-B, es comparen amb els nivells produïts per la mostra del mateix animal estimulada amb PPD-A o no estimulada. L'aplicació del mètode i el punt de tall per determinar la positivitat varia en funció de la situació epidemiològica de cada país amb la finalitat d'incrementar la sensibilitat o l'especificitat diagnòstica.

Diversos estudis han comparat la sensibilitat i l'especificitat de la IDTB front a la prova de l'IFN-γ. La gran majoria conclouen que la prova de l'IFN-γ és més sensible que la IDTB però no sempre és tan específica com la IDTB comparada (Wood et al., 1992; Domingo et al., 1995; de la Rua-Domenech et al., 2006; EFSA Panel on Animal Health and Welfare (AHAW). 2012).

La identificació d'antígens alternatius al PPD ha permès desenvolupar IGRAs més específics. Fonamentalment ESAT-6 i CFP-10 són els antígens específics de MTBC més estudiats per aquest propòsit (Pai et al., 2004; Pai et al., 2006) i s'han incorporat en dos kits de diagnòstic comercial: el QuantiFERON-TB GOLD Test (QFT, Cellestis Limited, Carnegie, Victoria, Austràlia) i l'assaig T-SPOT.TB (Oxford Immunotec, Oxford, Regne Unit). Aquests dos assajos han estat dissenyats específicament per diagnosticar la LTBI humana. El QFT és una prova equivalent al Bovigam® (ELISA detecció d'IFN-γ) però estimulant la sang perifèrica amb ESAT-6, CFP-10 i TB7.7 al mateix temps. Per la seva banda, el T.SPOT.TB quantifica cèl·lules productores d'IFN-γ després d'estimular cèl·lules mononuclears de sang perifèrica amb ESAT-6 i CFP-10. L'ús d'aquests antígens atorga una elevada especificitat a les dues tècniques (Ferrara et al., 2006).

3.4.3. Diagnòstic immunològic en caprins

La tuberculosi caprina encara no apareix en el llistat de malalties notificables de la OIE i no està sotmesa a campanyes d'eradicació de caràcter obligatori. El programa d'eradicació de tuberculosi bovina a l'estat espanyol contempla l'anàlisi obligatori del bestiar cabrum

que conviu, comparteix pastures o està epidemiològicament relacionat amb el bestiar boví. No obstant, en algunes Comunitats Autònomes existeixen programes voluntaris de qualificació sanitària de ramats respecte a la TB.

La proximitat biològica entre bovins i caprins permet que el bestiar cabrum pugui ser sotmès a les mateixes proves de diagnòstic *antermortem* que s'apliquen al bestiar vaquí (IDTB i prova de l'IFN-γ) (Gutiérrez et al., 1998; Liebana et al., 1998; Bezos et al., 2012) (Figura 12). Alguns estudis rebel·len que els resultats de sensibilitat i especificitat en diferents situacions de camp són similars als dels bovins (Álvarez et al., 2008; Bezos et al., 2012). En cabres co-infectades per paratuberculosi i TB, s'ha demostrat que la resposta contra PPD-A pot emmascarar la detecció de la infecció tuberculosa, tant per mitjà de la IDTB com per l'IGRA (Bezos et al., 2010). Per tal de validar les proves diagnòstiques en el bestiar cabrum encara manquen dades addicionals d'estudis de camp realitzats amb un número més elevat d'animals, així com estudis específics d'optimització de les proves en l'espècie caprina (dosis de tuberculina, punts de tall, etc.).

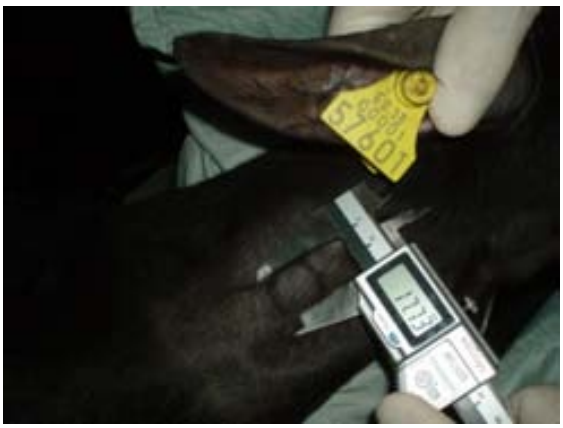

Figura 12. Detall del procés de lectura d'una reacció positiva a la IDTB en una cabra infectada amb *M. caprae.*

4. Prevenció i control de la tuberculosi: vacunes

Les mesures de prevenció de la TB, el diagnòstic precoç de la infecció i, pel que fa a les persones, el tractament, són els tres pilars fonamentals de l'estratègia de control i eradicació de la TB. Les vacunes dirigides tant a la profilaxi com a la immunoteràpia, juguen un paper central en aquesta estratègia.

4.1. Història de les vacunes contra la TB

La primera temptativa de vacuna contra la TB recau en el propi Robert Koch. Es tractava d'utilitzar el PPD del *M. tuberculosis* que ell mateix havia descrit com una vacuna amb finalitats terapèutiques. Koch va realitzar uns estudis preliminars en cobais amb resultats prometedors i el 1890 va realitzar un assaig clínic en 1700 pacients amb TB. En aquest estudi l'ús del PPD va resultar ser un fracàs com a teràpia antituberculosa (Koch. 1891) .

La primera vacuna amb finalitat preventiva no va veure la llum fins als anys 20 del segle passat. Va ser la BCG, desenvolupada a l'*Institute Pasteur* pel microbiòleg Albert Calmette i el veterinari Camile Guerin. Uns anys abans aquests científics havien aconseguit atenuar una soca virulenta de *M. bovis* després de múltiples subcultius seriats en medi de patata,

bilis i glicerina realitzats cada 3 setmanes durant més de 13 anys. La hipòtesi es basava en que per obtenir protecció era necessària la presència de bacils tuberculosos a l'organisme. La BCG va ser utilitzada per primera vegada en un nounat l'any 1921 i el 1927 es va realitzar el primer estudi d'eficàcia de la vacuna en més de vint mil recent nascuts (Calmette. 1927). Des d'aleshores la BCG ha estat (i encara és) l'única vacuna disponible contra la TB i més de 4000 milions de persones han estat immunitzades amb BCG des de la seva aparició (Kaufmann et al., 2010).

Després de vàries dècades sense temptatives fermes per a desenvolupar noves vacunes que milloressin l'eficàcia de la BCG, la recerca en aquest camp va patir un punt d'inflexió a partir de 1993. Aquest any l'OMS va declarar que la TB havia esdevingut una emergència global (World Health Organization. 1994) i uns anys més tard, a través de la pròpia OMS, es va donar un impuls molt important a la recerca contra la TB amb la creació de l'aliança internacional STOP-TB, que té per objectiu l'accés al diagnòstic i el tractament, així com potenciar la investigació de noves eines diagnòstiques, medicaments i vacunes (www.stoptb.org).

En la darrera dècada, s'ha produït un increment significatiu de la recerca sobre vacunes contra la TB humana i animal (Figura 13, Buddle et al., 2012). Aquest fet també s'ha vist traduït en avenços significatius en el desenvolupament de nous candidats vacunals contra la TB. En l'actualitat, 14 d'ells es troben en fase d'assajos clínics.

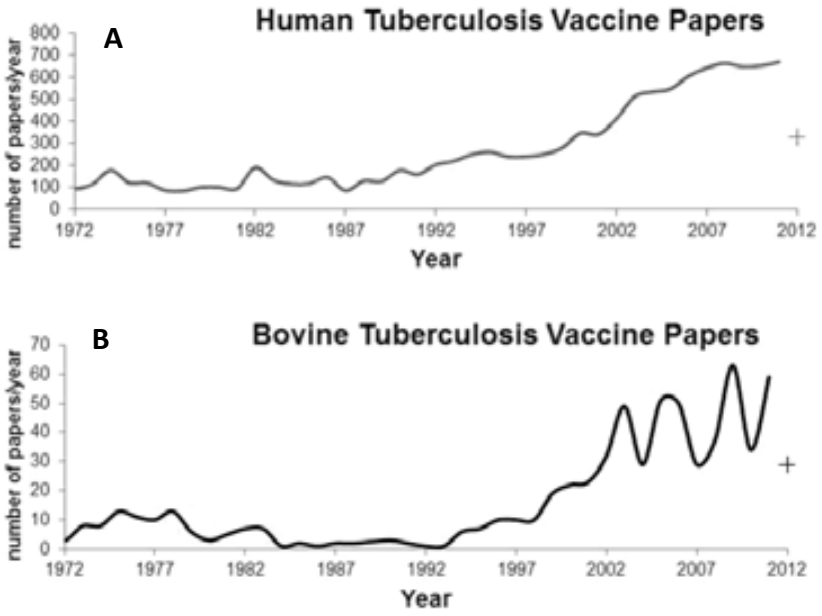

Figura 13. Evolució de les publicacions sobre vacunes contra la TB humana (A) i bovina (B) en revistes científiques indexades (Buddle et al., 2012).

4.2. La vacuna BCG

La soca atenuada de *M. bovis* BCG es va mantenir en subcultius seriats durant dècades produint-se diversos polimorfismes genètics que van anar donant lloc a una varietat de soques resultants (Behr and Small, 1999; Garcia Pelayo et al., May 2009) creant nous llinatges a partir de noves soques de la BCG (Taula 6). No obstant, actualment tan sols

està disponible la seqüència completa del genoma la soca original, també anomenada BCG *Pasteur* (Brosch et al., 2007).

Taula 6. Evolució dels llinatges derivats de la soca original *M. bovis* BCG Pasteur.

Any	Soca	Sinònim
1921	Pasteur	
1924	Moscou	Rússia
	Brasil	Moreau
	Tòquio	Japó
	Göteborg	Suècia
	Filadèlfia	Phipps
1931	Danesa 1331	Dinamarca
1934	Chicago	Tice
1937	Montreal	Frappier
1946	Birkhaug	

També s'han originat noves soques derivades d'aquets nous llinatges paral·lels, com és el cas de les soques Praga, Glaxo, Beijing i Xangai que deriven de la BCG Danesa 1331, o la soca Toronto (també anomenada Connaught) que deriva de la BCG Montreal (Behr and Small, 1999; Garcia Pelayo et al., May 2009).

Com s'ha esmentat en l'apartat anterior, la BCG és l'única vacuna disponible en l'actualitat contra la TB. La soca vacunal emprada és la BCG Danesa 1331, que s'utilitza en humans en una dosi de 5×10^5 Unitats Formadores de Colònia (UFC). Tot i que és eficaç en els casos més greus de TB, com la meningitis tuberculosa infantil, la seva eficàcia en protegir contra la TB pulmonar és molt variable (Fine. 1995). Les seves principals avantatges són: a) l'elevada eficàcia contra la TB extra pulmonar en nens, b) el baix cost (pot ser utilitzat en una dosi baixa) i c) la seva elevada seguretat. Mentre que les seves principals desavantatges són: a) la limitada eficàcia protectora contra la TB pulmonar, b) l'absència de protecció contra la LTBI, c) la manca d'efectes terapèutics i d) la positivitat d'alguns individus vacunats a la IDTB.

A nivell veterinari l'única espècie domèstica que ha estat sotmesa a estudis d'eficàcia de la BCG contra la infecció per *M. bovis* és el bestiar boví. Tal i com s'observa en l'ésser humà, la BCG és eficaç en protegir contra la TB vacunant vedells neonatals (Hope et al., 2005) i dosis entre 10^4-10^6 UFC confereixen un nivell similar de protecció (Buddle et al., 1995). Les soques BCG Pasteur 1173P2 (subcultiu número 1173, segon passi) i BCG Danesa 1331 confereixen similars nivells de protecció, però la BCG Pasteur produeix respostes d'IFN-γ front a PPD-B més elevades (Wedlock et al., 2007). La durada de la immunitat protectora es manté entre 12 i 24 mesos i després decau en estudis realitzats amb vedells neonatals vacunats amb la BCG Danesa 1331 (Thom et al., 2012).

Fins a la data no s'han realitzat estudis d'eficàcia de vacunes antituberculoses en el bestiar cabrum, tot i ser l'hoste principal de *M. caprae* (Aranaz et al., 1999) i probablement el reservori domèstic més important de TB bovina causada tant per *M.*

bovis com per *M. caprae* (Rodriguez-Campos et al., 2012; Napp et al., 2013; Zanardi et al., 2013).

Per contra darrerament s'han realitzat diversos estudis d'eficàcia de la BCG en les espècies de fauna silvestre que es consideren els principals reservoris silvestres de *M. bovis (*i/o *M. caprae*), com l'opòssum (Nova Zelanda), el teixó (Gran Bretanya i Irlanda), el porc senglar (Península Ibèrica) o el cérvol de cua blanca (Nord Amèrica). S'ha demostrat protecció en diferents graus de la vacunació oral amb la BCG en opòssums (Wedlock et al., 2005; Tompkins et al., 2009) i porcs senglars (Ballesteros et al., 2009; Garrido et al., 2011), de la vacunació oral, intranasal, subcutània i intramuscular en teixons (Lesellier et al., 2009; Corner et al., 2010; Chambers et al., 2011), i de la vacunació oral, parenteral i subcutània en cérvols de cua blanca (Palmer et al., 2007; Nol et al., 2008).

Tot i que la BCG és l'única vacuna actualment disponible contra la TB, els diferents estudis realitzats en persones i animals coincideixen que la seva eficàcia és molt variable (Fine. 1995; Waters et al., 2012a). El repte present de la recerca en vacunes antituberculoses és el desenvolupament de nous candidats i noves estratègies vacunals que reemplacin o que millorin l'eficàcia de BCG i allarguin la durada de la immunitat protectora que aquesta confereix. En el camp veterinari, encara queda pendent el desafiament addicional associat a l'ús de la BCG que és el desenvolupament d'eines de diagnòstic que permetin diferenciar animals infectats i vacunats (en anglès DIVA).

4.3. Noves estratègies vacunals

En l'actualitat es poden definir dues grans famílies de candidats a vacunes antituberculoses: les vacunes terapèutiques i les preventives. Les primeres estan concebudes per accelerar o complementar l'efecte de la quimioteràpia antituberculosa, mentre que el principal repte de les noves vacunes preventives contra la TB és el de millorar l'eficàcia i la durada de la immunitat protectora de la BCG.

Les dues principals aproximacions per desenvolupar noves vacunes profilàctiques contra la TB són el reemplaçament de la BCG i l'ús d'antígens immunodominants en una vacuna de reforç que és administrada després de la vacunació amb BCG. Així doncs, l'objectiu d'aquesta *segona generació* de vacunes profilàctiques és el d'establir un estat d'immunitat perllongat en el temps que permeti protegir individus contra una futura exposició a MTBC, o una reactivació de la LTBI. L'obtenció d'una vacuna esterilitzant "antiinfecciosa" no es considera una fita realista en l'actualitat, de manera que els esforços van més dirigits a generar una freqüència adequada de recirculació de cèl·lules T de memòria antigen-específiques que puguin entrar ràpidament en els llocs d'infecció, contenir la disseminació de la infecció i/o esterilitzar la lesió (Orme. 2005).

A llarg termini, es considera necessari el desenvolupament d'una *tercera generació* de vacunes profilàctiques concebudes per a prevenir l'establiment de la infecció. La principal estratègia alternativa que s'apunta per assolir aquest objectiu, es basa en la generació d'anticossos específics que evitin l'entrada de MTBC al teixit diana (teixit pulmonar). Aquesta generació de vacunes seran dissenyades per estimular una immunitat de mucoses potent, capaces d'induir la producció local d'immunoglobulines A (IgA) específiques contra

MTBC en l'espai alveolar (Kaufmann. 2006).

A hores d'ara hi ha 14 candidats a vacunes contra la TB en persones en fase d'assajos clínics que es distribueixen en 3 grans grups de vacunes: 4 són vacunes de reemplaçament de BCG, 8 són vacunes de reforç de BCG i 2 són vacunes terapèutiques. Els 3 grups de vacunes amb els seus respectius candidats es mostren a la Taula 7 (Kaufmann et al., 2010; Ottenhoff and Kaufmann, 2012; www.stoptb.org, juliol de 2012).

Taula 7. Noves vacunes contra la tuberculosi en assajos clínics.

Grup	Tipus de vacuna	Candidat	Descripció	Estat[a]
Reemplaçament de BCG	BCG recombinants (BCGr)	VMP 1002	BCGr que expressa listerolisina i amb delecció d'ureasa	Fase IIa
		rBCG30	BCGr que expressa Ag85B	Fase I
		AERAS-422	BCGr que expressa Ag85A, Ag85B, RV407 i perfringolisina	Fase I
	Micobacteris recombinants	MTBVAC	*M. tuberculosis* amb delecció de phoP i els gens fadD26	Fase I[b]
Reforç de BCG	Antígens expressats per vectors virals	MVA85A (AERAS-485)	*Vaccinia* Ankara modificat que expressa Ag85A	Fase IIb
		Crucell Ad35 (AERAS-402)	*Adenovirus* serotip 35 deficient en replicació que expressa Ag85A, Ag85B i TB10.4	Fase IIb
		AdAg85A	*Adenovirus* humà serotip 5 deficient en replicació que expressa Ag85A.	Fase I
	Proteïnes de fusió	M72	Fusió de Rv1196 i Rv0125; Adjunvant AS01 o AS02	Fase IIa
		H1-IC31	Fusió d'Ag85B i ESAT-6; Adjunvant IC31	Fase I
		H1-CAF01	Fusió d'Ag85B i ESAT-6; Adjunvant CAF01	Fase I
		H4-IC31 (AERAS-404)	Fusió d'Ag85B i TB10.4; Adjunvant IC31	Fase I
		H56-IC31	Fusió d'Ag85B, ESAT-6 i Rv2660c; Adjunvant IC31	Fase I
Terapèu-tiques	Micobacteris inactivats	*M. vaccae*	*M. vaccae* inactivat	Fase III
		RUTI	*M. tuberculosis* detoxificat en liposomes	Fase IIa

[a] A data juliol de 2012; [b] ha entrat en fase d'assajos clínics el 2013 (Martin et al., 2006).

4.3.1. Estratègia *prime-boost* heteròleg

Conceptualment hi ha 2 vies per incrementar l'eficàcia o allargar la durada de la immunitat de la BCG:

a) Amplificar o reforçar la immunitat induïda per la BCG en el moment de la vacunació (veure Figura 14, *amplify/boost*). Els candidats a vacunes antituberculoses basades en

BCG recombinant serien un exemple d'aquesta estratègia.

b) Induir una primera immunitat amb la BCG i reforçar-la posteriorment amb l'administració d'antígens immunodominants (veure Figura 14, *boost* 1). Aquest règim de vacunació es denomina estratègia d'inducció i reforç (o *prime-boost*) heteròleg. Entre les diferents aproximacions de desenvolupament de noves vacunes antituberculoses que es mostren a la Taula 7, aquesta és la que aglutina més candidats (8 en total).

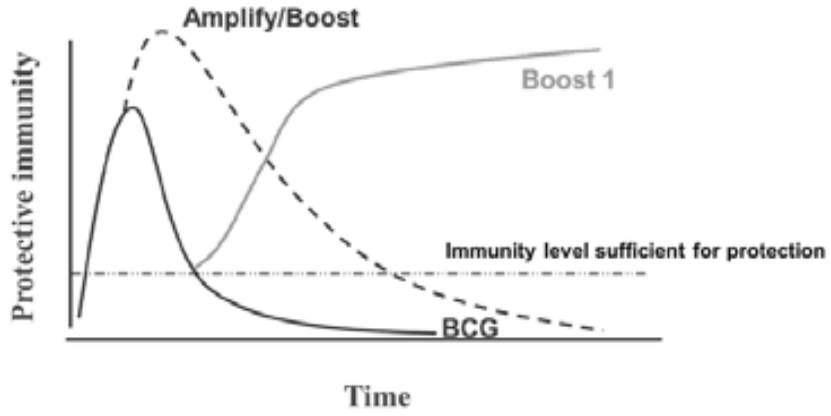

Figura 14. Esquema conceptual de les 2 estratègies per incrementar i allargar la immunitat induïda per la BCG (gris fosc): Estratègia d'amplificació o reforç de la BCG en una sola dosi (gris fosc discontinu) i estratègia de reforç heteròleg en una segona dosi (gris clar). El nivell d'immunitat necessari per conferir protecció es mostra en gris clar discontinu.

Com es pot veure a la Figura 14, a banda de reforçar la immunitat protectora front a la infecció tuberculosa, l'estratègia *primer-boost* heteròleg té l'avantatge d'allargar la durada d'aquesta immunitat protectora, en comparació amb l'estratègia d'amplificació de l'efecte de la BCG en una sola formulació.

L'estratègia *prime-boost* heteròleg utilitzada en la que s'utilitzen els actuals candidats vacunals en fase d'assajos clínics es basa sempre en fer el *prime* amb la BCG, per tant, els antígens requerits per a fer el *boost* han d'estar presents a les diferents soques de BCG i han d'estar altament conservats dins del MTBC. Els mecanismes pels quals aquesta estratègia indueix majors respostes de cèl·lules T antigen-específiques que la BCG en solitari no estan del tot elucidats però s'especula que un dels més importants és la immunodominància per cèl·lules T d'alguns epítops d'aquests antígens. Així doncs, en una primera exposició a l'antigen (*priming*) s'induiran les respostes de cèl·lules T contra els epítops més immunonodominants, i en una segona immunització (*boosting*), que s'efectuarà només amb alguns antígens seleccionats respecte a la primera immunització, s'expandiran preferentment les cèl·lules T de memòria induïdes en el *priming* i es focalitzarà la CMI en aquest tipus de resposta (McShane and Hill, 2005).

Tal i com es mostrava a la Taula 7, dins de les vacunes que segueixen l'estratègia *prime-boost* es distingeixen dos subgrups en funció del mecanisme "d'entrega" dels antígens: les vacunes subunitat basades en proteïnes i les vacunes subunitat basades en vectors virals. Les primeres són proteïnes de fusió que combinen dos o més antígens immunodominants

i són administrades conjuntament amb adjuvants sintètics inductors de la resposta de cèl·lules Th1 (Dietrich et al., 2005; Dietrich et al., 2006; Von Eschen et al., 2009; Aagaard et al., 2011). Per la seva banda, les vacunes basades en vectors virals utilitzen virus recombinants que mantenen la capacitat infectiva i d'expressar proteïnes però que han perdut la capacitat replicativa, com el poxvirus vaccina Ankara modificat (en anglès MVA) (McShane et al., 2005) o els adenovirus serotip 5 i 35 no replicatius (Xing and Charters, 2007; Radošević et al., 2007).

L'ús de virus recombinants com a vectors virals té l'avantatge de ser una tecnologia que permet clonar múltiples antígens immunodominants de diferents mides, obtenint títols molt elevats i, per tant, facilitant producció de vacunes. Emprant aquesta tecnologia, la MVA85A (MVA que expressa Ag85A) va ser la primera vacuna contra la TB després de la BCG que entrava en assajos clínics. En les primeres fases el règim *prime-boost* BCG-MVA85A va demostrar una bona eficàcia i immunogenicitat en diferents models animals (Williams et al., 2005; Verreck et al., 2009; Vordermeier et al., 2009). No obstant, recentment s'han publicat els resultats de la darrera fase d'assajos clínics. Aquests no demostren que la MVA85A confereixi una millora estadísticament significativa de la protecció respecte la BCG en 2794 nens sud-africans (Tameris et al., 2013).

Per la seva banda, l'ús d'adenovirus com a vacunes contra la TB presenta punts forts, com la seva potent immunogenicitat i el seu tropisme natural per l'epiteli respiratori (Wang et al., 2004; Santosuosso et al., 2006), mentre que el principal desavantatge, sobretot pel que fa a l'adenovirus humà serotip 5 (AdHu5), és la freqüència de la presència d'anticossos neutralitzants contra el vector viral en persones (Lasaro and Ertl, 2009). Evidentment aquesta limitació que suposa la immunitat basal contra adenovirus humans, desapareix quan es concep la vacuna per a l'ús veterinari. En aquest sentit, el candidat vacunal AdAg85A basat en un AdHu5 no replicatiu que expressa Ag85A, administrat com a reforç de la BCG, incrementa l'efecte protector d'aquesta en vedells posteriorment desafiats amb *M. bovis* (Vordermeier et al., 2009).

4.4. Diagnòstic diferencial i estratègia DIVA

La IDTB era l'única tècnica disponible per diagnosticar la TB en persones i animals durant la major part del segle passat. La seva principal limitació és que una proporció dels individus sensibilitzats amb micobacteris no tuberculosos o vacunats amb BCG, resulten falsos positius a la IDTB. Els actuals candidats a vacunes antituberculoses, tant els que utilitzen la BCG per fer el *priming*, com els que utilitzen micobacteris recombinants vius amb l'objectiu reemplaçar BCG, presenten aquest mateix inconvenient en relació al diagnòstic de la TB.

Per aquest motiu, en paral·lel al desenvolupament de vacunes, s'estan desenvolupant nous reactius de diagnòstic capaços de diferenciar els individus infectats del vacunats. Aquest concepte inicialment es va definir en el camp veterinari amb les sigles DIVA (*differentiation of infected from vaccinated animals*). Actualment, el terme DIVA també s'utilitza per referir-se més genèricament a la diferenciació d'individus vacunats i infectats. Es tracta d'identificar marcadors específics d'infecció i/o de vacunació que puguin ser

detectats per proves de diagnòstic (Figura 15)

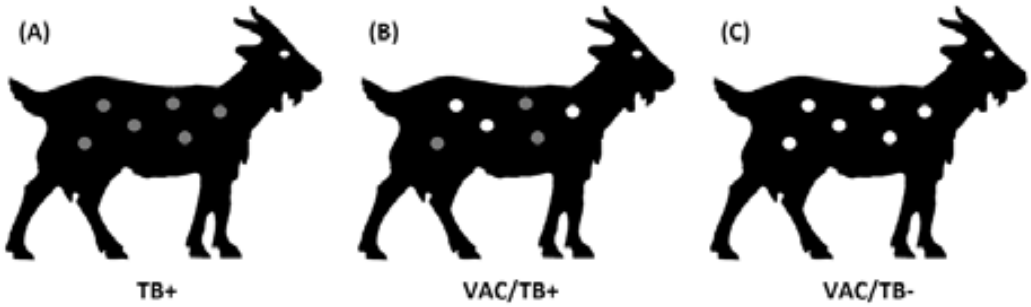

Figura 15. Representació esquemàtica dels diferents resultats d'una prova DIVA amb doble marcatge (infecció i vacunació). (A), animal infectat i no vacunat; (B), animal vacunat i infectat; (C), animal vacunat i no infectat. TB+, infectat; TB-, no infectat; VAC, vacunat.

Com s'ha descrit amb anterioritat, els IGRAs utilitzats per tal de diagnosticar la LTBI en persones, es basen en antígens absents al genoma de *M. bovis* BCG i d'altres micobateris no inclosos al MTBC. Aquestes proves han estat avaluades en diferents situacions clíniques i s'ha pogut demostrar la seva eficàcia en distingir individus infectats per TB d'individus vacunats amb la BCG (Diel et al., 2006; Pai et al., 2008). En contrast, els IGRAs actualment utilitzats per al diagnòstic de la TB en els remugants domèstics, encara continuen basant-ses en els PPDs. No obstant, seguint el camí dels IGRAs que ja s'utilitzen en persones, els antígens ESAT-6 i CFP-10, ja sigui formulats com una proteïna de fusió o com un còctel peptídic, han estat incorporats en els IGRAs i han estat àmpliament testats en proves de camp en bovins (Vordermeier et al., 2001; Waters et al., 2004; Cockle et al., 2006).

El còctel peptídic o proteic format per ESAT-6 i CFP-10, així com l'antigen Rv3615, també han estat testats en una prova intradèrmica per mesurar la DTH en el bestiar vaquí (Whelan et al., 2010; Casal et al., 2012) i recentment s'ha aconseguit millorar la sensibilitat afegint també l'antigen Rv3020c (Jones et al., 2012). De la mateixa manera, també s'ha desenvolupat una nova prova intradèrmica per diagnosticar la LTBI en persones, basada en ESAT-6 i CFP-10 que ja ha estat avaluada satisfactòriament en cobais (Weldingh and Andersen, 2008).

Tot i que la BCG no és pròpiament una vacuna marcadora, els IGRAs i les proves intradèrmiques basats en reactius DIVA han aconseguit incrementar significativament l'especificitat diagnòstica de la TB, permetent distingir els individus vacunats i no infectats dels infectats (vacunats o no, Figura 16). Tanmateix és cert que pel que fa a la millora de la sensibilitat, els resultats no són consistents en totes les poblacions i diferents situacions epidemiològiques estudiades quan es comparen amb les proves basades en els PPDs (Pai et al., 2008).

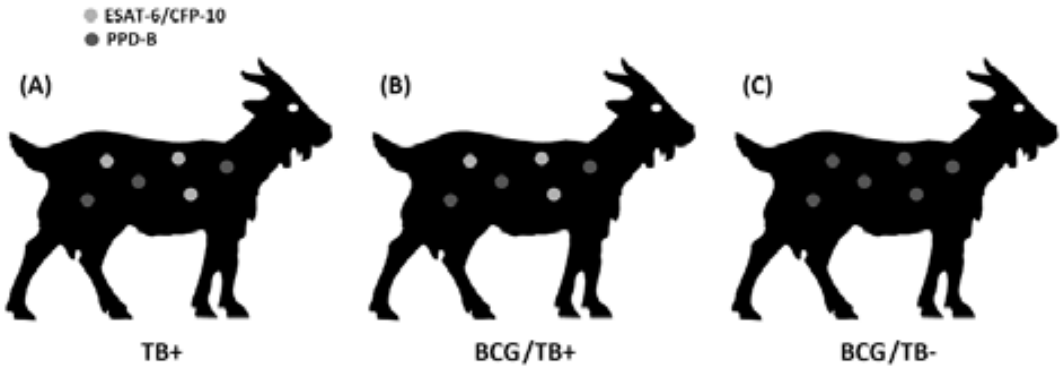

Figura 16. Representació esquemàtica dels diferents resultats diagnòstics en un estudi de vacunació i desafiament utilitzant reactius DIVA (còctel ESAT-6/CFP-10) i tuberculina bovina (PPD-B). (A), animal infectat i no vacunat; (B), animal vacunat amb la BCG i no protegit; (C), animal vacunat amb la BCG i protegit o no infectat. TB+, infectat; TB-, no infectat.

Els reactius DIVA basats en antígens expressats per gens codificats en la regió RD1 absent al genoma de la BCG (com ESAT-6 i CFP-10), o basats en antígens que són secretats per tots els MTBC però no per la BCG (com Rv3615c), poden ser aplicats en el diagnòstic de la TB conjuntament a la vacunació amb la BCG o a estratègies vacunals, basades en la BCG. Per aquest motiu, paral·lelament al desenvolupament de noves vacunes i nous tractaments contra la TB, una tercera línia de recerca prioritzada en l'actualitat, es basa en l'optimització d'aquests reactius de diagnòstic diferencial i la identificació de nous antígens que puguin complementar-los en pro d'incrementar la sensibilitat diagnòstica (Aagaard et al., 2006; Mustafa et al., 2008).

4.5. Biomarcadors

Els biomarcadors o marcadors biològics són indicadors d'un estat biològic derivat d'un procés fisiològic, d'un procés patogènic o d'una resposta farmacològica a un determinat tractament. A més a més, han de poder ser objectivament mesurables i avaluables. Pel que fa als processos patogènics, i més concretament de la infecció tuberculosa, es poden definir marcadors associats al patogen (especificitats de la soca, temps de cultiu negatiu post-infecció) o associats a l'hoste (resposta immunitària, clínica). En relació a la resposta immunitària durant el procés d'infecció tuberculosa s'han identificat biomarcadors immunològics específics d'infecció, de protecció i d'estadi clínic (LTBI o TB activa).

Els biomarcadors immunològics tenen un especial interès en estudis clínics perquè poden facilitar/accelerar el desenvolupament de vacunes. En aquest sentit podem distingir 3 tipus de biomarcadors (Vordermeier et al., 2012): a) predictors de protecció, que indiquen si una vacuna és efectiva després de la vacunació però abans de la infecció (no requereixen desafiaments experimentals); b) correlats de protecció, que indiquen si una vacuna és efectiva després de la vacunació i l'exposició a MTBC (requereix desafiaments experimentals); c) correlats d'infecció, que s'associen a l'augment de la patologia o la càrrega bacteriana durant la progressió de la infecció (després del desafiament).

Els principals marcadors immunològics són les citocines, les quimioquines i els anticossos.

4.5.1. Interferó-γ (IFN-γ)

L'IFN-γ és la citocina més àmpliament estudiada en relació a la infecció tuberculosa, i es considera un marcador d'immunogenicitat. S'ha descrit que l'IFN-γ és necessari per la defensa de l'hoste contra infeccions micobacterianes (Flynn et al., 1993), encara que és insuficient per si sol, per generar una resposta protectora contra la TB (Leal et al., 2001; Flynn. 2004). Així, paradoxalment, l'IFN-γ pot ser tant un marcador de predicció de protecció, com un marcador de correlació amb infecció activa. En aquest darrer cas, uns nivells elevats constants d'IFN-γ poden ser indicatius d'una immunitat ineficient (Andersen et al., 2007).

Per poder fer un ús discriminant de l'IFN-γ com a biomarcador de protecció o d'infecció és necessari estudiar la resposta antigen-específica mesurada després de l'estimulació *in vitro* de les cèl·lules que el produeixen. Així doncs, per exemple, l'IFN-γ ESAT-6-específic és un correlat d'infecció associat a la progressió de la infecció tuberculosa en persones i animals (Vordermeier et al., 2002; Lyashchenko et al., 2004; Andersen et al., 2007; Domingo et al., 2009). Per altra banda, l'IFN-γ Ag85B-específic pot ser tant un correlat d'infecció com un predictor de protecció, al veure's incrementat en persones infectades per *M. tuberculosis* o vacunades amb la BCG respectivament (Geluk et al., June 2007). Els mateixos resultats s'han observat pel que fa a l'IFN-γ TB10.4-específic en ratolins infectats per *M. tubeculosis* i vacunats amb la BCG (Dietrich et al., 2005). Les cèl·lules T de memòria productores d'IFN-γ Ag85A-específic s'han identificat, també, com un predictor de protecció però no un correlat d'infecció en bovins vacunats seguint un protocol *prime-boost* amb BCG-AdAg85A (Vordermeier et al., 2009).

Més enllà de la monitorització d'assajos clínics de vacunes, l'IFN-γ també és un bon biomarcador per estudiar la LTBI i la possible reactivació de la infecció en persones infectades latentment. Les respostes d'IFN-γ específiques per antígens relacionats amb la latència, s'han vist incrementades en pacients amb LTBI que eren reactors a la IDTB (Leyten et al., 2006). Per exemple, s'ha observat que les respostes d'IFN-γ Acr-específic no estan correlacionades amb la vacunació amb la BCG però sí amb la LTBI (Geluk et al., June 2007).

4.5.2. Altres biomarcadors immunològics

El balanç entre les respostes efectores i reguladores després de la infecció per MTBC és clau per la contenció o la disseminació de la infecció (o el pas de la LTBI a la TB activa). Així doncs, tot i que existeixen moltes evidències que la protecció contra la TB s'associa a la resposta efectora proinflamatòria, un sol marcador per si sol, no serà suficient per predir i/o monitoritzar l'eficàcia d'una vacuna en un estudi clínic (Vordermeier et al., 2012).

Diversos estudis de vacunació i posterior desafiament amb *M. bovis* en vedells, han permès estudiar l'expressió gènica de determinades citocines i quimioquines amb posterioritat a la vacunació i el desafiament. D'aquesta manera s'han obtingut biosignatures que podrien permetre predir l'eficàcia de la vacunació (Vordermeier et al., 2012). S'ha identificat que les respostes d'IL-17A i l'expressió gènica d'IL-22 són predictors de protecció en bovins vacunats (Aranday-Cortes et al., 2012b; Bhuju et al., 2012; Rizzi et

al., 2012; Vordermeier et al., 2012) i correlats d'infecció en bovins desafiats amb *M. bovis* (Blanco et al., 2011; Aranday-Cortes et al., 2012a). De la mateixa manera, la resposta immunitària participada per les subpoblacions de cèl·lules Th17 i Th22 també es veu induïda per controlar la infecció per *M. tuberculosis* en persones (Scriba et al., 2008).

Recentment també s'ha avaluat per a aquest propòsit la quimioquina CXCL10 o IP-10 (Proteina inductora d'IFN-γ 10) com a biomarcador de la TB. La IP-10 es detecta a l'interior del granuloma i actua atraient cèl·lules T al focus inflamatori i promovent la resposta de tipus Th1. Les respostes associades a IP-10 estan relacionades amb la progressió de la infecció tuberculosa en humans (Ruhwald et al., 2011) i en bovins (Waters et al., 2012b), mostrant gran potencial com a marcador de diagnòstic de la TB.

Finalment, tot i que no hi ha consens sobre el paper dels anticossos durant la infecció, sí que s'ha estudiat àmpliament la seva aplicació en el diagnòstic de la TB. S'ha demostrat que la IDTB en bovins vacunats o infectats, indueix a un increment dels títols d'anticossos front al PPD-B i a altres antígens específics de MTBC, principalment MPB83 (Lyashchenko et al., 2004; Palmer et al., 2006). A més a més, en bovins vacunats amb la BCG i desafiats amb *M. bovis*, el títol d'anticossos contra MPB83, mesurat immediatament abans del sacrifici dels animals i dues setmanes després de la realització de la IDTB, es correlacionava positivament amb la patologia i la càrrega bacteriana, suggerint que també poden ser utilitzats com a correlat d'infecció en estudis de vacunes (Lyashchenko et al., 2004).

4.6. Models animals per al desenvolupament de vacunes

Les característiques genètiques i fisiològiques de l'hoste, determinen la seva susceptibilitat i resposta immunitària enfront a la infecció tuberculosa. Els petits mamífers, fonamentalment el ratolí, el conill i el cobai, han estat els més extensament emprats en l'estudi de la infecció, el seu tractament i la seva profilaxi:

a) **Ratolí**. El model murí ha estat molt utilitzat per a l'estudi de l'eficàcia de candidats vacunals contra la TB. És un animal econòmic, de maneig fàcil i amb un ampli rang de reactius específics disponibles. A més a més, els ratolins es poden infectar eficientment per aerosols (la ruta natural de transmissió de la TB). El model murí pot reproduir la LTBI controlant inicialment la infecció amb una resposta immunitària de tipus Th1 (Orme. 2005). No obstant, la immunopatologia difereix sensiblement d'altres models animals i les persones. Els granulomes presenten poca necrosi intragranulomatosa i una reacció fibrosa molt discreta que retarda la encapsulació dels mateixos (Gil et al., 2006). Les cèl·lules T secretores de citocines proinflamatòries estan més uniformement distribuïdes a través de les lesions granulomatoses i, en conseqüència, els macròfags infectats tenen més accés als gradients de citocines que els activen i no degeneren, produint la necrosi característica del granuloma tuberculós en altres espècies (Orme. 1998).

b) **Cobai**. Comparteix amb el model murí la facilitat de reproduir la infecció per aerosolització dels micobacteris però, a diferència d'aquest, les lesions granulomatoses en el cobai s'organitzen de manera similar a la dels grans mamífers.

Els macròfags i els limfòcits ocupen concèntricament les regions externes de l'estructura del granuloma i la regió interna presenta major necrosi i degeneració cel·lular (Orme. 1998). Aquesta resposta inflamatòria, similar a la de les persones, converteix el cobai en un model útil per avaluar la reducció del dany tissular en teràpies antituberculoses i candidats vacunals. Per contra, una de les limitacions d'aquest model és que les pròpies característiques de la lesió, fan que els cobais presentin una gran susceptibilitat a la infecció i un temps relativament baix de supervivència (Orme. 2005).

c) **Conill**. Els primers estudis sobre la patogènesi de la tuberculosi es van realitzar en aquest model. Presenta una resposta similar a la del cobai però la necrosi progressa més ràpidament cap a la liqüefacció i formació de lesions cavitàries, comparable a la de etapes avançades de la infecció en humans (Orme. 1998). No obstant, tot i la presència de cavitats, els conills tenen capacitat per controlar la infecció i inclús poden evolucionar cap a la curació (Dannenberg. 2001).

La hipòtesi dels volums postula que com més gran és l'hoste (i major volum té el seu pulmó), menor és la tolerància a la infecció i major és la resposta inflamatòria contra el micobacteri (Cardona. 2006). Un hoste amb capacitat per destruir un major volum de parènquima, podrà aturar més eficientment el creixement bacterià (Figura 17).

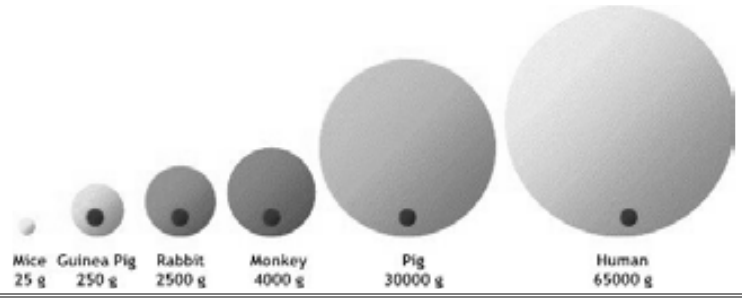

Figura 17. Comparació de diferents hostes segons el seu pes. El volum d'una lesió cavitària tuberculosa es mostra en una tonalitat més fosca (Cardona. 2006).

A la Figura 17 s'il·lustra que el ratolí no pot mantenir la seva supervivència, induïnt una resposta inflamatòria que el condueixi a la formació d'una cavitat equivalent al volum del pulmó. En el cas del cobai, que sí que pot induir la formació de cavitats, aquestes són tan grans respecte el volum del pulmó que comprometen la seva supervivència. Per aquest motiu, els models de TB en primats no humans i grans mamífers, resulten importants per valorar la seguretat i l'eficàcia de candidats vacunals contra la tuberculosi en humans:

d) **Primats no humans**. Els macacos s'han utilitzat per estudiar la LTBI i la TB activa, així com l'evolució de l'una a l'altra. Les espècies més utilitzades són el *Cynomolgus* (*Macaca fascicularis*) (Lin et al., 2009) i el *Rhesus* (*M. mulatta*) (Sharpe et al., 2010b). La proximitat filogenètica amb l'ésser humà i la capacitat d'extrapolar-ne resultats és el punt fort d'aquest model. Entre els desavantatges hi trobem el seu cost elevat, per aquest motiu, s'utilitza en candidats vacunals que ja han superat estudis en altres models. No obstant, els macacos presenten limitacions similars als petits mamífers esmentats anteriorment. La principal és que, a diferència de mamífers de major mida

(com porcins, remugants o humans), els pulmons no estan dotats d'estructures interlobulars i intralobulars de teixit connectiu que podrien tenir un paper en la contenció de la disseminació de les lesions pulmonars (Gil et al., 2010).

e) **Porcins**. Els porcs infectats per MTBC desenvolupen lesions molt similars a les dels humans (Bolin et al., 1997). Un estudi recent d'infecció experimental de *minipgs* amb *M. tuberculosis,* demostra que aquests reprodueixen la LTBI humana i la capacitat de controlar la infecció per mitjà d'una potent resposta immunològica Th1, conjuntament amb una potent resposta fibròtica local que permet l'encapsulació dels granulomes, limitant la constant inducció de noves lesions (Gil et al., 2010).

f) **Bovins**. Els bovins són hostes naturals de la TB. Per aquest motiu presenten diverses avantatges, com una elevada capacitat de transmissió horitzontal i la possibilitat d'estudiar l'eficàcia de vacunes sobre el mateix tàndem hoste-patogen en que es dóna a la infecció natural. La patogènesi i la resposta immunològica és molt similar a la dels humans (Hewinson et al., 2003) i la malaltia (aparició de símptomes clínics) pot trigar anys a manifestar-se tal i com succeeix a les persones. No obstant, tot i que els animals infectats desenvolupen lesions macroscòpiques en la majoria de casos, no hi ha una cavitació evident de les lesions tuberculoses com sí succeeix en la majoria de casos de pacients humans amb TB activa (Buddle et al., 2005b). El model boví també té en comú amb l'ésser humà el fet que la vacunació amb la BCG és més eficaç en vedells neonatals que en vaques. Per tots aquests motius el boví ha s'ha proposat com un model adequat per a un segon cribratge de candidats vacunals (Buddle et al., 2005b).

4.6.1. El model caprí

Les principals desavantatges de l'ús de bovins en infeccions experimentals, són el seu elevat cost de manteniment i les seves grans dimensions que dificulten el maneig i restringeixen sensiblement les característiques d'instal·lacions adequades per dur a terme els protocols experimentals. En canvi els caprins, que també són hostes naturals de la TB, presenten algunes avantatges respecte els bovins: a) maneig més fàcil, b) manteniment més econòmic, c) no precisen d'instal·lacions estabulades excessivament grans i d) produeixen lesions cavitàries similars als casos de TB activa en humans (Sanchez et al., 2011). Aquests són elements favorables, que converteixen el bestiar cabrum en una espècie candidata per a ser potencialment utilitzada com a model per al desenvolupament de vacunes antituberculoses per persones i/o per animals (Domingo et al., 2009).

OBJECTIUS

La present tesi doctoral és una aproximació multidisciplinària (clínica, immunològica, patològica i bacteriològica) enfocada a la recerca de noves vacunes antituberculoses i de noves eines diagnòstiques que s'hi adeqüin, utilitzant el model experimental de TB en la cabra domèstica. La tesi està orientada a la recerca de solucions integrals des de la perspectiva de salut pública i de sanitat animal. Per assolir aquest propòsit es van establir cinc objectius específics:

1. Desenvolupar un model d'infecció experimental per *M. caprae* en caprins dirigit a avaluar l'eficàcia de vacunes mitjançant la caracterització quantitativa i qualitativa dels principals trets immunològics, patològics i bacteriològics (*Estudi I*).

2. Avaluar l'eficàcia de la vacuna BCG i de dues vacunes de reforç heteròleg de la BCG en caprins posteriorment desafiats amb *M. caprae* (*Estudi II i III*).

3. Avaluar la interferència diagnòstica i la protecció creuada en caprins vacunats contra la paratuberculosi abans i després de ser infectats amb *M. caprae* (*Estudi IV*).

4. Estudiar comparativament l'ús dels reactius de diagnòstic actualment disponibles i de nous reactius DIVA, per diferenciar caprins infectats per tuberculosi de caprins vacunats contra la tuberculosi o la paratuberculosi (*Estudi I-IV*).

5. Identificar marcadors immunològics associats amb la progressió de la infecció i l'eficàcia de les vacunes en el model caprí de TB (*Estudis I-IV*).

STUDIES

STUDY I

Experimental model of tuberculosis in the domèstic goat after endobronchial infection with *Mycobacterium caprae*

Abstract

Caprine tuberculosis has increased in recent years, highlighting the need to address the problem this infection poses in goats. Moreover, goats may represent a cheaper alternative for testing of prototype vaccines in large ruminants and humans. With this aim, a *Mycobacterium caprae* infection model has been developed in goats. Eleven 6-month old-goats were infected by the endobronchial route with 1.5 x 10^3 cfu, and two other goats were kept as non-infected controls. Animals were monitored for clinical and immunological parameters throughout the experiment. After 14 weeks, goats were euthanized and detailed post-mortem analysis of lungs lesions performed by Multi-Detector Computed Tomography (MDCT) and direct observation. Respiratory lymph nodes were also evaluated and cultured for bacteriological analysis.

All infected animals were positive to single intradermal comparative cervical tuberculin (SICCT) test at 12 weeks post-infection (wpi). IFN-γ antigen-specific responses were detected from 4 wpi until the end of the experiment. Humoral response to MPB83 was especially strong at 14 wpi (13 days after SICCT-boost). All infected animals presented severe TB lesions in lung and associated lymph nodes. *M. caprae* was recovered from pulmonary lymph nodes in all inoculated goats. MDCT allowed a precise quantitative measure of TB lesions. Lesions in goats induced by *M. caprae* appeared to be more severe than those induced in cattle by *M. bovis* over a similar period of time. The present work poses a reliable new experimental animal model for a better understanding of caprine tuberculosis and future development of vaccine trials in this and other species.

1. Introduction

Tuberculosis (TB) in the domestic goat (*Capra hircus*), mainly caused by *Mycobacterium caprae* (Aranaz et al., 2003), is an endemic disease in the Iberian Peninsula. *M. caprae* is widespread in goat herds and is an emerging infectious agent in cattle (Duarte et al., 2008; Rodriguez et al., 2011). Infected goat herds can constitute a reservoir of TB-inducing mycobacteria in the field, posing a risk of infection to cattle and wildlife (Erler et al., 2004; Rodriguez et al., 2011). Furthermore, caprine TB not only may hamper the eradication campaigns of bovine TB in affected areas, but may be also responsible for cases of TB in humans (Kubica et al., 2003; Prodinger et al., 2005; Cvetnic et al., 2007; Rodríguez et al., 2009)(Prodinger et al., 2002; Kubica et al., 2003; Cvetnic et al., 2007; Cvetnic et al., 2007).

In the last decade, interest on vaccines against bovine TB has been renewed, as a tool for controlling infection in cattle and in wildlife (Buddle et al., 2006) in areas where eradication by the test and slaughter scheme alone is not considered feasible. Moreover, ruminant and porcine models of TB may be useful for screening prototype vaccines for humans, due to their similar lesional pattern and immunological responses to mycobacteria (Buddle et al., 2005b; Domingo et al., 2009; Gil et al., 2010). Standardization of the goat as a model of TB would improve our understanding of TB in this species, which in turn could help develop new strategies to combat this disease in goat flocks. Similarly, it could be used as an animal model for TB vaccine development in humans.

Caprine and bovine TB are closely related in regards to the immune response and pathological characteristics. In natural infections, such as in cattle, TB in goats is primarily a lower respiratory tract disease, with lesions in the lungs and associated lymph nodes. Occasionally tuberculous lesions may also be found in the upper respiratory tract lymph nodes and other organs like spleen, liver or mesenteric lymph nodes (Daniel et al., 2009; Quintas et al., 2010). Histologically, lesions are similar to those observed in cattle and humans. Typical tuberculous granulomatous necrotizing lesions are observed, characterized by central caseous necrosis often with some mineralization, surrounded by macrophages, foamy macrophages, numerous giant cells, lymphocytes and a fibrotic capsule. Acid-fast bacilli are usually present inside the caseous necrosis, but in very low number (Cvetnic et al., 2007).

Several TB diagnostic tests currently available for use in cattle, such as the tuberculin skin test or the interferon-gamma (IFN-γ) assay, can be also applied with minor modifications for diagnosis of TB in goats (Gutiérrez et al., 1998; Liebana et al., 1998). Refinement of specificity of these tests has been achieved in recent years for their use in humans, based on the detection in peripheral blood of effector T-cells reacting against antigens secreted by active growing bacilli, such ESAT-6 and CFP-10, which are not induced by BCG vaccination (Pai et al., 2004). As it has been observed previously in cattle (Vordermeier et al., 2002), we have recently shown that an IFN-γ-ESAT-6 specific response also occurs in goats naturally infected with *M. caprae,* which is positively correlated with the severity of the pathological changes (Domingo et al., 2009). A peptide cocktail containing ESAT-6 and CFP-10 has been also successfully used for diagnosis of TB in naturally infected goats (Bezos et al., 2011). In cattle, it has been shown that the route of challenge can have a significant influence on infection outcome (Pollock et al., 2006). The endobronchial route of inoculation has been used successfully in several experimental models of TB infection in cattle (Dean et al., 2005), brushtail possums (Buddle et al., 1994) or European badgers (Corner et al., 2007) for its capacity to mimic the natural infection. In adult goats, an infection model of transthoracic inoculation of *M. caprae* has been previously described (Bezos et al., 2010), demonstrating the potential of this species as a research model for TB.

Qualitative or semiquantitative scoring systems of gross lesions have been used to assess efficacy of vaccines, based on lesion distribution and extension. Improvement in this scoring system into a more precise quantitative system would be of benefit to allow better comparison between treatment groups and experiments. Recently, magnetic resonance imaging (MRI) has been used in to measure disease burden in macaques experimentally infected with *M. tuberculosis* (Sharpe et al., 2009; Sharpe et al., 2010), with promising results. The aim of the present work was to reproduce experimentally TB infection in young goats by inoculation with *M. caprae* through the endobronchial route, to characterize the immune response, and to standardize methods for quantifying pathological changes in target tissues, including the assessment of Multi-Detector Computed Tomography (MDCT) to measure the magnitude of lesions in pulmonary tuberculosis. To our knowledge, this is the first study aimed at comprehensively characterising the effect on endobronchial infection of goats with *M. caprae*.

2. Materials and methods

2.1. Experimental animals

Thirteen Murciano-Granadina female 6 months-old goats obtained from an officially TB-free herd were used. Goats were negative to the single intradermal comparative cervical tuberculin (SICCT) test and the IFN-γ assay (Bovigam™, Prionics, Schlieren, Switzerland) as well as seronegative for paratuberculosis (Paratub.Serum-S™, Institut Pourquier, Montpellier, France). The herd was not vaccinated against Paratuberculosis.

Eleven goats were housed in appropriate containment accommodation for a week, prior to infection with *M. caprae*. Two additional goats were kept uninfected in an outdoor box throughout the experiment. All experimental procedures with animals were in agreement with the European Union Laws for protection of experimental animals and were approved by the Animal Welfare Committees of the *Universitat Autònoma de Barcelona* and the *Generalitat de Catalunya*.

2.2. *Mycobacterium caprae* cultures and experimental infection

The *M. caprae* SB0416 (www.mbovis.org) field strain used as inoculum was originally isolated from a tuberculous goat from Catalonia. The isolate was subcultured in Middlebrook 7H11 solid media (BD Diagnostics, Spark, USA) and bacteria were resuspended in Brain Heart Infusion broth with 20% of glycerol at a concentration of 2×10^6 colony forming units (cfu)/ml (calculated by plating dilutions on Middlebrook 7H11 media). The suspension was stored at -80 ◦C in 0.5 ml aliquots. The inoculum was prepared to the required final concentration by diluting the suspension with sterile phosphate buffered saline (PBS).

For infection, goats were pre-anaesthetized with 0.05 mg/kg of acepromacin (Calmo Neosan®) and 0.2 mg/kg of butorphanol tartrate (Torbugesic®) co-administered by intramuscular injection; after 30 minutes a catheter was placed into the left cephalic vein and 4-6 mg/kg of propophol (Propofol Lipuro®) and 0.2 mg/kg of midazolam (Dormicum®) were both administered intravenously. Goats were then intubated with an endotracheal tube and were placed in right lateral decubitus. A plastic cannula (3.3 mm outer diameter) was passed through the endotracheal tube to the level of the carina. For inoculation a thinner cannula (2.1 mm outer diameter) was passed through the thicker one to a bronchus, and then 0.5 ml of *M. caprae* inoculum was injected into the inner cannula, followed by flushing with 5 ml of 0.9%-saline. The inoculum was titrated after the inoculation in duplicate by ten-fold serial dilution in Middlebrook 7H11 solid media; accordingly, each goat received 1.5×10^3 cfu of *M. caprae*. The animals recovered from anaesthesia in sternal decubitus.

2.3. Clinical signs and sampling

Before and during the experimental infection goats were observed for clinical signs. Rectal temperature was measured weekly and weight every two weeks. Blood samples were collected every two weeks from the jugular vein in heparinized blood tubes for immunological studies and isolation of mycobacteria. Also, two nasal swabs were collected from each animal at the same time points, one was decontaminated and subsequently cultured and the other

one was submerged in ultrapure water 1 hour at 75 ºC for mycobacteria inactivation and stored at -80 ºC until a specific *M. tuberculosis* complex (MTBC)-PCR assay was performed.

2.4. Antigens and peptides

Bovine (PPD-B) and Avian (PPD-A) tuberculins were obtained from CZ Veterinaria (Porriño, Galicia, Spain). ESAT-6/CFP-10 and Rv3615c peptide cocktails synthesized as described earlier (Vordermeier et al., 2001; Sidders et al., 2008) were received from Dr. H.M. Vordermeier, Veterinary Laboratories Agency (Weybridge, UK). Recombinant MPB83 was obtained from Lionex (Braunschweig, Germany). Phytohemagglutinin (PHA) (Sigma-Aldrich, Steinheim, Germany) was used as a positive control.

2.5. Skin Test

SICCT test was performed in all goats at 12 weeks post-infection (wpi, 2 weeks before sacrifice) by inoculating 0.1 ml of both PPD-B and PPD-A on the left and the right side of the neck respectively. The preinoculation skin-fold thickness was recorded before PPD injection, and the skin-fold thickness was measured again after 72h. The goats were considered positive if the increase of skin-fold thickness after PPD-B application was higher than 2 mm and higher than the increase after PPD-A application.

2.6. Whole-blood IFN-γ assay

Blood samples were collected at the time points described above, preserved at room temperature and processed in less than two hours after collection. One ml of whole blood was stimulated in 96-well cell culture plates with PPD-A, PPD-B and PHA at a final concentration of 10 µg/ml, or with peptide cocktails, each one at a final concentration of 5 µg/ml. PBS was used as non-stimulated control. Plasma supernatants were collected after 24h of culture at 37ºC and 5% CO_2 and were stored at -20ºC, and thawed just before performing the Bovigam IFN-γ enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. ELISA results are reported as OD_{450}. Specific reaction was expressed as ΔOD_{450} (OD_{450} of antigen-stimulated sample minus OD_{450} of non-stimulated control). A sample was classified as positive when the PPD-B ΔOD_{450} was higher than 0.05 and OD_{450} of PPD-B was higher than OD_{450} of PPD-A, according to the manufacturer's interpretation.

2.7. Serology

Plasma samples of all animals were analyzed in duplicate for antibodies to mycobacteria using ELISA, as described previously (Gil et al., 2010) with minor modifications. The 96-well plates were coated with PPD-B (2 µg/ml) or MPB83 (1 µg/ml) diluted in carbonate/bicarbonate buffer and incubated overnight at 4ºC. After blockade of 45 min. at 37ºC with PBS containing 0.05% Tween 20 (PBS-T20) with 0.5% casein, plasma samples (at 1/200 dilution in PBS-T20 with 1% casein) were added in duplicate and incubated 1h at 37ºC. After washing, a combination of Protein A and Protein G conjugated with peroxidase (Sigma-Aldrich, Steinheim, Germany) was added at a final concentration of 50 ng/ml and 100 ng/ml respectively. Plates were then read in a spectrophotometer and ΔOD_{450} was calculated as sample OD_{450} minus background OD_{450} (unspecific absorbance in wells where antigen had not been added). A sample was classified as

positive when the ΔOD_{450} was higher than the cut-off point, calculated as the mean of background OD_{450} + 3SD.

2.8. Post-mortem examination

All goats were euthanized at 14 wpi by intravenous injection sodium pentobarbital and carefully examined in order to evaluate the extension of tuberculous lesions in lungs and respiratory lymph nodes (LN).

2.8.1. Lungs

Lung gross lesions were recorded first by palpation and external observation of the different lobes. LN were removed for bacteriological investigation, taking special care of not incising the pleural surface. The heart and pericardium were removed, and then, whole lungs were fixed with 10%-buffered formalin by pouring the fixative into the trachea while holding the lungs in a vertical position, until the trachea was filled up with fixative. After that, the trachea was tied, and formalin-flooded whole lungs were immersed into a container with formalin as previously described (Gil et al., 2010), for two months. After complete fixation, lungs were scanned by using a high-resolution 64-slice Multi-Detector Computed Tomography (MDCT) scanner (Brillance CT 64-channel, Philips Medical Systems, Cleveland, Ohio, USA). MDCT data were analyzed and post-processed on a workstation (Aquarius Station, TaraRecon, Foster City, California, USA). Tuberculous lesions were defined as the following 4 lesion-types in respect to their density patterns: calcified lesions, cavitary lesions, solid lesions and complex lesions. The total pulmonary volume and volume of lesions were measured.

MDCT quantification of lesions was compared with conventional visual inspection with the aid of image analysis software. For that purpose, lungs were sliced at 4-5 mm width intervals. Each slice was photographed and gross lesions were subsequently quantified in pictures with the aid of an image analyzer (ImageJ 1.43u, National Institutes of Health, USA). Approximate volume of granulomas was calculated for each slice (area of lesion × slice thickness). Total volume of granulomas of each lobe was calculated adding slice-partial volumes. Data obtained by applying both the MDCT and the visual direct scoring were compared in order to evaluate the correspondence between the two methods. Representative sections of gross lesions were also processed for histopathological examination (haematoxylin-eosin staining and Ziehl–Neelsen staining for acid-fast bacilli) to confirm the tuberculous nature of the lesions.

2.8.2. Lymph nodes (LN)

The number and diameter of the macroscopic lesions were recorded in cranial mediastinal LN, tracheobronchial LN, caudal mediastinal LN as well as both right and left retropharyngeal LN. The approximate volumes of gross lesions were calculated as $4/3 \times \pi \times r^3$ assuming that most lesions showed fairly spherical morphology. The same pathologist performed all evaluations in order to ensure homogeneous application of the scoring criteria. After pathological measures each LN was processed entirely for bacterial enumeration.

2.9. Culture of *M. caprae*

2.9.1. Lymph nodes

To calculate the bacterial load (cfu/g) of each LN, the weight was recorded before homogenization. Then, the LN were mechanically sliced using dissection scissors and automatically homogenized in 10 ml of sterile distilled water in a Masticator (IUL Instruments, Barcelona, Catalonia, Spain). The homogenate was decontaminated with a final concentration of 0.35% w/v hexadecylpyridinium chloride (HPC) (Corner and Trajstman, 1988) for 15 min. in orbital shaking after which it was centrifuged at 2471×*g* for 30 min. The supernatant was discarded and the pellet was resuspended in 10 ml of PBS containing 0.05% Tween 80. Viable bacterial enumeration was determined by plating 0.1 ml of ten-fold serial dilutions of LN homogenates on Middlebrook 7H11 agar and incubated at 37 ºC for 28 days.

2.9.2. Peripheral blood

Whole blood (5 ml) from each goat was inoculated to BacT/ALERT MB flasks (Biomérieux España, Madrid, Spain) at the time points described above and incubated for 30 days before being considered negative, as recommended by the manufacturer.

2.9.3. Nasal swabs

Nasal swabs were decontaminated for 30 min. with 0.35% w/v HPC and subsequently cultured on Coletsos and pyruvate-enriched Löwenstein-Jensen media (Biomérieux España, Madrid, Spain); cultures were incubated for 60 days before being considered negative.

2.10. DNA amplification

The DNA from inactivated samples from nasal swabs was extracted using DNA purification kit (Promega Biotech Iberica, Madrid, Spain). A seminested-PCR was run under standard conditions. Two consecutive PCR reactions were performed using oligonucleotide primers described previously (IS-F: 5'-CCTGCGAGCGTAGGCGTCGG-3', IS-R1: 5'-TCAGCCGCGTCCACGCCGCCA-3') (Plikaytis et al., 1991) adding another reverse primer for the second reaction (IS-R2: 5'-CTCGTCCAGCGCCGCTTCGG-3') (Eisenach et al., 1990). These primers are specific for the MTBC-IS*6110* insertion sequence.

2.11. Data analysis

Differences in the mean rectal temperature between weekly measures were compared by employing analysis of variance (ANOVA) with Student-Newman-Keuls multiple comparison test and are reported with the 95% confidence interval (C.I.). Comparison of bacterial loads (log_{10} cfu/g) between pulmonary LN, as well as comparison of IgG or IFN-γ ELISA absorbance values (ΔOD_{450}) between antigens within the infected group, were analyzed by non-parametric Friedman test with the post-hoc Mann-Withney-Wilcoxon test. Correlation between MDCT and direct visual measure of macroscopic lesions was performed by non-parametric Spearman rank test. Immune responses and log_{10}-transformed pathological and bacteriological data were compared by applying linear regression, or by the non-parametric Spearman rank test depending on whether experimental units passed the Shapiro-Wilk normality test. Analysis of the data was performed using SPSS statistical package version 17.0.

3. Results

3.1. Clinical observations

Few clinical signs were observed in goats infected with *M. caprae* throughout the experiment. A significant increase in mean rectal temperature was detected at 4 wpi (39.6 ºC, 39.5-39.7, 95% C.I.) compared with the rest of time points (mean of 39 ºC, 39.0-39.1, 95% C.I.) ($p < 0.05$). Coughing was observed in 3 out of 11 (27 %) infected goats at six wpi; the majority of goats (9/11) showed coughing at the end of experiment (14 wpi). One goat also showed tachypnea.

3.2. Immunological response

The immunological response to infection with *M. caprae* was characterized using both cell-mediated and humoral immunological tests. Goats were subjected to the SICCT test at 12 wpi. The mean increase of skin-fold thickness 72h after PPD-B and PPD-A application were 21.3 mm (19.4-23.2, 95% C.I.) and 10.9 mm (9-12.7, 95% C.I) respectively. All infected goats were positive to PPD-B according to the official interpretation criterion described above.

Cell-mediated immunity (CMI) was measured throughout the course of the experiment by the release of IFN-γ from whole blood stimulated with PPD-B, PPD-A, ESAT-6/CFP-10 or Rv3615c (see Fig. 18). According to the standard interpretation of the Bovigam assay (considering the PPD-B stimulated well), all infected goats were negative from the day of infection to the 2nd wpi, but all goats became positive at 4 wpi (individual data not shown) and remained positive throughout the experiment with the exception of two goats which were negative at 10 wpi (and turned to be again positive at 12 and 14 wpi). Peak mean value of PPD-B stimulation was reached at 8 wpi, and from that point onwards, a progressive decrease on the mean PPD-B absorbance was observed until the end of the experiment (Fig. 18A). Uninfected goats remained negative through the experiment (individual data not shown). Production of IFN-γ in blood cultures in response to stimulation with PPD-A was also first detectable at 4 wpi and it was maintained until 14 wpi, however the mean ΔOD_{450} was significantly lower to that observed in cultures stimulated with PPD-B at 4 wpi ($p < 0.005$) (Fig. 18A). As expected, no avian reactors (Bovigam readout) were observed at any time point.

The release of IFN-γ to both ESAT-6/CFP-10 and Rv3615c peptide cocktails followed similar kinetics but was also significantly weaker than to PPD-B at 10 wpi and 14 wpi ($p < 0.05$) (Fig. 18B). If the standard cut-off point for positivity of the Bovigam assay is used for these antigens (OD_{450} of stimulated sample – OD_{450} of unstimulated control > 0.05), all goats reacted positive from the 4 wpi onward, with the exception of the same two goats which were negative for PPD-B at 10 wpi, that resulted also negative at the same time point using both peptide cocktails. Moreover, one of these goats was also negative at 14 wpi and, in addition, another goat was negative at 14 wpi using Rv3615c (individual data not shown). Therefore, considering the three time points analyzed between 6-14 wpi 29/33 samples from infected goats were positive for the two peptide cocktails (sensitivity of 88 %), whereas 31/33 samples were positive for PPD-B at the same time points (sensitivity of 94 %). The two uninfected goats remained negative to all IFN-γ tests during the trial (data not shown).

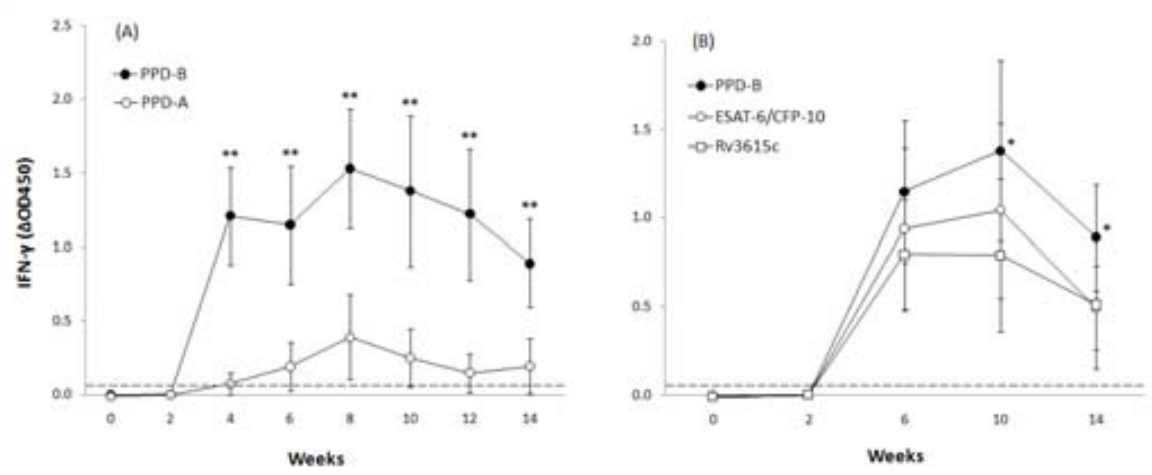

Figure 18. Kinetics of IFN-γ responses in infected goats. The release of IFN-γ was measured by ELISA after in vitro stimulation of whole blood with different antigens. Results are expressed as mean ΔOD_{450} responses with 95 % CI. Dashed horizontal line, cut-off point for positivity. (A) PPD-B (closed circles) and PPD-A (open circles). (**) $p < 0.005$, significant differences determined by non-parametric Mann-Withney-Wilcoxon test. (B) PPD-B (closed circles), ESAT-6/CFP-10 (open circles) and Rv3615c (open squares). (*) $p < 0.05$, significant differences determined by non-parametric Friedman test with post-hoc Mann-Withney-Wilcoxon test.

To analyze the IgG response to infection with *M. caprae*, plasma samples of all goats were tested by ELISA every two weeks after infection, in plates coated with PPD-B or the mycobacterial antigen MPB83. All goats were seronegative to both antigens before *M. caprae* infection, and uninfected goats remained seronegative through the experiment. After infection, goats remained seronegative to PPD-B at all time points before the tuberculin-boost at 12 wpi, but all seroconverted at 14 wpi (at 13 days after SICCT test, individual data not shown). By contrast, seropositivity to MBPB83 after infection appeared earlier in some animals, but it was weak and inconstant, with a total of 7/11 goats positive at 8, 10 or 12 wpi (Table 8).

Table 8. Detection of antibodies to MPB83 in plasma of goats infected or not with *M. caprae*. ELISA results at different time points post-infection (Week 0 represents day of infection) are expressed according to the cut-off described above as: +, positive or -, negative. Goats were subjected to SICCT test 13 days before the blood sampling at 14 wpi.

Group	Goat	Weeks postinfection							
		0	2	4	6	8	10	12	14
Infected	572	-	-	-	-	-	-	-	+
	605	-	-	-	+	+	+	-	+
	571	-	-	-	-	+	+	-	+
	567	-	-	-	-	-	-	+	+
	607	-	-	-	+	+	-	-	+
	563	-	-	-	-	+	+	+	+
	597	-	-	-	+	+	+	+	+
	568	-	+	-	-	-	-	+	+
	562	-	-	-	-	-	+	+	+
	565	-	-	-	+	+	+	+	+
	577	-	-	+	+	+	+	+	+
Non-infected	162	-	-	-	-	-	-	-	-
	187	-	-	-	-	-	-	-	-

All infected goats showed strong responses to MBPB83 after SICCT test, indeed pronounced differences of mean ΔOD_{450} were found between both antigens used, with much higher IgG responses to MBPB83 than to PPD-B ($p < 0.005$, Fig. 19).

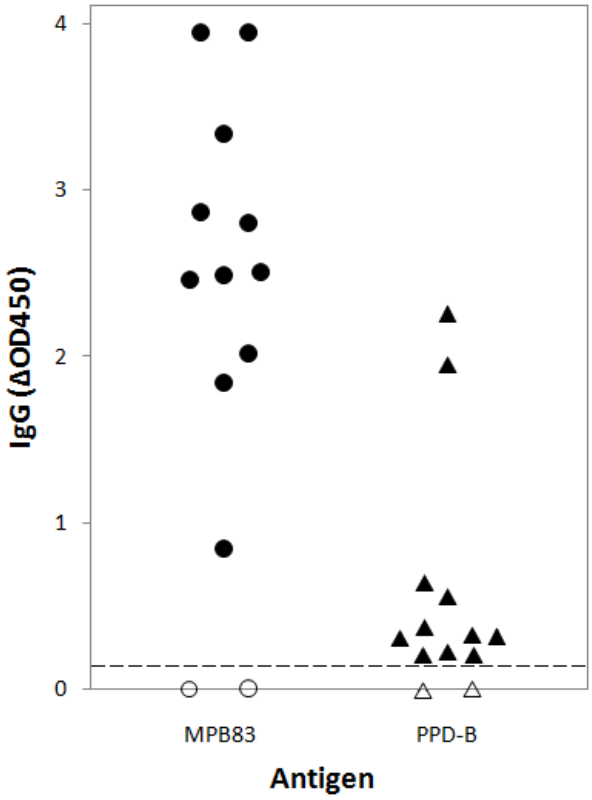

Figure 19. Humoral responses to MPB83 and PPD-B at 14 weeks post-infection.OD_{450} absorbance of total IgG to MPB83 (circles) and PPD-B (triangles) from individual goats infected or not with *M. caprae*. Results are expressed as ΔOD_{450} (OD_{450} of antigen-stimulated wells minus OD_{450} of non-stimulated wells). Filled symbols, infected animals; open symbols, non-infected control animals; dashed horizontal line, cut-off point for positivity.

3.3. Pathology

The pathological findings were mainly restricted to thoracic cavity. All the infected goats showed granulomatous caseous-necrotizing lesions in the lungs and in lung-associated lymph nodes. With few exceptions, the majority of lung lesions were located at right lobes, being the right diaphragmatic lobe affected in most goats.

Seven out of eleven goats showed well-developed cavitary lesions. MDCT scan technology allowed a 3-D representation of the lungs, and a cross-sectional visualization and analysis of lesions (Fig. 20).

Figure 20. Gross pathology analysis of a goat case. (A) MDTC-3D representation of the whole lung after excluding air and TAC's table (H, head; F, foot, L, left, R, right). The total volume of lung is calculated in cm^3 and is shown in red-dashed box at the bottom. (B) Volume rendering image of the lung showing different tissue densities discriminated by colour: water in grey, air in black and calcium in white. The volume of affected lung is also showed. (C) Pathological areas identified by segmentation in axial (see at the top; A, anterior; P, posterior), coronal and sagital planes (see at the bottom). (D) Formalin fixed, 5 mm-sections of left diaphragmatic lobe which showing a large cavitary lesion. Cranial to caudal sections are represented as bottom-up and left-right in the picture. Bar = 3 cm.

The comparison of the volume of granulomatous-necrotizing lesions in the lungs measured by MDCT and by image analysis of photographs of lung sections (direct observation) is shown in Table 9. By MDCT, volume of TB lesions for each goat ranged from 18.8 to 182 cm^3, with a mean of 60 cm^3. The MDCT allowed calculation of percentage of lung volume occupied by TB lesions (Table 9), which ranged from 1.1 to 14.3% (mean value of 5.3%). The extension of lesions in the lung was also measured by recording number of affected lung lobes in each infected goat (visual inspection), and these values are also shown in Table 9.

Table 9. Quantification of gross lesions in lungs in goats infected with *M. caprae*. Total volume of lesions and percentage of affected lungs were calculated using 64-slice Multi-Detector Computed Tomography (MDCT) and were compared to direct visual quantification by slicing, photography, and image analysis.

	64-MDCT		Direct visual observation	
Goat	Volume of	Volume ratio	Volume of lesions	No. of lobes with
572	26.8	1.8	5.4	1/7
605	97.1	8.3	39.5	2/7
571	35.5	3.8	17.2	2/7
567	100	10.6	108.9	6/7
607	59.3	5	25	4/7
563	22.3	1.9	3.2	1/7
597	24.1	2.5	9.8	3/7
568	66.3	6.5	58.3	6/7
562	182	14.3	104.8	6/7
565	31	1.1	9.3	4/7
577	18.8	2.4	0.9	2/7
Mean (95% C.I.)	60.3 (30.8-89.8)	5.3 (2.8-7.8)	34.7 (11.4-58.1)	3.4/7 (2.3-4.4/7)

Significant positive correlation was observed between volume values obtained by MDCT and by direct observation (Spearman rho = 0.955, $p < 0.001$) (Fig. 21) although volume values were higher for MDCT data (see Table 9).

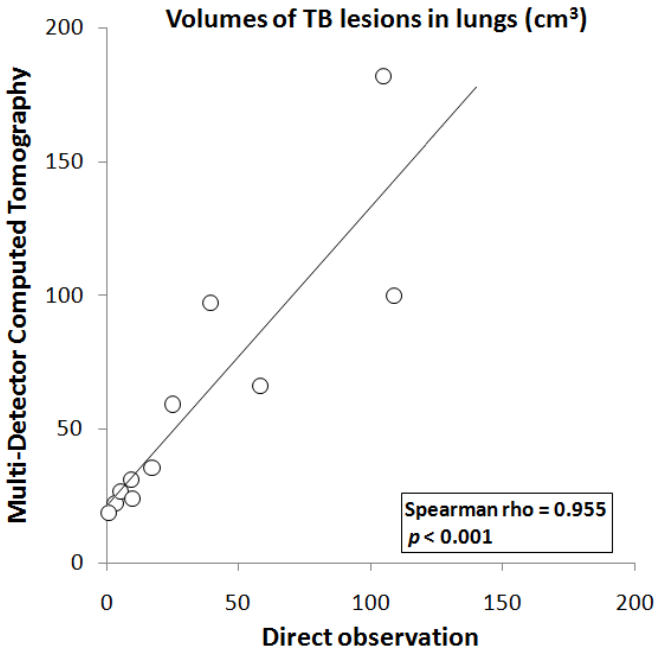

Figure 21. Correlation between volumes of lesions in lungs measured by two quantitative methods. Visible lesions in lungs calculated by Multi-Detector Computed Tomography (MDCT) and by image analysis of photographs of lung sections (direct observation) in infected goats (n = 11). Statistical analysis conducted with non-parametric Spearman rank test.

Pulmonary LN involvement was also extensive. All infected animals presented gross lesions in caudal mediastinal LN, whereas 10/11 and 8/11 goats presented lesions in tracheobronchial and cranial mediastinal LN respectively. Also, two animals showed lesions in retropharyngeal LN. TB lesions in mesenteric LN were also recorded in other two animals, one of them also showing TB lesions in spleen. In total, 4/11 goats showed extrapulmonary TB-lesions. The volumes of gross lesions in LN as well as the number of affected LN in each goat are shown in Table 10.

Table 10. Pathological findings in pulmonary lymph nodes (LN) of infected goats.

Goat	Volume of lesions (cm^3)			
	crm[a] LN	tb[b] LN	cdm[c] LN	Total
572	0	0.3	5.8	6.1
605	3.6	0.003	50.1	53.7
571	0.3	1.7	4.7	6.8
567	0	4.6	15.7	20.2
607	0.05	0	2.5	2.5
563	0	0.001	1.3	1.3
597	0.07	0.9	2.8	3.8
568	0.08	0.6	3.4	4.1
562	0.2	1.2	57.5	58.9
565	1.2	6.8	8	16
577	0.1	0.1	4.2	4.5
Mean (95% CI)	0.5 (0-1.2)	1.5 (0.2-2.8)	14.2 (2.3-2.6)	16.2 (3.9-28.4)

[a]crm: cranial mediastinal, [b]tb: tracheobronchial, [c]cdm: caudal mediastinal.

3.4. Bacteriology

M. caprae was isolated from post-mortem tissue samples from all inoculated animals, but it was not detected by PCR nor mycobacterial isolation from any of the nasal swabs or blood samples taken during the experiment. Mycobacteria were recovered from caudal mediastinial LN in all goats, from tracheobronchial LN of 10/11 goats, and from cranial mediastinial LN of 7/11 goats. In contrast, mycobacteria were isolated from retropharyngeal LN only in two goats. *M. caprae* was also isolated in all TB lesions observed in extra-respiratory organs. The bacterial load of *M. caprae* per gram of cultured pulmonary LN tissues and the total bacterial load of each LN are shown in Table 11.

The mean bacterial load (log_{10} cfu/g) in the cultured pulmonary LN was 3.5 log_{10} cfu/g (3.3-3.8, 95% C.I.), with a range between animals of 2.8 to 4.1 log_{10} cfu/g. Some differences were also found among bacterial load of each pulmonary LN. In caudal mediastinal LN bacterial load was of 3.3 log_{10} cfu/g (3-3.6, 95% C.I.), significantly higher than in cranial mediastinal LN (2 log_{10} cfu/g, 1-2.9, 95% C.I.) ($p < 0.05$) and in tracheobronchial LN (2.4 log_{10} cfu/g, 1.9-2.9, 95% C.I.) ($p < 0.01$). When the total bacterial count of respiratory LN was considered, this value ranged from 3.8 to 5.3 log_{10} cfu, with a mean value of 4.6 (4.3-4.8, 95% C.I.). Also, whole bacterial load was higher for the caudal mediastinal LN than for other LN (Table 11).

Table 11. Bacterial load in pulmonary lymph nodes (LN) of infected goats.

Goat	Bacterial load (Log_{10} cfu)							
	crm[a] LN		tb[b] LN		cdm[c] LN		Total	
	g[d]	LN[e]	g	LN	g	LN	g	LN
572	-	-	2.4	3.2	3	4.1	3.1	4.2
605	3.2	3.8	2.1	2.7	3.1	4.4	3.5	4.5
571	2.5	3.2	3	4	3.3	4.2	3.5	4.4
567	-	-	2.9	4.3	4	5.3	4	5.3
607	-	-	2.8	4	2.5	3.6	3	4.1
563	-	-	-	-	2.8	3.8	2.8	3.8
597	3.3	3.6	2.4	3.3	3.6	4.6	3.8	4.6
568	3.3	3.9	2.6	3.5	3	4.1	3.5	4.4
562	2.9	3.4	2.5	3.4	3.4	4.8	3.6	4.8
565	3.4	4.3	3.2	4.2	3.3	4.5	3.8	4.8
577	3	3.3	2.4	3.2	4.1	5.2	4.1	5.2
Mean	2	2.3	2.4	3.3	3.3	4.4	3.5	4.6
(95% CI)	(1-2.9)	(1.2-3.4)	(1.9-2.9)	(2.6-4)	(3-3.6)	(4.1-4.7)	(3.3-3.8)	(4.3-4.8)

[a]crm: cranial mediastinal, [b]tb: tracheobronchial, [c]cdm: caudal mediastinal.
[d]bacterial count per gram of tissue, [e]bacterial count in the whole lymph node.

3.5. Cross-sectional analysis

Association between cellular and humoral immune responses, pathology and bacteriology were evaluated transversally combining data obtained from all experimental goats (n = 13). In LN, a positive correlation was found between pathology (volume of lesions as log_{10} mm^3) and bacterial load (log_{10} cfu/g) (Pearson r = 0.858, $p < 0.001$). Positive correlations were also found between bacterial load in LN and IFN-γ specific responses to PPD-B (Pearson r = 0.528, p = 0.032) and to ESAT-6/CFP-10 (Pearson r = 0.579, p = 0.019), but not to Rv3615c (Spearman rho = 0.296, p = 0.163), at 14 wpi. However, only IFN-γ responses to PPD-B at 14 wpi were correlated significantly with volume of gross lesions in lungs determined by MDCT (Pearson r = 0.540, p = 0.028).

Humoral immune responses to MPB83 at 14 wpi correlated positively with both bacterial load in LN (Pearson r = 0.775, p = 0.001) and volume of gross lesions in lungs determined by MDCT (Pearson r = 0.685, p = 0.007), whereas IgG responses to PPD-B did not correlate significantly with bacterial load (Spearman rho = 0.322, p = 0.141) and were slightly positively correlated with volume of gross lesions in lungs (Spearman rho = 0.481, p = 0.048).

4. Discussion

Recent interest on development of TB vaccines in domestic ruminants and wildlife, as badgers and wild boar, has driven research to standardize infection models in domestic animals like ruminants (Buddle et al., 2005b) and pigs (Gil et al., 2008). Modelling TB in goats may be of great value to increase our knowledge of infection in this species, and at the same time the model can be used for research of TB in cattle. With these aims we have established an

efficient experimental goat model of TB, with slight clinical signs (coughing at the end of the experiment) and a relatively fast progression of lesions, very similar to natural disease. Gross TB lesions were reproduced in all the infected goats, which is an advantage over the previous existing model in adult goats (Bezos et al., 2010). It is well known from experiments in calves and other models that the route and dose of challenge can be very relevant for the pathological outcome of infection (see (Pollock et al., 2006) for a review). A high challenge dose (higher than 10^6 cfu), by non-natural routes (as intravenous or subcutaneous) may lead to systemic dissemination of infectionwith lesions that are not representative of natural field cases (Waddington and Ellwood, 1972). Using a relative low challenge dose of 1.5×10^3 cfu by the endobronchial route we have been able to reproduce typical granulomatous caseous-necrotizing lesions in lung and lung-associated LN in 11 out of 11 experimentally infected goats, resembling those observed in naturally-infected goats (Daniel et al., 2009; Quintas et al., 2010), and as seen sometimes in natural cases, the majority of our goats had liquefactive necrosis and cavernous lung lesions, which is a feature of tuberculosis in humans. In a previous study in goats experimentally infected with *M. caprae* (Bezos et al., 2010), adult goats were infected transthoracically with 10^2-10^3 cfu, achieving infection in all 6 infected goats (as demonstrated by mycobacterial culture), but with absence of macroscopic lesions in lung parenchyma in two goats, in spite of a much longer duration of the infection (nine months). This difference could be due to the use of 6-month-old goats in our study compared to adult animals, and is clearly an advantage over a model with adult goats. Extension of the infection with production of gross lesions in extrapulmonary sites is often included in scoring systems to assess vaccine efficacy, and therefore, an inoculation route that conveys the challenge dose to a circumscribed area, mimicking natural infection, should be preferred to models that disperse mycobacteria into different systems or mucosal surfaces. In this respect, transthoracic inoculation drops inoculated mycobacteria directly in the lung parenchyma, but may cause also pleuritis (according to our own personal observation), and local infection of the thoracic wall at inoculation point, with mycobacteria draining to regional LN like the axillary nodes, thus complicating the assessment of extrapulmonary dissemination. In our study four animals had extension of the infection from thoracic primary focus to extrapulmonary tissues, like medial retropharyngeal or mesenteric lymph nodes, and to the spleen (one case, indicative of systemic circulation of mycobacteria). Probably, pulmonary lesions allow the dissemination to upper respiratory/head and mesenteric lymph nodes, by mycobacterial shedding in tracheobronchial secretion and its subsequent ingestion. Similarly to results in calves inoculated with a dose of 10^4 cfu of *M. bovis* (McCorry et al., 2005) nasal shedding of *M. caprae* was not detected in our study, showing that, if nasal shedding occurs, it should happen at a very low load, or intermittently. As expected, blood culture was also negative through the whole experiment in all goats, indicating that bacteremia is not a feature of TB in goats, at least in the early phase of the infection.

The data obtained here strengthen the hypothesis that young goats seem to be highly susceptible to infection by *M. caprae.* Pathological and bacteriological findings point to a fast progression of lesions, which reached relatively large size in some animals (more than 5% of lung affected). In natural cases of TB, such large lesions with liquefactive necrosis and caverna formation are usually associated to a long period of lesion progression in herds not subjected to eradication (Daniel et al., 2009; Domingo et al., 2009; Quintas et al., 2010). In contrast, in

trials carried out in other species like badgers (Corner et al., 2007) and calves (Vordermeier et al., 2009) using the endobronchial route of infection, lesions progressed slowly, resembling what is observed in naturally cases of TB in these species. Particularly, in the low challenge dose experiments in calves, big coalescent lesions were not usually found, in contrast our model appears to be faster in the progression of lesions, which can be considered an advantage.

Assessment of vaccine efficacy in experimental trials by non-immunological parameters has used semiquantitative scoring systems based in number of pulmonary lobes affected and size of lesions in lung and pulmonary LN, as well as bacterial load in LN in cattle (Vordermeier et al., 2002), in rabbits (Jassal et al., 2011), and in macaques (Lin et al., 2009). A drawback of these scoring systems is that the intrapulmonary extension of lesions to one or more lobes may be strongly influenced by the inoculation procedure, and consequently, this may also influence the extension to lymph nodes (which depends of the drainage of the lobes affected). A clear evidence of this is in our study the direction of the inoculum to the right lung by inoculation of the goats in the right decubitus position. To avoid this drawback and increase the usefulness of the pathological assessment, in our study we have attempted to express severity of lung lesions in a quantitative way, to allow better comparisons between treatment groups and different experiments. The use of high-resolution 64-slice Multi-Detector Computed Tomography (MDCT) can resolve the whole burden of lung lesions to a volume, and the ratio of affected lung can be calculated. Additionally we made an effort to compare results obtained with MDCT with a conventional visual inspection of sliced lung, photography and calculation of area of lesions in each picture by image analysis. If sections of lung are similar in thickness, an approximate volume of lesions can be obtained by adding volumes of lesions in each slice. This is a time-consuming task, although it also provides with an approximate total volume of lesion per lung. We have shown that results of visual inspection had a strong correlation with those obtained by MDCT, although in general were lower. Interestingly, MDCT seems to have the capacity to detect small changes in density patterns due to inflammatory reactions around the granuloma that may be not visible by direct macroscopic observation. This, together with error introduced by the use for calculation of the same thickness for all lung slices, could explain the slightly higher but homogenous animal-to-animal volume values obtained by using MDCT in respect to direct observation measures. Therefore, the MDCT can be a far more precise method, in comparison to the usually applied pathology scoring systems, to assess the severity of lesions or their reduction in future vaccine efficacy assays. A similar approach to the measurement of lung lesions was followed recently by Sharpe et al. in macaques (Sharpe et al., 2009). These authors measured the total volume of lung lesion in relation to the whole lung volume after fixation by immersion in formalin by using magnetic resonance imaging (MRI) stereology. They concluded that the ratio of lung lesion to whole volume was superior to thoracic radiography or pathology scores for measuring disease burden. Also, in their aerosol model of infection, the total volume of lesions accurately reflected differences in challenge dose in different groups.

Methodologically, irrespective of whether MRI or MDCT technologies are used, it is worth stressing the importance of insufflation of lung with formalin to distend the lung to approximately the same volume as they would have in the pulmonary cavity. This renders the ratio of lesion volume to total lung volume comparable between different experiments and

research groups. The use of this ratio corrects also for slight differences that could exist in size of the animals and of the lungs, even in age matched animals. We believe that this very precise quantitative data set offers the possibility of a better assessment of vaccine efficacy in TB studies. The same conclusion has been drawn by Sharpe et al. (Sharpe et al., 2009), who stressed the benefits of MRI stereology as an accurate and quantifiable assessment, easy to standardize and comparable between laboratories, suggesting that it will be an essential component of pathology assessment in vaccine efficacy studies.

Our experimental model may be useful for assessing the performance of diagnosing techniques in caprine TB. The infection was detected satisfactorily at 12 wpi with SICCT test, the official ante-mortem diagnostic tests currently used for bovine TB eradication campaigns, and all infected goats were also positive to the standard IFN-γ assay from 4 wpi, confirming the usefulness of these techniques also for diagnose of caprine TB as described previously by others (Gutiérrez et al., 1998; Liebana et al., 1998).

Intriguingly, the kinetics of cell-mediated immune responses to infection with *M. caprae*, measured as anamnestic IFN-γ secretion, was slightly different to described previously in the calf model. In goats, for all antigens used the levels of specific IFN-γ were unappreciable until 4 wpi while experimental infections in cattle with similar mycobacterial dose usually showed a significant specific IFN-γ response at 2 wpi, especially in samples stimulated with PPD-B (Vordermeier et al., 2002; Buddle et al., 2003; Vordermeier et al., 2009). Nevertheless, the appearance of detectable levels of IFN-γ a week later has been also reported in cattle infected with a low dose of *M. bovis* (Dean et al., 2005). Unexpectedly, a decrease of IFN-γ responses seemed to occur in infected goats at 10 wpi, whereas in a long-term cattle infection these responses were maintained in their intensity for at least 20 weeks (Buddle et al., 2003). This phenomenon, if confirmed in further long-term studies, could correlate to the fast progression of infection in our goat model as deduced from the extent of lesions observed, coincident with a decline of activity of effector IFN-γ producing cells.

Peptide cocktails ESAT-6/CFP-10 and Rv3615c are being considered as new DIVA reagents for use in cattle (Vordermeier et al., 2001; Sidders et al., 2008). The usefulness of ESAT-6/CFP-10 has been successfully demonstrated in the field, showing high sensitivity and specificity in comparison to tuberculins in cattle that have been naturally infected with *M. bovis* (Cockle et al., 2006) and more recently in goats infected naturally with *M. caprae* (Bezos et al., 2011). Interestingly, the sensitivity obtained in our study for the two peptide cocktails (88 %) would increase to 91 % if combining the results obtained for the two cocktails, the same theoretic sensitivity that was reported previously for cattle infected with *M. bovi*s (Sidders et al., 2008). Moreover, the IFN-γ response to ESAT-6/CFP-10 but not to Rv3615c correlates positively with the bacterial burden in LN, although an even higher correlation has been described previously for IFN-γ responses to ESAT-6 and bacterial burden in *M. bovis*-infected cattle (Lyashchenko et al., 2004). These findings are consistent with the concept that bacterial load in infected tissues is proportional to host IFN-γ responses against antigens secreted by active growing mycobacteria such ESAT-6 and CFP-10 (Pai et al., 2004), but these responses get lower at the end of the experiment, so the correlation should be considered at each stage of the disease. The capacity of ESAT-6/CFP-10 to predict the disease status, the increment of sensitivity when the two peptide cocktails are used and their DIVA capability in animals vaccinated with *M.*

bovis bacillus Calmette-Guérin (BCG), could make them a useful tool for vaccine trials to distinguish vaccinated-protected and infected animals.

Serology is another important tool for assessing infection or exposure to mycobacteria and could be another useful biomarker to determine disease status; although it is not yet clear whether antibody responses play a role in controlling TB. In recent years serological tests have been assessed in trials in cattle and wild mammals, and most of them have concluded that MPB70, and especially MPB83 are serodominant, being recognised in early stages of infection (Lyashchenko et al., 2004; Waters et al., 2006; Lyashchenko et al., 2008). The serodominance of MPB83 described in other species is also consistent with our findings, as most of the goats (10/11) were seropositive at least at one time point before the boost effect of the SICCT test (12 wpi). Moreover, two weeks after boosting with PPDs, all animals reacted strongly increasing dramatically the sensitivity of the ELISA as has been shown in cattle (Vordermeier et al., 2009). In contrast, the IgG-ELISA with PPD-B as antigen failed to detect any positive animals before the boost effect of the SICCT test, after which, antibody responses were positive, although very weak in comparison to MPB83 IgG-ELISA. This result suggests that serology to MPB83 could be a useful tool to detect infected animals in farms, as well as to monitor the progression of the infection in experimental trials.

Summarizing, our goat TB infection model may be useful in TB research for the understanding of pathogenesis of TB in goats and for testing of therapeutic and immunoprophylactic treatments and of new diagnostic tools. The use of MDCT for quantification of volume of lesions and their ratio to the whole lung volume may serve for a quantitative evaluation of pathology in vaccination trials. Research in human TB can also benefits from large animal models different from non-human primates, due to the similarities with the human disease, and its lower cost (Vordermeier et al., 2009; Young. 2009).

Also, reports of caprine TB have increased in recent years, and studies are needed to validate whether control measures used in cattle can be applied to goat herds. Vaccination based on BCG has been developed for use in wild species that act as reservoirs of *M. bovis* and could represent a control tool for caprine TB and to limit its transmission to cattle and humans.

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STUDY II

Goats primed with *Mycobacterium bovis* BCG and boosted with a recombinant adenovirus expressing Ag85A show enhanced protection against tuberculosis

Abstract

This is the first efficacy study using the experimental goat model, a natural host of tuberculosis (TB), to evaluate the efficacy of heterologous *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) prime followed by boosting with a replication-deficient adenovirus expressing the antigen Ag85A (AdAg85A). Three experimental groups of 11 goat kids each were used: BCG vaccinated, BCG vaccinated and AdAg85A boosted, and non-vaccinated. Twenty-two goat kids were vaccinated with ~5 x 10^5 CFU of BCG (week 0), and 11 of them were boosted at week 8 with 10^9 PFU of AdAg85A. At week 14, all goats were challenged by the endobronchial route with ~1.5 x 10^3 CFU of *Mycobacterium caprae*. The animals were euthanized at week 28. Cellular and humoral immunity induced by vaccination and *M. caprae* infection was measured throughout the study.

After challenge BCG-AdAg85A-vaccinated animals exhibited reduced pathology compared to BCG-vaccinated animals in lungs and in pulmonary lymph nodes. There were significant reductions in bacterial load in both groups of vaccinated goats, but the reduction was more pronounced in prime-boosted animals. Antigen-specific gamma interferon (IFN-γ) and humoral responses were identified as prognostic biomarkers of vaccination outcome depending on their correlation with pathological and bacteriological results. As far as we know, this is the first report using multi-detector computed tomography (MDCT) to measure vaccine efficacy against pulmonary TB in an animal model. The use in vaccine trials of animals that are natural hosts of TB may improve research into human TB vaccines.

1. Introduction

Tuberculosis (TB) in goats can be caused by *Mycobacterium caprae* or *Mycobacterium bovis*, which are both members of the *Mycobacterium tuberculosis* complex (MTBC) (Aranaz et al., 2003), and are endemic throughout the Iberian Peninsula (Duarte et al., 2008; Rodriguez et al., 2011). Apart from being a reservoir of TB for animals, infected goats present a zoonotic risk for human infections (Kubica et al., 2003; Prodinger et al., 2005; Cvetnic et al., 2007; Rodríguez et al., 2009).

Eradication of TB infection from domestic animals seems to be unachievable in areas where wildlife is endemically infected with *M. bovis* (Duarte et al., 2008; Rodriguez et al., 2011, and vaccination against *M. bovis* infection in domestic and wild animals has become a field of intensive research (Corner et al., 2008; Lesellier et al., 2009; Vordermeier et al., 2009*. M. bovis* Bacillus Calmette-Guerin (BCG) is the basis of most new vaccine strategies assayed in animals, and there is a large body of knowledge regarding its use against *M. tuberculosis* in humans. In this regard, BCG shows protection against severe and disseminated forms of childhood TB, but protection against pulmonary TB in adults is limited (Brewer. 2000). BCG has been shown to confer a degree of protection in cattle challenged with *Mycobacterium bovis* (Buddle et al., 1995; Vordermeier et al., 2002; Hope et al., 2005). Attempts to increase this protection by boosting with BCG in BCG-primed calves have failed (Buddle et al., 2003). Therefore, in recent years, new vaccination protocols regimes to improve upon BCG vaccination have been developed. Heterologous prime-boost strategies have been established as promising approaches combining BCG priming with virus-vectored subunit vaccines boosting. These

strategies have been applied to humans (McShane and Hill, 2005; Xing and Charters, 2007) and cattle (Vordermeier et al., 2006; Vordermeier et al., 2009). In this regard, a molecular construct based on a recombinant replication-deficient human type 5 adenovirus expressing the MTBC protein Ag85A (AdAg85A) was developed and assessed as a booster vaccine in BCG-primed mice (Wang et al., 2004) and cattle (Vordermeier et al., 2009). In both cases, this approach provided greater protection than BCG alone.

Neither BCG vaccination against TB nor heterologous prime-boost vaccination regimes have yet been assessed as a prophylactic treatment against TB in goats. In a study carried out in goats experimentally infected with *M. caprae*, we observed that a short time after infection (14 weeks), the majority of goats had developed typical caseous-necrotizing granulomas, often with liquefactive necrosis and cavitary lung lesions (Pérez de Val et al., 2011), features similar to active TB in humans (Domingo et al., 2009). In this study we assessed quantitative methods for measuring pathological changes after *M. caprae* infection.

Attempts to improve the semi-quantitative scoring systems used until now, employing image technologies and quantitative reading of macroscopic lesions, have been carried out recently. Magnetic Resonance Imaging (MRI) has been used in macaques experimentally infected with *M. tuberculosis* for scoring of pathological changes in a precise and quantitative way (Sharpe et al., 2009; Sharpe et al., 2010) and volumetric Computed Tomography (CT) has been applied for scanning the thorax of humans in TB vaccine clinical trials (Sander et al., 2009). We have also shown that Multi-Detector Computed Tomography (MDCT) represents a rigorous quantitative method to measure the magnitude of lung TB lesions in goats experimentally infected with *M. caprae* (Pérez de Val et al., 2011).

The aim of the present work was to compare the relative protection of goats vaccinated with BCG alone or in combination with AdAg85A against *M. caprae*. MDCT was used for assessing magnitude of pulmonary pathological changes, and commonly used immunological tests were performed to determine immunological responses to vaccination and challenge.

2. Materials and Methods

2.1. Experimental animals

A total of 33 three-month-old murciano-granadina female goats obtained from an official TB-free flock were used in the study. All of them were reconfirmed negative for TB being negative to the single intradermal comparative cervical tuberculin (SICCT) test and the IFN-γ assay (Bovigam™, Prionics, Schlieren, Switzerland). Also, the goats were seronegative for Paratuberculosis (Paratub.Serum-S™, Institut Pourquier, Montpellier, France).

All experimental procedures were approved by the Animal Welfare Committee of the *Universitat Autònoma de Barcelona* and the *Generalitat de Catalunya*, and were in agreement with the European Union Laws for protection of experimental animals.

2.2. Vaccination and challenge procedures

Goats were divided randomly in three experimental groups of eleven animals each. The first group (BCG group) was vaccinated with BCG at week 0; a second group (BCG-Ad group) was

vaccinated at the same time with BCG, and boosted at week 8 with a recombinant adenovirus expressing Ag85A (AdAg85A); a third group of unvaccinated goats was kept as a control group. The BCG Pasteur strain (ATCC, Ref. 35734™) was subcultured in Middlebrook 7H9 media (BD Diagnostics, Sparks MD, USA). For immunization, BCG growth was diluted to 5 x 10^5 CFU by suspension in 0.5 ml of phosphate buffered saline (PBS), and injected subcutaneously in the right axilla. The AdAg85A inoculum was prepared at 10^9 PFU in 0.5ml of PBS, without adjuvant, as previously described (Wang et al., 2004), and injected intramuscularly into the left brachiocephalic muscle. Rectal temperatures were recorded before and at 6, 24, 48 and 72h after boosting with AdAg85A.

At week 14, goats were anaesthetized with 4-6 mg/kg of propophol (Propofol Lipuro®) and 0.2 mg/kg of midazolam (Dormicum®) administrated intravenously and then challenged with a *M. caprae* field strain from Catalonia (SB0416, www.mbovis.org) by endobronchial inoculation of 1.5 x 10^3 CFU suspended in 0.5 ml of PBS further confirmed by plating dilutions on Middlebrook 7H11 medium (BD Diagnostics, Sparks MD, USA) as described previously (Pérez de Val et al., 2011).

2.3. Antigens and peptides

Tuberculins of *M. bovis* (PPD-B) and *M. avium* (PPD-A) were obtained from CZ Veterinaria (Porriño, Galicia, Spain). Immunodominant MTBC peptide cocktail ESAT-6/CFP-10 (E/C) and Rv3615c (ESAT-6 system 1 substrate protein C) were received from Animal Health and Veterinary Laboratories Agency (Weybridge, UK) and were synthesized as described previously (Vordermeier et al., 2001; Sidders et al., 2008). Recombinant MTBC-specific antigens ESAT-6, Ag85A and MPB83 were obtained from Lionex (Braunschweig, Germany). Phytohemagglutinin (PHA) obtained from Sigma-Aldrich (Steinheim, Germany) was used as a positive control of blood stimulation assays.

2.4. Skin Test

SICCT test was performed in all goats at week 26 (2 weeks before sacrifice) by inoculating 0.1ml of both PPD-B and PPD-A on the left and the right side of the neck respectively. The pre-inoculation skin-fold thickness was recorded before PPD injection and the skin-fold thickness was measured again after 72h. The goats were considered positive if the increase of skin-fold thickness after PPD-B application was greater than 2 mm and greater than the increase in the site of PPD-A application.

2.5. Whole-blood IFN-γ assay

The progression of the infection was followed with the IFN-γ assay by collecting whole blood samples from the jugular vein in heparinized blood tubes. Whole blood was stimulated in 96-well cell culture plates with the following final concentrations of stimuli: 10 μg/ml of PPD-B and PHA, and 5 μg/ml of Ag85A, ESAT-6, E/C and Rv3615c. PBS was added to cultures used as non-stimulated controls. Plasma supernatants were collected after 24h of culture at 37ºC and 5% CO_2 and were stored at -20ºC; samples were thawed just before performing the Bovigam™ IFN-γ enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. ELISA results were obtained as Optical Density determined at 450 nm (OD_{450}).

Specific reaction was expressed as ΔOD_{450} (OD_{450} of antigen-stimulated sample minus OD_{450} of non-stimulated control). A sample was classified as positive reactor to any antigen when specific ΔOD_{450} was higher than 0.05. The assay was performed every two weeks throughout the experiment using PPD-B, ESAT-6 and Ag85A as stimuli. Peptide cocktails were also included at weeks 14, 16, 20, 24 and 28.

2.6. Serology

The presence of antibodies to mycobacterial antigens was analyzed in plasma samples using IgG indirect ELISA as described previously (Pérez de Val et al., 2011). Flat bottom 96-well plates (Nunc Multisorp™, Thermo Fisher Scientific, Roskilde, Denmark) were coated with MPB83 (1 µg/ml) and Ag85A (2 µg/ml) separately. The antigens were diluted in carbonate/bicarbonate buffer and incubated overnight at 4ºC. Plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T20) and then incubated 45 min. at 37ºC with PBS-T20 with 0.5% casein (blocking solution). After washing, goat sera were added in duplicate (at 1/200 dilution in PBS-T20 with 1% casein and incubated 1h at 37ºC. Plates were washed again and a mixture of Protein A (50 ng/ml) and Protein G (100 ng/ml), conjugated with peroxidase (Sigma-Aldrich, Steinheim, Germany) was added to each well. Plates were then read in a spectrophotometer and ΔOD_{450} was calculated as sample OD_{450} minus background OD_{450} (unspecific absorbance in wells where antigen had not been added). A sample was classified as positive when the ΔOD_{450} was higher than the cut-off point, calculated as the mean of background OD_{450} + 3SD. Samples were analyzed at weeks 0, 14 and 28 for the MPB83-ELISA and at weeks 0, 4, 8 and then at rest of time points (every two weeks) for the Ag85A-ELISA.

2.7. Postmortem examination

All goats were euthanized at week 28 (14 weeks post-infection) by intravenous sodium pentobarbital overdose and carefully examined in order evaluate the extension of tuberculous lesions in lungs and respiratory lymph nodes (LN). TB lesions in non-respiratory organs were also recorded.

2.7.1. Lungs

Lung gross lesions were first evaluated by palpation and visual examination of each lobe. Then whole lungs were fixed with 10%-buffered formalin by pouring the fixative into the trachea while sustaining it in a vertical position. After that, the trachea was tied, and whole lungs were immersed into a container with formalin. After complete fixation, lungs were scanned with a high resolution 64-slice Multi-Detector Computed Tomography (MDCT) scan (Brillance CT 64-channel, Philips Medical Systems, Cleveland, Ohio, USA), and sequential slices were analyzed on a workstation (Aquarius Station, TeraRecon, Foster City, California, USA) as described previously (Pérez de Val et al., 2011), allowing calculation of the total volume of granulomatous lesions and the whole lung volume.

2.7.2. Lymph nodes (LN)

The diameter of gross lesions in pulmonary (caudal and cranial mediastinal, right and left tracheobronchial) and right and left retropharyngeal LN was also recorded by direct observation after slicing. Degree of pathology in LN was determined by adding together the

approximated volume of granulomas in respiratory LN of each animal. The volume was calculated as $4/3 \times \pi \times r^3$ considering a sphere-like morphology of the lesions found. The measurement of gross lesions was performed by the same pathologist in order to ensure the same criterion for all samples.

2.8. Bacterial count

After pathological evaluation, the whole pulmonary and retropharyngeal LN were homogenized in 10 ml of sterile distilled water in a Masticator (IUL Instruments, Barcelona, Catalonia, Spain). The homogenate was decontaminated with a final concentration of 0.35% w/v hexadecylpyridinium chloride (HPC) (Corner and Trajstman, 1988) for 15 min. in continuous shaking, after which it was centrifuged at 2471 × *g* for 30 min. The supernatant was discarded and the pellet was resuspended in 10 ml of PBS containing 0.05% Tween 80. The viable bacterial enumeration was determined by plating 0.1 ml of serial dilutions of LN homogenates on Middlebrook 7H11 agar plates. The inoculated plates were incubated at 37ºC for 28 days. After that, the total count of CFU of each LN was calculated.

2.9. Data analysis

Differences in antigen-specific IFN-γ responses, as well as Ag85A-specific IgG responses among treatment groups were compared by using non-parametric Kruskal-Wallis test with the post-hoc Mann-Withney test, whereas analysis of variance (one-way ANOVA) with Student-Newman-Keuls (SNK) multiple comparison test was applied to compare SICCT results and MPB83-specific IgG responses among treatment groups. Rectal temperatures changes after AdAg85A vaccination were also compared by one-way ANOVA with the post-hoc SNK test. All postmortem data (pathological parameters and $\log_{10}$-transformed bacterial counts) were compared by Kruskal-Wallis test with the post-hoc Mann-Withney test. Correlations between immune responses and postmortem parameters were assessed by employing non-parametric Spearman rank test. Data analysis was performed using SPSS for Windows statistical package version 17.0 (IBM Inc, Chicago, IL, USA).

3. Results

3.1. Effect of different vaccination regimens on cell-mediated antigen-specific responses

The release of IFN-γ after *ex vivo* stimulation of whole blood with PPD-B, Ag85A, and ESAT-6 was measured by ELISA every two weeks throughout the course of the experiment whereas responses to ESAT-6/CFP-10 (E/C) or Rv3615c peptide cocktails were measured at weeks 14, 16, 20, 24 and 28 (Fig. 22, data for Ag85A are not shown). Ag85A-specific IFN-γ responses were not detectable in any of the groups and at any time point during the study. BCG vaccination induced an increase in mean IFN-γ responses to PPD-B in vaccinated groups with a peak at week 4 followed by a progressive decrease until week 12 but still significantly higher in comparison to unvaccinated control group responses ($p < 0.001$).

After *M. caprae* challenge (at week 14), all goats showed a considerable increase in the levels of IFN-γ to PPD-B from week 18. The two vaccinated groups responded with similar intensity, but considerably lower than the unvaccinated control group at weeks 18 to 22 and 26 (Fig. 22A). Responses to ESAT-6 after challenge with *M. caprae* were slightly lower in AdAg85A-

boosted goats (BCG-Ad group) than in goats vaccinated with BCG alone (BCG group), although this difference was statistically significant only at week 26 ($p < 0.05$). As occurred with PPD-B, responses to ESAT-6 in unvaccinated goats were also stronger than in the vaccinated groups (Fig. 22B), being significantly higher than in BCG-Ad group at weeks 18 to 28 and in the BCG group at weeks 18 to 26 ($p < 0.05$). The peptide cocktails E/C and Rv3615c induced an ESAT-6-like pattern of IFN-γ responses (Fig. 22C and Fig. 22D respectively) with higher responses in control than in vaccinated groups ($p < 0.05$ at weeks 20 and 28 for E/C, and at weeks 20, 24 and 28 for R3615c).

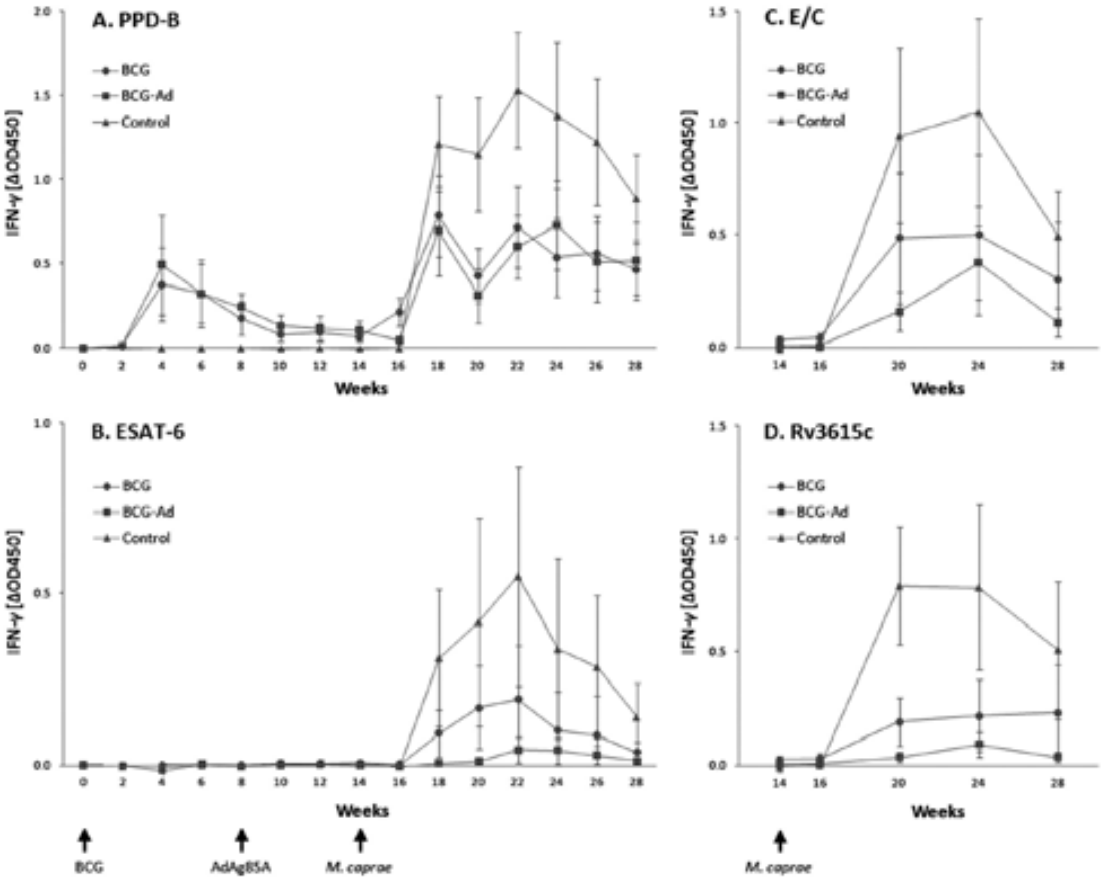

Figure 22. Antigen-specific IFN-γ responses in goats after vaccination and *M. caprae* challenge. ●, BCG group. ■, BCG-AdAg85A prime-boosted group. ▲, Unvaccinated control group. Whole blood stimulated in vitro with (A) PPD-B, (B) ESAT-6, (C) E/C and (D) Rv3615c. Results are expressed as mean of ΔOD450 ± 95% CI.

The results of the IFN-γ assay at weeks 14, 16, 20, 24 and 28 in terms of positive/negative using PPD-B, ESAT-6, E/C and Rv3615c as diagnostic reagents are shown in Table 12. The highest and the lowest number of positive animals were found when using PPD-B and ESAT-6 respectively. However, if results of E/C and Rv3615c are combined, the number of animals scoring positive in the IFN-γ assay was similar to results with PPD-B from week 20 to 28. Moreover, 11 out of 22 BCG-vaccinated goats were positive prior to *M. caprae* challenge (week 14) when using PPD-B, whereas all of them were negative when using ESAT-6.

At week 26 all goats were positive at the SICCT test (individual data not shown). The mean increase of skin-fold thickness 72h after the application of PPD-B and PPD-A respectively presented minor differences among groups: 19.3 mm (17.6-21, 95% CI) and 9.7 mm (8-11.3,

95% CI) in BCG group, 17.3 mm (15.7-19, 95% CI) and 8.7 mm (6.9-10.4, 95% CI) in BCG-Ad group, and 21.3 mm (19.4-23.2, 95% CI) and 10.9 mm (9-12.7, 95% CI) in control group. PPD-B-induced increase of skin-fold thickness in BCG-Ad group was significantly lower compared to that obtained in control group ($p < 0.05$).

Table 12. Number of goats of each treatment group positive to the IFN-γ assay (Bovigam®) using different antigens.

Group	Antigen	Week				
		14[a]	16	20	24	28
BCG (n=11)	PPD-B	6	9	8	9	11
	ESAT-6	0	0	4	2	2
	E/C[b]	0	0	7	8	5
	Rv3615c	0	0	5	4	5
	E/C + Rv3615c	0	0	9	9	9
BCG-Ad (n=11)	PPD-B	5	3	9	11	11
	ESAT-6	0	0	1	2	1
	E/C	0	0	8	10	6
	Rv3615c	0	0	2	5	3
	E/C + Rv3615c	0	0	10	11	8
Control (n=11)	PPD-B	0	0	11	11	11
	ESAT-6	0	0	7	4	7
	E/C	0	0	10	9	10
	Rv3615c	0	0	11	9	9
	E/C + Rv3615c	0	0	11	9	10

[a] Prior to challenge with M. caprae.

[b] E/C: ESAT-6/CFP-10.

3.2. Humoral responses to MPB83 and Ag85A

All goats were seronegative in the MPB83 indirect ELISA prior to vaccination (week 0) whereas two goats of the BCG group seroconverted at week 14 (just before challenge). At week 28 (2 weeks after SICCT test), all goats were seropositive (data not shown). At this time point, the mean ΔOD_{450} found in vaccinated groups was significantly lower than in the control group ($p < 0.05$, individual data not shown), with mean ΔOD_{450} of 1.614 (0.895-2.334, 95% CI) in BCG group, 1.502 (0.932-2.071, 95% CI) in BCG-Ad group, and 2.644 (2.105-3.183, 95% CI) in control group. Thus, the overall levels of MPB83-specific antibody responses showed a similar temporal pattern as seen with *M. caprae* antigen-specific T cell responses (Fig. 22).

The IgG responses to Ag85A were determined at weeks 0, 4, 8 and then every 2 weeks (Fig. 23). Goats in the control group and BCG group were seronegative at all time points during the experiment. Goats in the BCG-Ad group were also negative prior to AdAg85A inoculation. Two weeks after that, the mean values of ΔOD_{450} reached a peak, followed by a progressive decrease until week 28, in which the boost effect of the SICCT test (applied at week 26)

dramatically raised again the mean value of ΔOD_{450}. These values of ΔOD_{450} were significantly higher than values in the other groups ($p < 0.001$).

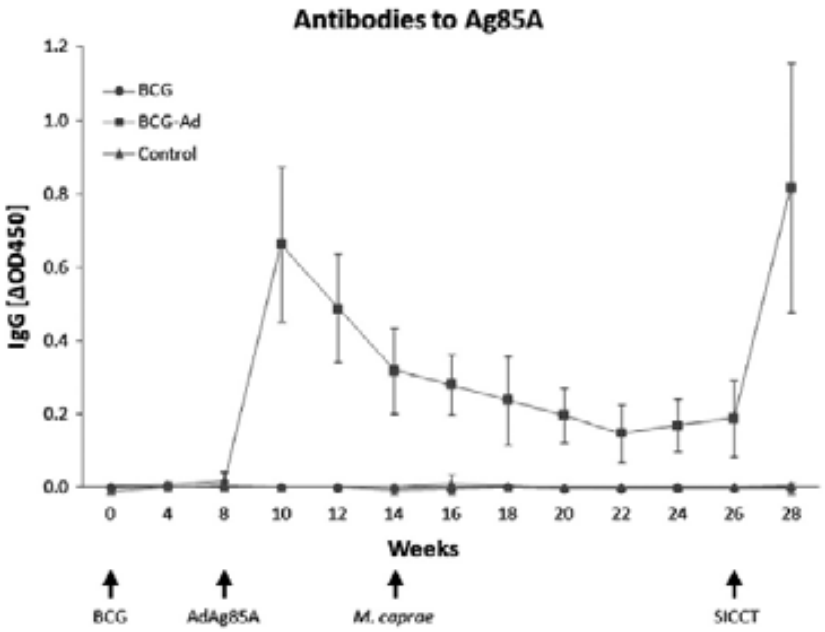

Figure 23. Temporal kinetics of goat IgG responses to Ag85A protein. ●, BCG group. ■, BCG-AdAg85A prime-boosted group. ▲, Unvaccinated control group. Results are expressed as mean of ΔOD_{450} ± 95% CI.

3.3. Clinical, pathological and bacteriological findings

A mild but not statistically significant increase of mean rectal temperature was detected at 6h after BCG inoculation (+0.31°C [0.13-0.49, 95% CI]) followed by normalization at 24h and subsequent time points (data not shown). After AdAg85A inoculation, significant change in mean rectal temperature was observed at 24h in comparison to the mean basal temperature (+1.47°C [1.11-1.84, 95% CI], $p < 0.001$), which returned to normal at 48h. Inoculation with *M. caprae* produced only minimal clinical signs. Occasional coughing was observed from the week 20 (6 weeks after challenge) until the end of the experiment in 8 out of 11 goats of the BCG group, 7 out of 11 goats of BCG-Ad group, and 9 out of 11 unvaccinated goats. The main weight gain from challenge (week 14) to the end of the experiment (week 28) was 6.5 kg (5.4-7.7, 95% CI) in BCG group, 6.5 kg (6.0-7.0, 95% CI) in BCG-Ad group and 6.4 kg (5.2-7.5, 95% CI) in control group. Therefore, there was no difference in weight gain during the challenge period among the three groups.

At necropsy, lungs and respiratory lymph nodes were processed separately. Pathological findings were restricted to thoracic cavity in vaccinated animals whereas the disease was more disseminated in 4 out of 11 control goats, which presented TB extrathoracic lesions in retropharyngeal LN, mesenteric LN or spleen.

The mean volume of TB lesions in formalin-fixed lungs was compared among groups by MDCT. All goats showed granulomatous necrotizing lesions in lung parenchyma and in lymph nodes, and although none of the lungs was completely free of TB lesions, AdAg85A-boosted goats showed much less gross lung lesions than goats belonging to BCG-group and unvaccinated group (see group-representative MDCT lung images at Fig. 24). As shown in Table 13, the

volume of TB lung lesions in BCG-Ad group was very much lower than in BCG-group ($p < 0.005$) and in control group ($p < 0.001$). Also, percentage of lung involvement (volume of TB-lesions related to the volume of the whole lung) was much lower in BCG-Ad group than in BCG group ($p < 0.005$) and control group ($p < 0.001$).

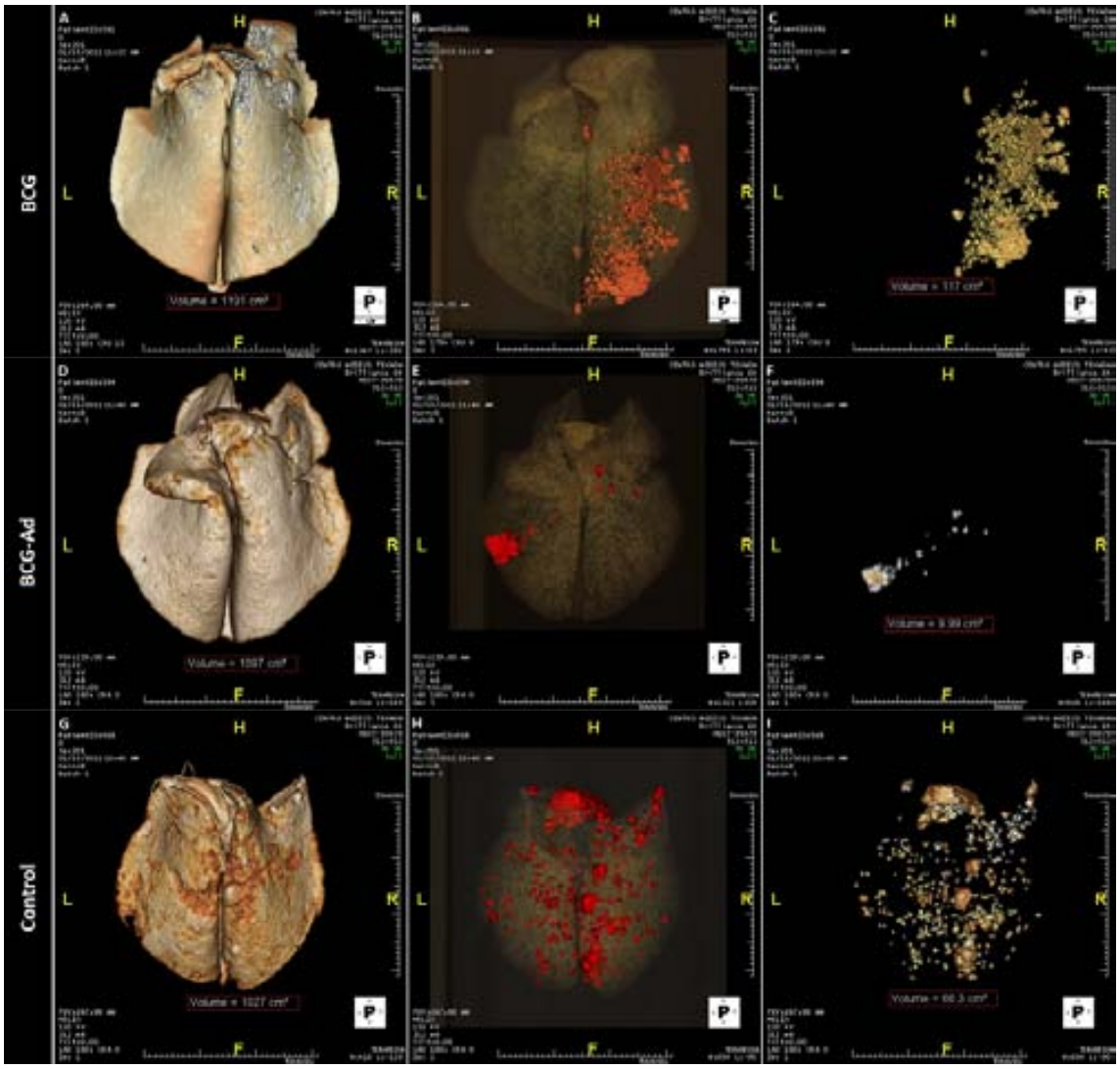

Figure 24. Multi-detector computed tomography analysis of gross lung lesions. Representative lungs from goats in treatment groups: BCG (A to C), BCG-AdAg85A prime-boost (D to F), and Control (G to I). (A, D and G) Three-dimensional reconstruction of the whole lung. (B, E, and H) Volume-rendering images of the whole lung with the different tissue densities discriminated by colour: water in grey, air in black and TB lesions reds. Total volumes of the lungs are shown in red-dashed boxes. (C, F, and I) Volume-rendering images of the affected lung showing only lesions. Total volumes of the affected lungs are shown in red-dashed boxes. Lung orientation is indicated as: H, head; F, foot, L, left, R, right.

Individual data and median values of the volume of TB-lesions are plotted in Fig. 25A. The number of affected lung lobes (of a total of 7 in goats) has been used frequently to assess the spread of the infection within the lung. The extent of scoring for lung lobe involvement was similar for both vaccinated groups, and lower than in the control unvaccinated group, where TB lesions were more widespread (Table 13). Gross lesions in pulmonary and retropharyngeal LN were also analysed by measuring the volume of TB lesions by direct visual assessment. In the BCG-Ad group there was a strong reduction of mean volume of TB lesions in comparison to

the unvaccinated control group ($p < 0.001$), and the volume of lesions was also smaller in the BCG group compared with the control group ($p < 0.005$); whereas, difference between both vaccinated groups was not statistically significant (Fig. 25B).

Table 13. Pathological and bacteriological findings at postmortem in vaccinated and control goats[a].

Group	Lungs			LN	
	Volume of VL (cm^3)	Volume of VL/ lung volume (%)	Number of lobes with VL[b]	Volume of VL (cm^3)	Bacterial load (log CFU)
BCG	80 (44-157)	8.1 (4-13.5)	2/7 (1-3/7)	0.2 (0-4.1)*	3.7 (2.6-4.3)*
BCG-Ad	4 (0-22)**	0.5 (0.1-1.8)**	1.9/7 (1.4-2.5/7)	0.1 (0-0.2)**	3.2 (2.3-
Control	36 (27-94)	3.8 (2.4-8.1)	3.4/7 (2.3-4.4/7)	6.1 (2.3-30.1)	4.5 (4.3-4.9)

[a] The volume of visible lesions (VL) in lungs was calculated using Multi-Detector Computed Tomography and those for lymph nodes (LN) by direct visual observation. Values are medians. Interquartile ranges in parentheses. Significant differences between groups are shown as follows: *, $p < 0.005$; **, $p < 0.001$ (Kruskal-Wallis test with the *post-hoc* Mann-Withney test).
[b] Values are means.

In order to investigate the effect of the different vaccination protocols, the mycobacterial burden was also assessed in respiratory LN. Median values of total burden of mycobacteria (expressed as log CFU/pulmonary LN) of each group are shown in Table 13. The amount of mycobacteria recovered in respiratory LN was significantly lower in both BCG and BCG-Ad groups in comparison to the control group ($p < 0.005$ and $p < 0.001$ respectively, Fig. 25C).

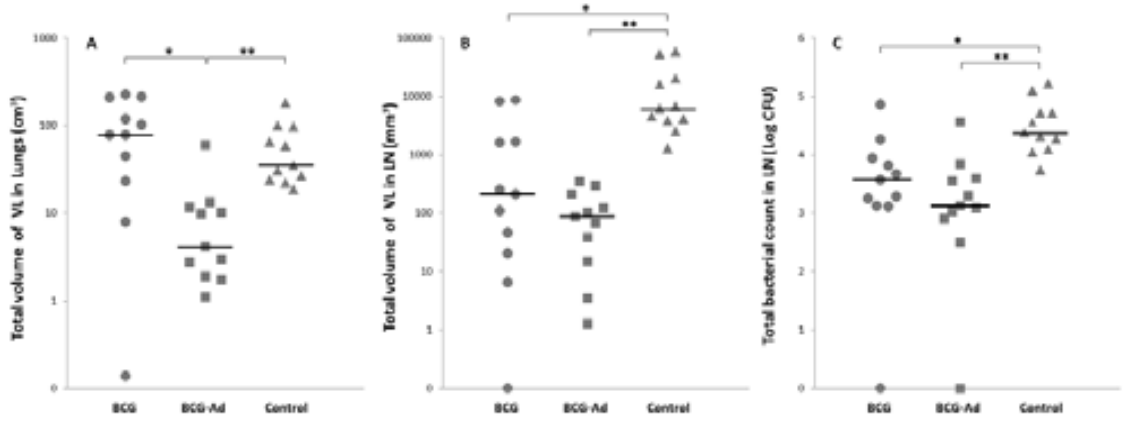

Figure 25. Postmortem results of individual goats for the three treatment groups. Volume of visible lesions (VL) in lungs (A) and LN (B) expressed as cm^3 and mm^3 respectively. (C) Total bacterial count in the pulmonary LN expressed as log_{10}-transformed CFU. ●, BCG group. ■, BCG-AdAg85A prime-boosted group. ▲, Unvaccinated control group. Horizontal lines indicate median values. Significance determined by Kruskal-Wallis test with the post-hoc Mann-Withney test: *, $p < 0.005$; **, $p < 0.001$.

3.4. Immunological responses as predictors of vaccine efficacy and disease status

Immunological parameters were correlated with pathological and bacteriological parameters in order to assess their predictive value as biomarkers of vaccine efficacy or disease status of individual goats.

Antigen-specific IFN-γ responses were compared with pathological and bacteriological parameters (volume of VL and log_{10} CFU in LN respectively) at weeks 20, 24 and 28 (Table 14). Significant positive correlations between IFN-γ to all antigens (PPD-B, ESAT-6, E/C and Rv3615c) and the two postmortem parameters were only found at week 20, whereas inconstant and less significant correlations were found in subsequent time points.

Table 14. Correlation of antigen-specific IFN-γ released in whole blood measured by ELISA (ΔOD_{450}) with postmortem parameters as measured by volume of visible lesions (VL) and log_{10}-transformed bacterial counts[a].

	Week 20		Week 24		Week 28	
Antigen	Volume of VL	log CFU	Volume of VL	log CFU	Volume of VL	log CFU
PPD-B	0.461**	0.533**	0.211	0.310*	0.148	0.133
ESAT-6	0.388*	0.363*	0.181	0.212	0.380*	0.361*
E/C[b]	0.451**	0.554***	0.303*	0.368*	0.077	0.014
Rv3615c	0.446**	0.561***	0.202	0.319*	0.401*	0.487**

[a] Tabulated are results of Spearman rank test. Significance is shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
[b] E/C: ESAT-6/CFP-10.

Humoral responses were also compared with post-mortem parameters. A significant inverse correlation was found between Ag85A-induced IgG responses at weeks 10 to 28, and the total volume of VL found postmortem ($p < 0.005$, data not shown), whereas the negative correlations found between the bacterial counts in LN and Ag85A-specific IgG responses were only statistically significant at weeks 10, 12, 16, 18, 20, 24 and 26 ($p < 0.05$, data not shown). On the contrary, at week 28 (just prior to sacrifice) we found a significant positive correlation between MPB83-induced IgG responses and both total volume of VL (Spearman $\rho = 0.312$, $p < 0.05$) and bacterial counts (Spearman $\rho = 0.389$, $p < 0.05$).

4. Discussion

Research in vaccines against human TB also benefit from large animal models based on natural TB hosts where histopathological features are similar to those in human disease (Young. 2009). We have reported recently the development of a TB infection model in goats and found the induction of TB lesions in all the infected animals after inoculating *M. caprae* by the endobronchial route (Pérez de Val et al., 2011). In the present work we have used this model for testing a human and cattle vaccine candidate against TB, based on BCG priming and subsequent boost with an adenoviral vector expressing the immunogenic antigen Ag85A (AdAg85A), followed by challenge with *M. caprae*. To our knowledge, this is the first attempt to assess safety and efficacy of a prophylactic vaccination strategy against TB in goats.

The rationale for this strategy is based on the previous studies in cattle showing only partial efficacy of BCG or Adenoviral vectored vaccines alone, but improved results when a combined prime-boost strategy was used. In cattle, homologous prime-boost vaccination strategies failed to confer protective immunity to *M. bovis*, either by using BCG-BCG or AdAg85A-AdAg85A (Buddle et al., 2003; Vordermeier et al., 2006). In contrast, heterologous BCG-AdAg85A prime-boost procedures showed strong improvement in protection, when compared to vaccination with BCG alone (Wang et al., 2004; Santosuosso et al., 2006; Vordermeier et al., 2009).

In our goat model, BCG vaccination followed by AdAg85A boosted protection to a challenge with *M. caprae*, leading to a reduction of lung and lymph node lesions, and reduction of mycobacterial burden in respiratory lymph nodes in the vaccinated group in comparison to control non-vaccinated goats.

The strong reduction of pathological and bacteriological parameters observed in BCG-Ad group is in agreement with the results obtained previously in mice (Santosuosso et al., 2006) and, particularly, with those reported in a recent study in calves challenged with *M. bovis* (Vordermeier et al., 2006; Vordermeier et al., 2009). In the latter study, with design analogous to the present study, 4 out of 10 BCG-vaccinated and AdAg85A-boosted calves did not show visible pulmonary lesions at the necropsy. Main difference between the two studies is that we did not find any goat free of VL. This could be due to the use of a highly sensitive, quantitative evaluation method of pulmonary lesions (MDCT) which allowed us to find TB lesions in all goats, in some cases even smaller than 1 mm^3, lesions that would be difficult to observe by direct macroscopic evaluation. However, another possible explanation for this difference in pathological expression of TB between both experiments could be that different species of host and mycobacteria were used and a faster progression of TB infection in goats compared to cattle, as it has been postulated recently (Pérez de Val et al., 2011).

Unexpectedly, the mean volume of lung lesions of the BCG group, measured by MDCT, was slightly higher than the control group (without statistically significant differences). This results contrast with that obtained in calves and badgers vaccination studies, where a significant reduction of pulmonary pathology score was reported in BCG (Pasteur strain) vaccinated and *M. bovis* challenged animals (Buddle et al., 2005a; Wedlock et al., 2007; Corner et al., 2008; Lesellier et al., 2009). Nevertheless, the different scoring system used in our study precludes us from making firm conclusions about a different response to BCG vaccination in goats and other species. In cattle experiments, size and distribution of gross lesions were integrated in a semiquantitative pathology scoring, whereas we have quantified lung lesion volumes, and have evaluated extension to the different lung lobes and extrapulmonary involvement separately.

Considering the intrapulmonary distribution of lesions, measured as number of affected lobes, we observed better containment of internal dissemination of infection within the lung in the two vaccinated groups compared to unvaccinated goats. Also, both gross lesions and bacterial burden in pulmonary drainage lymph nodes were significantly higher in the control group, corroborating the higher disease severity in untreated goats. In addition, extrathoracic TB lesions were only found in unvaccinated goats (4 out of 11), showing that vaccination mitigates hematogenous dissemination of mycobacteria. This finding is consistent with the widely reported capacity of BCG to prevent extrapulmonary TB in children (Rodrigues et al., 1993; Colditz et al., 1995).

With regard to the relationship between CMI and vaccination outcome, we found strong positive correlations between post-mortem parameters and IFN-γ produced in peripheral blood effector T-cells in response to MTBC antigens at week 20 (6 weeks postinfection). However, very weak or no correlations were found at subsequent time points. It is well-known that IFN-γ is a necessary Th1 cytokine for host defense against mycobacterial infection (Flynn

et al., 1993), although its production alone is insufficient for a protective response to TB (Flynn. 2004). Thus, paradoxically, IFN-γ response is also a biomarker of the protective immunity failure (reviewed in Andersen et al., 2007). In fact, a constant high secretion of IFN-γ by ESAT-6-specific T cells is associated with uncontrolled mycobacterial replication and it is considered a predictor of active TB in humans (Andersen et al., 2007) and cattle (Vordermeier et al., 2002).

By contrast, we observed a decrease of both single ESAT-6 and E/C cocktail specific IFN-γ responses at 14 weeks postinfection in all treatment groups. This IFN-γ kinetic is not in accordance with those observed in calf experiments with similar infective dose of *M. bovis* (Vordermeier et al., 2002; Vordermeier et al., 2009). Notwithstanding, in our study the IFN-γ decrease does not seem to be related to the control of infection, but it might be an immune response failure or even an early anergic process. Therefore, the use of antigen-specific IFN-γ as a prognostic biomarker of control/severity of the infection must be employed with caution. It may be useful to determine vaccine success in the early stages of infection (< 8-10 weeks) but it is inconclusive with regard to infection status of an animal at subsequent weeks.

Ag85A-specific IFN-γ production was not detected during the experiment. However, we found indirect correlations of Ag85A-specific humoral responses with postmortem parameters prior to challenge and after SICCT booster effect. The kinetic of antibody responses to Ag85A confirmed the successful recognition of this antigen by the goat immune system and its usefulness as a biomarker of Ag85A expression.

We have confirmed the usefulness of DIVA-candidate peptides as novel diagnostic reagents for use in goats. None of the vaccinated goats produced detectable IFN-γ to the DIVA antigens prior to challenge, whereas half of the vaccinated goats were positive in the bovine tuberculin-based IFN-γ assay, an assay taht is being applied in some bovine TB eradication programmes (de la Rua-Domenech et al., 2006). The usefulness of whole-blood IFN-γ release assays (IGRAs) using ESAT-6 and CFP-10 to distinguish infected from vaccinated individuals has been reported previously for humans and cattle (Pai et al., 2004; Cockle et al., 2006). In goats infected with *M. caprae* we found that tuberculin-based IFN-γ assay and DIVA-based IFN-γ assays, where results for E/C and Rv3615c IFN-γ assays were taken together, had similar sensitivities. In this sense, the capacity of these antigen-specific assays to increase the detection of infected animals when they are used together was as previously reported for cattle (Sidders et al., 2008) and goats (Pérez de Val et al., 2011).

Vaccination is the best strategy to control TB in humans and may be a valid approach in domestic and wild animal species when eradication is not feasible. Employing for the first time a goat model of TB using *M. caprae* infection, the goat being a natural host of this organism, we have demonstrated enhanced protection after boosting BCG-primed goats with an adenoviral vector expressing the antigen Ag85A. Furthermore, we have identified specific IgG response to be an immunomarker of protection and disease severity useful for monitoring vaccine efficacy. We believe that our current study serves as an important step to investigating immune mechanisms of protection in the goat model of TB.

5. Acknowledgments

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STUDY III

A multi-antigenic adenoviral-vectored vaccine improves BCG protection of goats against pulmonary tuberculosis infection and prevents disease progression

Abstract

The "One world, one health" initiative emphasizes the need of new strategies to control human and animal tuberculosis (TB) based on their shared interface. A good example would be the development of novel universal vaccines against *Mycobacterium* tuberculosis complex (MTBC) infection. This study uses the goat model, a natural TB host, to assess the protective efficacy of a new vaccine candidate in combination with Bacillus Calmette-Guerin (BCG) vaccine.

Thirty three goat kids were divided in three groups of 11 each: 1) vaccinated with BCG (week 0), 2) vaccinated with BCG and boosted 8 weeks later with a recombinant adenovirus expressing the MTBC antigens Ag85A, TB10.4, TB9.8 and Acr2 (AdTBF), and 3) unvaccinated controls. Afterwards, an endobronchial challenge involving a low dose of *M. caprae* was performed (week 15). After necropsy (week 28), the pulmonary gross pathology was quantified by using high resolution Computed Tomography. Small granulomatous pulmonary lesions (< 0.5 cm diameter) were also evaluated through a comprehensive qualitative histopathological analysis. *M. caprae* CFU were counted from pulmonary lymph nodes.

The AdTBF improved the effects of BCG reducing gross lesions volume and bacterial load, as well as increasing weight gain. Protection and infection biomarkers were also evaluated. Interferon-γ producing memory T-cells were assessed as predictors of vaccine efficacy. Specific cellular and humoral responses were measured throughout the 13-week post-challenge period, and they were correlated with the severity of lesions.

Unvaccinated goats reproduced the main pathological features of the active tuberculous disease in humans, while vaccinated goats showed very small lesions, resembling those observed in human latent TB infection (LTBI). This study strongly evidences that the multi-antigenic adenoviral-vectored vaccines against TB deserve further attention in clinical trials.

1. Introduction

Tuberculosis (TB), mainly caused by *Mycobacterium* tuberculosis, is the first cause of infectious disease mortality and morbidity worldwide (Lienhardt et al., 2012). Moreover, it is estimated that one-third of the world's population has latent tuberculosis infection (LTBI), making it one of the most prevalent human infections (World Health Organization. 2009). On the other hand, *M. caprae* and *M. bovis*, also members of *M. tuberculosis* complex (MTBC), are the main causative agents of caprine TB, an emerging disease in a number of European countries, causing an increase of economic losses to the livestock sector (Daniel et al., 2009; Rodriguez et al., 2011; Shuralev et al., 2012).

Goats infected with *M. caprae* may be a source of infection for cattle, acting as domestic reservoirs of bovine TB (Napp et al., 2013). *M. caprae* has been also isolated from a wide range of wildlife species (Erler et al., 2004; Rodriguez et al., 2011; Domogalla et al., 2013), and has also been isolated from TB cases in humans (Kubica et al., 2003; Prodinger et al., 2005; Rodríguez et al., 2009). In endemic areas, vaccination is seen as the best long-term prospect for controlling TB in livestock (Krebs and The Independent Scientific Review Group, 1997).

Reducing the prevalence of the disease prior to start an eradication program based on test and sacrifice would reduce economic costs for the producers and the public sector.

Bacillus Calmette-Guerin (BCG), the only currently available vaccine, displays variable efficacy against human and animal TB (Buddle et al., 1995; Fine. 1995). In recent years new subunit vaccines have been developed for use after a previous immunization with BCG or other live vaccines (Skeiky 2006). The viral delivery system of these subunit vaccines has been widely used (McShane and Hill, 2005; Xing and Charters, 2007). Particularly, the use of adenoviruses as vectors for TB vaccines has the advantage of their natural tropism for the respiratory epithelium, as well as the strong immunity induced (Wang et al., 2004; Santosuosso et al., 2006). Boosting BCG with a recombinant replication-deficient adenovirus expressing the antigen Ag85A showed enhanced protection against TB in small laboratory animals (Santosuosso et al., 2006; Xing et al., 2009), cattle (Vordermeier et al., 2006; Vordermeier et al., 2009), and goats (Pérez de Val et al., 2012).

Apart from Ag85A, additional immunoprotective antigens are candidates to be included in multi-antigenic formulations. Among them, the MTBC antigens TB10.4, TB9.8 and Acr2 (http://tuberculist.epfl.ch) have been recently selected for this purpose on the basis of the induction of an early-CMI in calves after *M. bovis* infection or after BCG vaccination (Wilkinson et al., 2005; Mustafa et al., 2006; Dean et al., 2008).

The aim of this study was to assess the efficacy of a novel recombinant adenoviral booster vaccine expressing four MTBC-antigens (Ag85A, TB10.4, TB9.8 and Acr2) to improve BCG protection against TB in goats challenged with *M. caprae*, their natural TB causing agent (Aranaz et al., 1999). Exhaustive postmortem analysis based on Computed Tomography (CT) and a comprehensive histopathological evaluation were used for assessing vaccine efficacy.

2. Materials and Methods

2.1. Study design

2.1.1. Animals and experimental schedule

Thirty-three female goat kids (3 month old Murciano-Granadina) obtained from an official TB-free herd of Murcia Region (South West of Spain) were selected on the basis of negative results to single intradermal comparative cervical tuberculin (SICCT) test and the Bovigam IFN-γ assay (Prionics, Schlieren, Switzerland). Subsequently, animals were randomly divided in three treatment groups of eleven goats each: Group 1, vaccinated with BCG; Group 2, vaccinated with BCG and boosted with a recombinant adenovirus expressing a fusion protein which contained four MTBC antigens (BCG-AdTBF); and Group 3, unvaccinated controls.

Animals were followed-up daily for clinical signs and weighted every two weeks during the experiment. All animal experimental procedures were undertaken in accordance with the European Union Laws for protection of experimental animals, and were approved by the Animal Welfare Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (Permit Number: 6332)

2.1.2. Vaccines

For the BCG inoculum preparation, *Mycobacterium bovis* BCG Danish 1331 strain (ATCC, Ref. 35733™) was sub-cultured in Middlebrook 7H9 media (BD Diagnostics, Sparks MD, USA) supplemented with 0.5% (v/v) Tween 80, 40nM sodium pyruvate (Sigma-Aldrich, Steinheim, Germany) and 10% (v/v) albumin dextrose catalase enrichment (BD Diagnostics). It was incubated for 28 days at 37°C. An aliquot of growth culture was titrated by platting 10-fold dilutions in phosphate buffered saline containing 0.05% Tween 80 (PBS-T80) on 7H11 media (BD Diagnostics) for 28 days at 37°C. The remaining aliquots were stored at -80°C prior to use. After bacterial count, growth culture was diluted to 10^6 CFU/ml by suspension in phosphate buffered saline (PBS). A dose of 0.5 ml of this suspension was inoculated subcutaneously in animals of groups 1 and 2 at week 0 of the experiment.

The adenoviral booster construct (AdTBF) was developed by the Animal Health Veterinary Laboratories Agency (AHVLA, Weybridge, UK) and consisted in a replicant-deficient human adenovirus of serotype 5 which expresses a fusion protein containing four MTBC antigens: Ag85A (Rv3804c), TB10.4 (Rv0288), TB9.8 (Rv0287) and Acr2 (Rv0215c) (http://tuberculist.epfl.ch). One mL of AdTBF diluted in PBS at a concentration of 109 PFU/ml was injected intramuscularly in animals of group 2 at week 8 of the experiment.

2.1.3. *M. caprae* challenge

A field isolate of *M. caprae* SB0416 (www.Mbovis.org) was sub-cultured in Middlebrook 7H9 supplemented media for 28 days at 37°C. After that, an aliquot of growth was platted on 7H11 media and cultured again for 28 days at 37°C. Then the bacterial count was performed.

One week prior to challenge, goats were housed in Bio-Safety Level 3 boxes for acclimatization. For challenge (week 15), all animals were anesthetized with 4-6 mg/kg of propophol (Propofol Lipuro®) and 0.2 mg/kg of midazolam (Dormicum®) administrated intravenously. Subsequently the animals were challenged by the endobronchial route with a suspension of approximately 1.5×10^3 CFU of *M. caprae* as previously described (Pérez de Val et al., 2011).

2.2. Postmortem studies

All goats were euthanized at week 28 by intravenous sodium pentobarbital overdose and necropsied. Pulmonary lymph nodes (LN) were aseptically removed making sure the pleural lung surface was not sectioned, and were used to measure bacterial load. Number, extension, and distribution of tuberculous lesions in the lungs were recorded visually and by Computed Tomography (CT). Microscopic features of small lung granulomatous lesions were assessed by histopathological examination.

2.2.1. Lungs

Lungs were fixed in toto by perfusion with 10%-buffered formalin through the trachea while being sustained in a vertical position. After complete lung distension by the fixative, the trachea was tied, and whole lungs were immersed into a container with 10%-buffered formalin. Six month after, lungs were scanned with a high resolution 64-slice Multi-Detector CT

scan (Brillance CT 64-channel, Philips Medical Systems, Cleveland, Ohio, USA), and sequential slices were analyzed on a work station (Aquarius Station, TaraRecon, Foster City, California, USA) as described previously (Pérez de Val et al., 2011), allowing calculation of the total volume of granulomatous lesions and the whole lung volume.

Once scanned by CT, lungs were sliced in approximately 1 cm sections to visually examine the lesions. Animals were classified in three groups depending on the maximum diameter of the granulomas found in their lungs. Three granuloma diameter size intervals were defined: < 0.5 cm, Small Granulomas (SG); 0.5-2 cm, Medium Granulomas (MG); and 2 cm, Large Granulomas (LG). The number of affected lung lobes was also recorded.

Five SG (when present) from each animal were selected for histopathological study to determine possible qualitative morphological differences between groups. Granulomas were sectioned through its maximum diameter and embedded in paraffin. Then, sections of 4 µm were stained with hematoxylin and eosin (HE). Slides were microscopically examined blindly and separately by two pathologists. The following parameters were scored: 1) Stage of granuloma development according to the four stages previously defined by Wangoo et al.: Stage I (Initial), Stage II (Solid), Stage III (Minimal necrosis) and Stage IV (Necrosis and mineralization) (Wangoo et al., 2005); 2) Number of satellite granulomas (SatG) surrounding the central lesion (as shown in Fig. 26); 3) Presence of multinucleated giant cells (MNGC) as previously described (Garcia-Jimenez et al., 2013): 0, 1 to 10 and > 10 MNGC; 4) Central necrosis extension (absence of necrosis, necrosis comprising < 50%, and ≥ 50% of granuloma surface); and 5) Mineralization degree: absence, low and high.

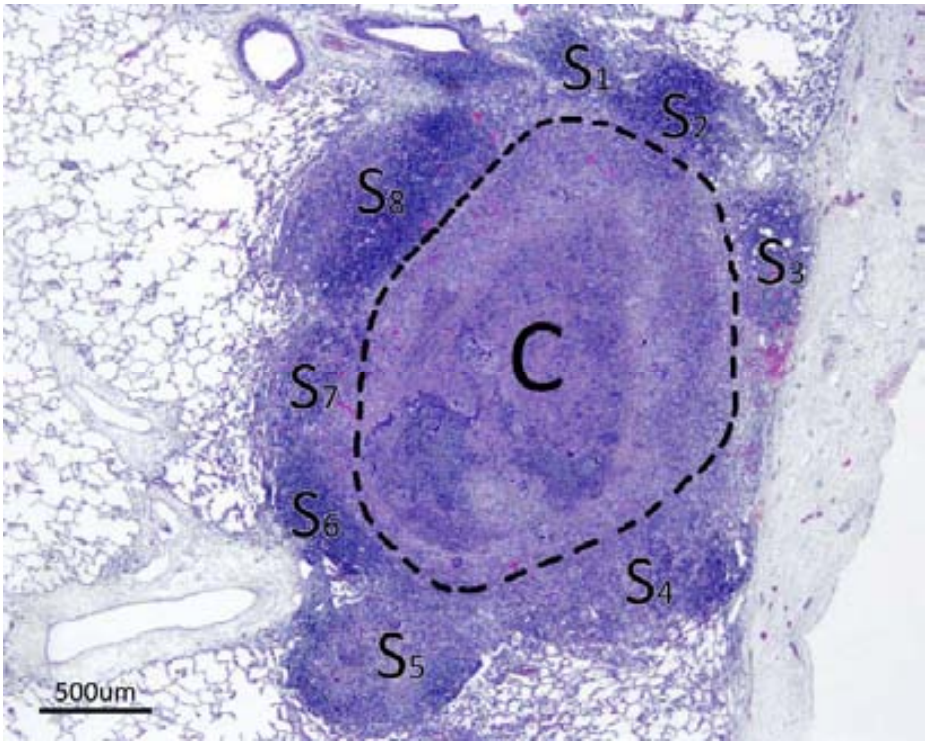


Figure 26. Evaluation of satellite granuloma (SatG) formation. Example of TB small lesion consisting of a central granuloma (C) surrounded by 7 smaller SatG (S1-7) characterized by variably sized clusters of inflammatory cells contiguous to the central granuloma, sometimes with presence of MNGC and central necrosis (e.g. S5). The number of SatG, upon histopathological assessment of small granulomas, was used as a parameter to evaluate the tendency of lesions to disseminate.

2.2.2. Pulmonary LN

Gross lesions in pulmonary LN (caudal and cranial mediastinal, right and left tracheobronchial) were measured as previously described (Pérez de Val et al., 2011). The volume of visible lesions of each animal was calculated as $4/3 \times \pi \times r^3$ (where r is the lesion radius) taking into account the sphere-like morphology of the lesions found. Recording of all granuloma diameters was performed by the same pathologist in order to ensure the same criterion was followed for all samples.

After gross pathological evaluation, whole pulmonary LN were homogenized and decontaminated as previously described (Pérez de Val et al., 2011). The viable bacterial count was determined by plating 0.1 ml of serial dilutions of LN homogenates on 7H11 agar plates (BD Diagnostics). The inoculated media were incubated at 37°C for 28 days. After that, the total CFU count of each LN was calculated.

2.3. Assessment of immune responses

2.3.1. Antigens and peptides

Bovine tuberculin (PPD-B) and Avian tuberculin (PPD-A) were obtained from CZ Veterinaria (Porriño, Spain), mycobacterial proteins Ag85A, Acr2 and MPB83 were obtained from Lionex (Braunschweig, Germany), and ESAT-6/CFP-10 (E/C) peptide cocktail, Rv3615c, TB9.8 and TB10.4 were supplied by the AHVLA.

2.3.2. IFN-γ release assays (IGRAs)

2.3.2.1. Whole blood assay

Blood samples were collected from the jugular vein in heparinized blood tubes. One ml of whole blood was stimulated in 96-well cell culture plates with PPD-B at 10 µg/ml, and Ag85A, E/C and Rv3615c at 5 µg/ml. Blood samples stimulated with phytohemagglutinin (Sigma-Aldrich) at 10 µg/ml and PBS were used as positive and negative controls respectively. Plasma supernatants were collected after 24h of culture at 37°C and 5% CO_2 and were stored at -20°C until tested by Bovigam IFN-γ enzyme-linked immunosorbent assay (ELISA), which was performed according to the manufacturer's instructions. ELISA results were obtained as Optical Density determined at 450 nm (OD_{450}). Specific reaction was expressed as ΔOD_{450} (OD_{450} of antigen-stimulated sample minus OD_{450} of non-stimulated control). The assay was performed every two weeks throughout the experiment using PPD-B and Ag85A as stimuli. Peptide cocktail E/C and Rv3615c were also included at weeks 15, 20, 26 and 28.

2.3.2.2. Cultured ELISPOT assay

At week 15 (prior to challenge), Peripheral Blood Mononuclear Cells (PBMC) were isolated from blood of a subset of 10 randomly selected vaccinated goats (5 of BCG group and 5 of BCG-AdTBF group). Blood samples were diluted 1:1 in PBS, separation of blood cell populations was performed with a gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). PBMC were then stimulated in 24-well plates (2×10^6 cells/well) with 10 µg/ml of PPD-B in cell culture medium: RPMI 1640 medium (Sigma-Aldrich) supplemented with 10 % fetal calf serum

(Sigma-Aldrich), non-essential acids (Sigma-Aldrich), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin sulphate. Cell culture plates were incubated at 37°C 5% CO_2. Cultured ELISPOT assay was based on a previously described method (Vordermeier et al., 2009). Recombinant human IL-2 (Sigma-Aldrich) was added to cell cultures at a final concentration of 10 U/ml at days 5 and 8. Half of supernatant was replaced with fresh cell culture medium at days 10 and 12. On day 13, 5×10^4 cultured cells/well were added in ELISPOT plates (MultiScreen HTS, Merck Millipore, Darmstadt, Germany) previously coated overnight at 4°C with bovine monoclonal IFN-γ antibody (Acris Antibodies GmbH, San Diego, USA) and then blocked with 10% fetal calf serum in RPMI medium. Cells were stimulated with 10 µg/ml of PPD-B, 5 µg/ml of Ag85A, TB10.4 and TB9.8, and incubated 24h at 37°C 5% CO_2. Cultures were performed in the presence of autologous antigen presenting cells (obtained from PBMC isolated at week 15, as described in Vordermeier and Whelan, 2012). After that, biotin labelled bovine monoclonal IFN-γ antibody (Acris Antibodies GmbH) and phosphatase-conjugated streptavidin (Life Technologies S.A., Madrid, Spain) were added for developing spots. Spot-Forming Cells (SFC) were revealed and counted as previously described (Vordermeier et al., 2009), and the number of SFC/ml was calculated.

2.3.3. Serological tests

Humoral responses to vaccination and *M. caprae* infection were studied by carrying out IgG indirect ELISAs to the MTBC protein MPB83 and the four antigens expressed by the adenoviral vaccine (Ag85A, Acr2, TB9.8 and TB10.4). Ninety-six-well plates were coated for each of the five antigens separately (Nunc Maxisorp; Thermo Fisher Scientific, Roskilde, Denmark) at a final concentration of 0.5 µg/ml each, diluted in carbonate/bicarbonate buffer. The plates were incubated overnight at 4°C. For the MPB83-specific ELISA, plasma samples from all goats were analysed at weeks 0, 4, 8, 15 and then every two weeks, whereas for the four AdTBF-antigens ELISAs plasma samples were analyzed at weeks 0, 8, 9, 10 and then every two weeks. The IgG ELISAs were performed as described previously (Pérez de Val et al., 2012). PPD-B and PPD-A intradermal inoculations were performed at week 26 following the standard procedures for SICCT test in order to generate a boost-effect on humoral responses at week 28.

2.4. Statistical analyses

One-way ANOVA with Student-Newman-Keuls multiple comparison test was used for comparisons among groups in terms of differences in weight increase, volume of lesions, number of affected lobes, number of SatG and bacterial load (log10-transformed data). Whole blood IFN-γ responses and serological tests were compared by non-parametric Kruskal-Wallis test with *post hoc* Mann-Whitney or Wilcoxon test, whereas IFN-γ SFC differences between vaccinated groups were compared by Student's unpaired two-sample T-test. Differences in observed frequencies of qualitative histopathological features among groups were assessed by chi-squared test. Correlations between postmortem parameters, as well as between IFN-γ cultured ELISPOT results and postmortem parameters were assessed by using Pearson's correlation, whereas the rest of immunological responses were compared with postmortem parameters by employing non-parametric Spearman rank test. Data analysis was performed using SPSS statistical package version 19.0 (IBM Inc. Chicago, IL, USA).

3. Results

3.1. Clinical signs and body weight

Coughing was observed in 7/11 BCG, 2/11 BCG-AdTBF and 6/11 unvaccinated control goats throughout the experiment. The mean body weight increase during this period was significantly lower in unvaccinated control animals (249 g/week, 95% CI: 230-269) in comparison to BCG-AdTBF group (403 g/week, 95% CI: 383-423, $p < 0.001$) and BCG group (351 g/week, 95% CI: 331-370, $p < 0.05$). The weight increase during the post-challenge period of the different groups is shown in Fig. 27.

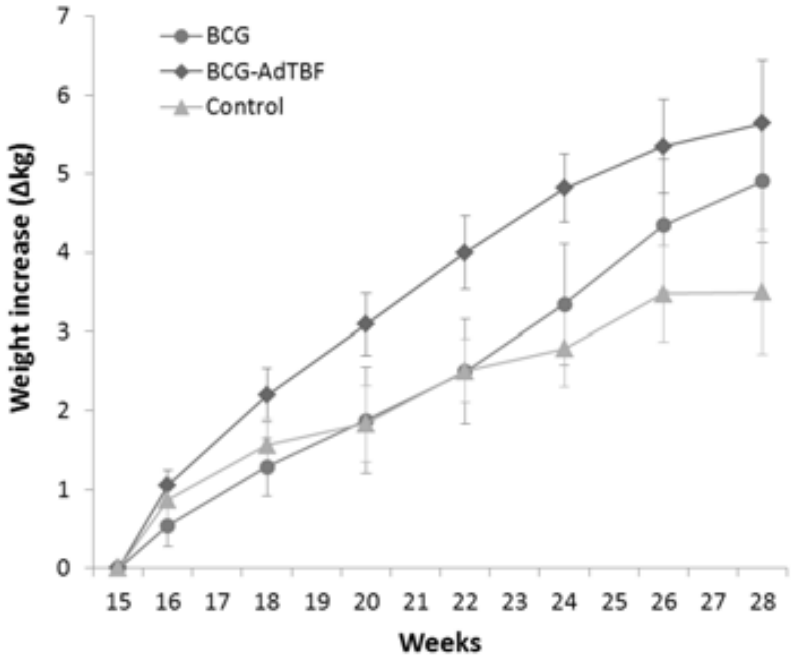


Figure 27. Body weight after *M. caprae* infection. The goats were challenged with 1500 CFU *M. caprae* at week 15 and animals were weighted weekly up to the end of the experiment (week 28). Each line represents the cumulative weight increases (in kg ± 95% CI) in the three treatment groups from the challenge to the end point.

3.2. Gross pathology and bacteriology

All animals presented macroscopic TB lesions at necropsy, but showing a wide range of intensity between groups. Lesions were mainly restricted to the respiratory system, although 4 unvaccinated goats also showed extra-pulmonary TB lesions in liver (n=3), pericardium (n=2) and spleen (n=1).

The sum of gross lesions volume of lung and LN and the bacterial load in respiratory LN, are shown in Fig. 28 (A-B). Vaccinated groups showed a significant reduction of gross lesions (mean volume of gross lesions) and bacterial load (mean Log_{10} CFU/LN) in comparison to the unvaccinated group ($p < 0.001$). Significant differences were also found in the volume of gross lesions between the two vaccinated groups. The BCG-AdTBF group showed lower volume of gross lesions in lungs (mean Log_{10} cm^3: 0.9, 95% CI: 0.7-1.2; $p < 0.05$) and LN (mean Log_{10} mm^3: 2.3, 95% CI: 2.1-2.5; $p < 0.05$) compared with BCG group (mean Log_{10} cm^3: 1.3, 95% CI: 1.1-1.4; and mean Log_{10} mm^3: 2.9, 95% CI: 2.6-3.2; respectively). A direct correlation between bacterial load and total volume of gross lesions was only found in unvaccinated animals (r = 0.684, $p <$

0.05, Fig. 28C). In vaccinated animals, however, the gross lesions volume reduction was more pronounced than was the bacterial load reduction.

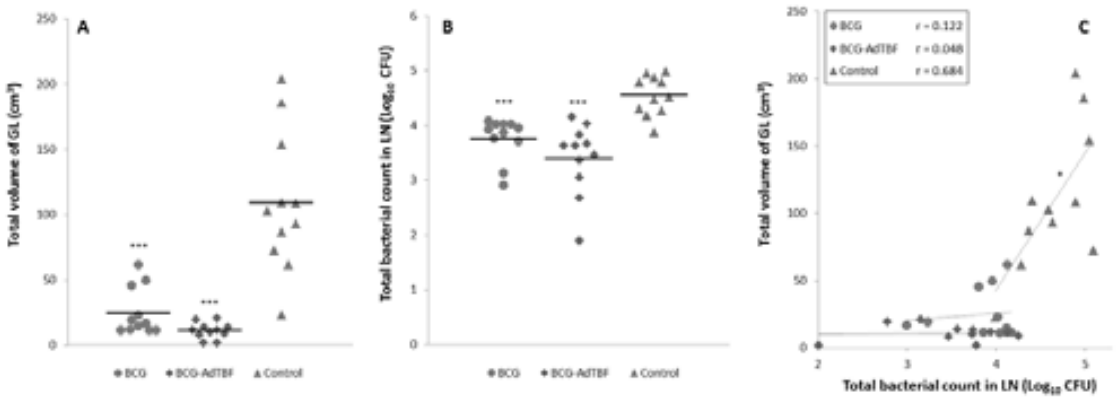
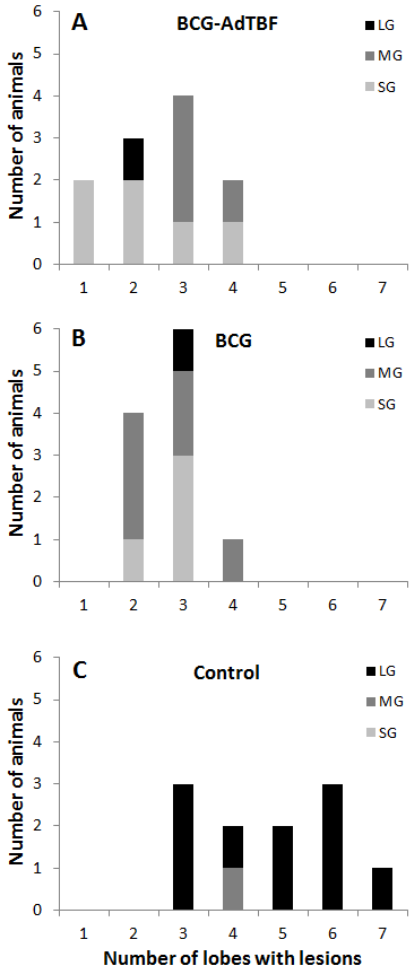


Figure 28. Quantification of pulmonary pathology and bacterial burden. (A and B) Individual volume of gross lesions (GL) in lungs (measured by CT) and thoracic LN (measured by direct visual examination) are expressed in cm^3, and the bacterial counts in thoracic LN are expressed as Log_{10}-transformed CFU. Horizontal lines indicate mean values. *p < 0.05, ***p < 0.001, one-way ANOVA/Student-Newman-Keuls multiple comparison test. (C) Correlation between the volume of VL and bacterial counts. Results are divided according to treatment groups (BCG, circles; BCG-AdTBF, diamonds; and Control, triangles). *p < 0.05, Pearson's correlation (r).

The extension of gross lesions in the lung was also assessed to classify animals depending on the maximum sized granuloma found in their lungs. Ten animals (4 BCG and 6 BCG-AdTBF) showed only SG, 11 animals (6 BCG, 4 BCG-AdTBF and 1 Controls) showed at least one MG and no LG, and 12 animals (1 BCG, 1 BCG-AdTBF and 10 controls) presented at least one LG. Classification of goats by maximum granuloma size revealed significant differences between vaccinated and unvaccinated groups (p < 0.001).



Vaccinated goats also presented lower dissemination amongst lung lobes. The mean number of affected lung lobes was of 2.7 (95% CI: 2.3-3.2) in the BCG group and of 2.5 (95% CI: 1.9-3.2) in the BCG-AdTBF group, both of them significantly lower than that found in the control group (4.7, 95% CI: 3.8-5.7, p < 0.001). The distribution of goats on each group with regard to maximum granuloma size classification and the dissemination of gross lung lesions (number of affected lobes) is shown in Fig. 29.

Figure 29. Individual relationship between the number of lung lobes with TB lesions (out of 7) and the classification according to the maximum sized granuloma found in the lungs (SG, Small Granuloma: < 0.5 cm diameter, clear grey; MG, Medium Granuloma: 0.5-2 cm diameter, grey; and LG, Large Granuloma: > 2 cm diameter, black). Results are divided according to the three groups: BCG-AdTBF (A), BCG (B) and Control (C).

3.3. Histopathological analysis

Five SG were collected from each animal and processed for histopathology, with the exception of 1 goat of the BCG-AdTBF group, in which only 4 granulomas were found. Thus, a total of 164 granulomas were analyzed (55 from BCG group, 54 from BCG-AdTBF group and 55 from Control group). Table 15 shows a qualitative granuloma evaluation of each group. Vaccinated goats had a higher proportion of SG with higher grade of necrosis (83%, $p < 0.05$), as well as more mineralization (38%, albeit not statistically significant) in comparison to unvaccinated controls (64% and 20% respectively). BCG and BCG-AdTBF groups showed a significant higher frequency of SG in advanced developmental stages (IV and III respectively, $p < 0.05$). Examples of representative SG of vaccinated groups are shown in Fig. 30 (A-D). By contrast, unvaccinated animals presented a higher proportion of SG at early stages (24% at stages I and II), showing poorly delimitated microscopic lesions with a higher number of SatG (51% with ≥ 4 SatG, $p < 0.01$, Fig. 30E-F). The mean number of SatG in each animal correlated positively with both the volume of gross lesions and the number of affected lung lobes (r = 0.418, $p < 0.05$; r = 0.546, $p < 0.01$; respectively).

Table 15. Histopathological analysis of pulmonary small granulomas (< 0.5 cm diameter).

		No. of granulomas (%) of each group		
Granuloma feature		BCG[a]	BCG-AdTBF[b]	Control[a]
Necrosis	Low or none	10 (18)	9 (17)	20 (36)
	High	45 (82)*	45 (83)*	35 (64)
Mineralization	Low or none	34 (62)	34 (63)	44 (80)
	High	21 (38)	20 (37)	11 (20)
Developmental stage	I	1 (2)	0 (0)	1 (2)
	II	6 (11)	6 (11)	12 (22)
	III	25 (45)	38 (70)*	26 (47)
	IV	23 (42)*	10 (19)	16 (29)
No. of multinucleated giant cells	< 10 or none	23 (42)	10 (19)	16 (29)
	≥ 10	32 (58)	44 (81)*	39 (71)
No. of satellite granulomas	None	15 (27)	9 (17)	7 (13)
	1-3	28 (51)	28 (52)	20 (36)
	≥ 4	12 (22)	17 (31)	28 (51)**

[a] Five granulomas /animal (N = 55).

[b] One animal only presented 4 granulomas (N= 54).

* $p < 0.05$, ** $p < 0.01$ (Chi-squared test).

Vaccinated animals showed a higher proportion of SG at later stages with partial or complete encapsulation (i.e. stages III and IV, Fig. 30A-D). These lesions showed highly organized structure containing a very low proportion of lymphocytes and macrophages surrounding the central necrotic area. On the contrary, microscopic lesions observed in many unvaccinated

animals showed SG at early-stages, with a poorly organized mixed inflammatory infiltrate composed of lymphocytes, macrophages and neutrophils and lower necrosis extension (see Fig. 30E-F).

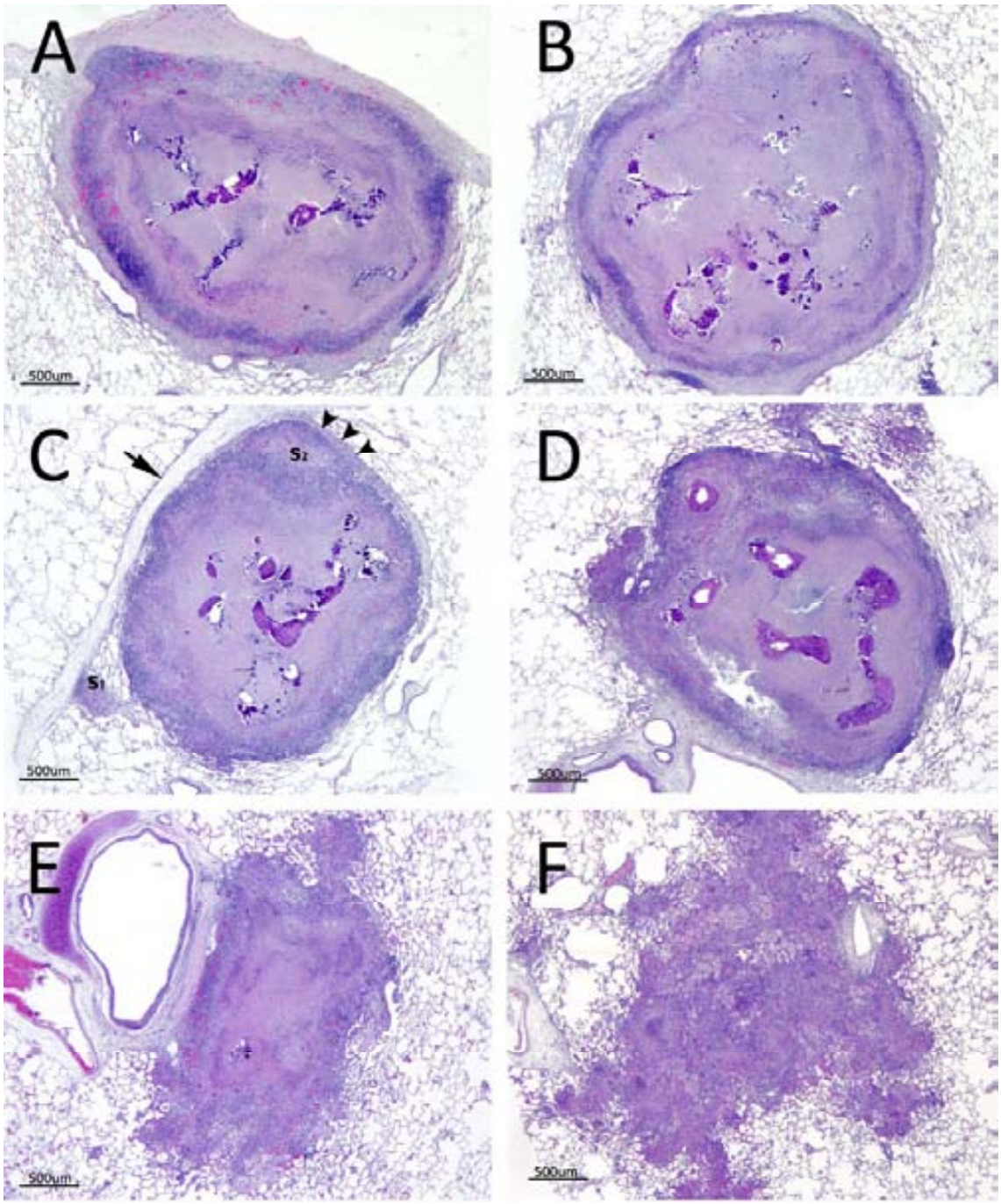


Figure 5. Staging of small granulomas (SG) by histopathological analysis. Representative developmental stages of the SG found in formalin-fixed lungs. (A-B) Stage IV. SG from 2 BCG-vaccinated goats. Encapsulation is complete. Extensive central necrosis with high degree of mineralization. (C-D) Stage III. SG from 2 BCG-AdTBF-vaccinated goats. Uncomplete encapsulation. Macrophages and lymphocytes surrounding the central necrosis and mineralization. Note the formation in C of 2 satellite granulomas (SatG, S_1 and S_2). S_2 is surrounded by a thin mantle of fibroblasts (arrowheads) which is connected to the intralobular septa (arrow). (E) Stage II. SG from an unvaccinated goat, presenting an irregular outline, central necrosis without mineralization and SatG formation. (F) Stage I. SG from an unvaccinated goat. Unstructured lesion presenting a diffuse mixture of inflammatory cells, without a distinguishable necrotic core.

Unvaccinated goats frequently showed SG which opened to the bronchiolar lumen, emptying its content into the airways leading to the formation of small cavities (Fig. 31A). Additionally, all unvaccinated goats presented conspicuous macroscopic cavitary lesions (Fig. 31B).

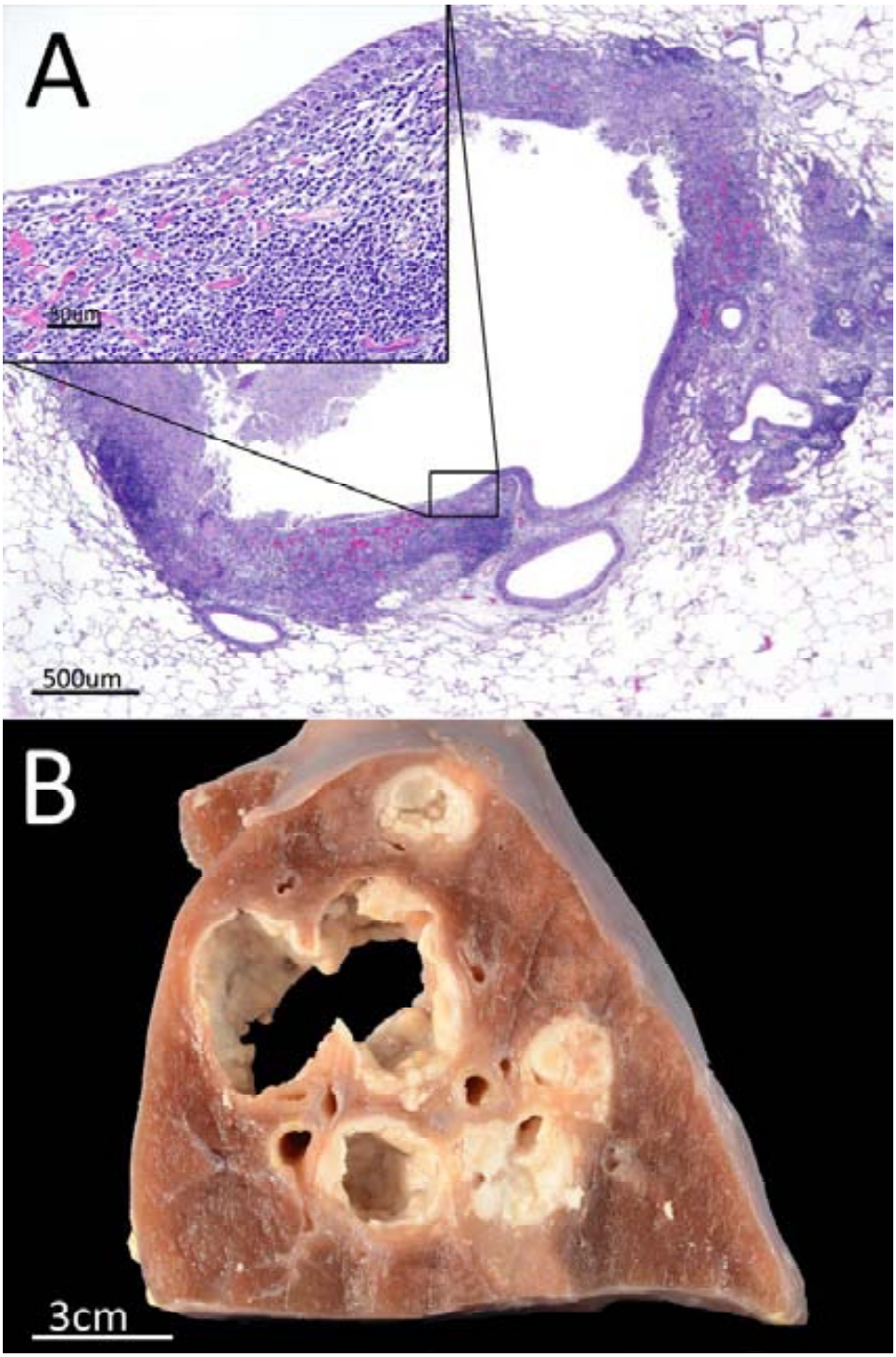


Figure 31. Progression of caseous necrosis to cavitary lesions. (A) The caseous necrosis in the centre of the granuloma progresses to liquefaction and, if not effectively encapsulated, the necrotic reaction destroys the epithelium of an adjacent bronchiole, emptying the content of the liquefied lesion into the airways. (B). Large cavitation of pulmonary parenchyma, originates from progressive liquefaction of confluent granulomatous lesions, observed macroscopically in all unvaccinated goats.

3.4. Cell-mediated immune responses

The whole blood IFN-γ responses to E/C and Rv3615c-specific showed significant differences among groups at week 20, when the peak response was reached in unvaccinated animals (Fig. 32). At this time point, the mean E/C-specific IFN-γ responses in the control group were significantly higher than in the BCG ($p < 0.01$) and BCG-AdTBF ($p < 0.05$) groups. Also, the Rv3615c-specific IFN-γ responses in the control group were significantly higher than in both vaccinated groups ($p < 0.05$). At the rest of time points, responses were not significantly different among groups.

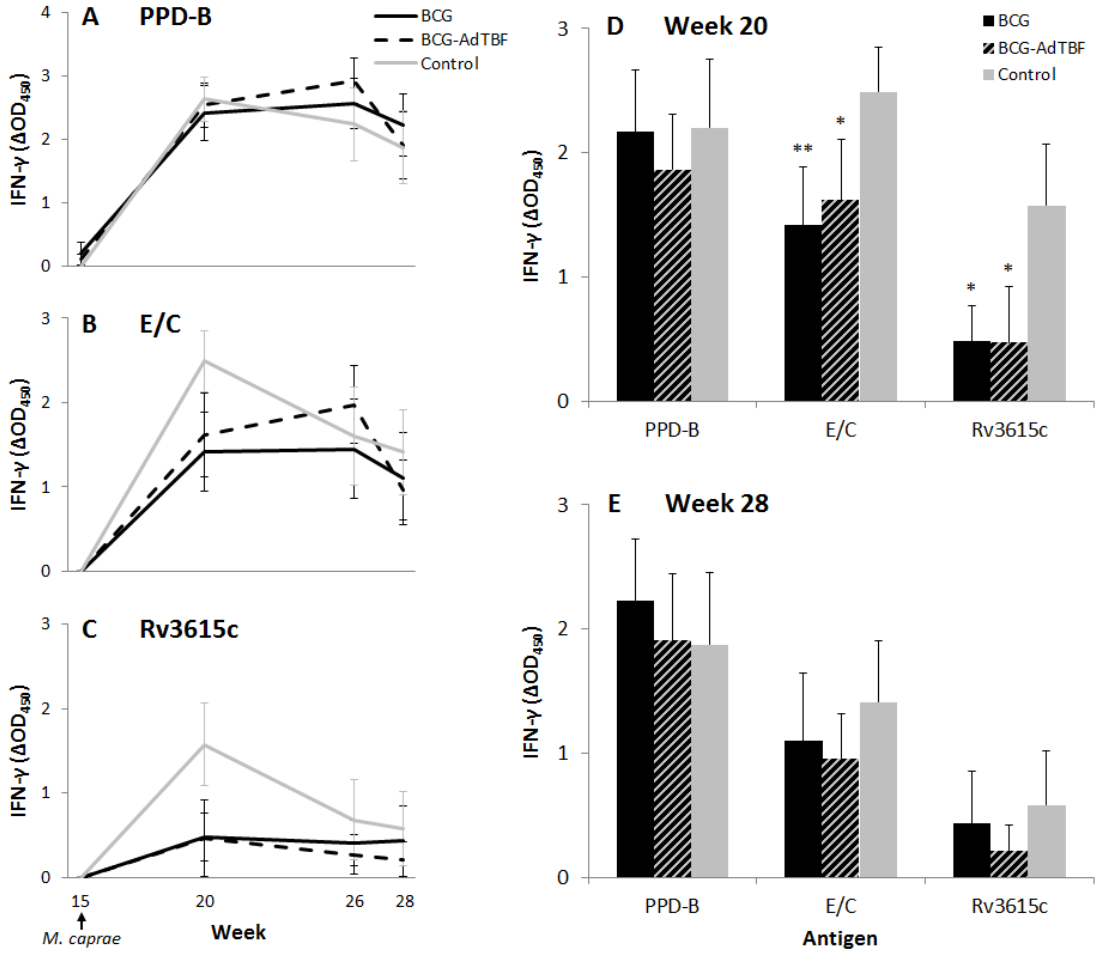


Figure 32. Antigen-specific IFN-γ responses after *M. caprae* challenge. Determination by ELISA of the IFN-γ released after peripheral blood stimulation with: (A), bovine PPD (PPD-B); (B), ESAT-6/CFP-10 peptide cocktail (E/C); and (C), Rv3615c protein. (D) Results at week 20 (5 weeks postinfection). (E) Results at the end point (week 28, 13 weeks postinfection). The results are expressed as mean ΔOD_{450} ± 95% CI for the 3 treatment groups (BCG, BCG-AdTBF and Control). *$p < 0.05$, **$p < 0.01$, Kruskal-Wallis/Mann-Withney *post hoc* test.

Ag85A-specific IFN-γ responses were undetectable throughout the experiment except at two weeks after AdTBF inoculation (week 10), when they were significantly higher in BCG-AdTBF group (mean ΔOD: 0.085, 95% CI: 0.021-0.149) compared with BCG (mean ΔOD: 0.025, 95% CI: 0-0.05, $p < 0.05$) and control (mean ΔOD: 0.007, 95% CI: 0.04-0.01, $p < 0.01$) groups.

The results of the cultured ELISPOT carried out prior to challenge did not show significant differences of antigen-specific IFN-γ SFC between both vaccinated groups (data not shown).

3.5. Antibody responses

Mean IgG responses to AdTBF-encoded antigens in BCG-AdTBF prime-boosted goats are shown in Fig. 33. Two peaks were observed, one at two weeks after AdTBF-immunization and the second two weeks after PPD-B and PPD-A intradermal inoculations (weeks 10 and 28 respectively, Fig. 33A). At these time points significant differences were found among the different antigen-specific antibody responses (Fig. 33B). In addition Acr2-specific antibody responses increased progressively after challenge (Fig. 33C). Humoral responses to AdTBF-encoded antigens were undetectable in the other two groups throughout the experiment.

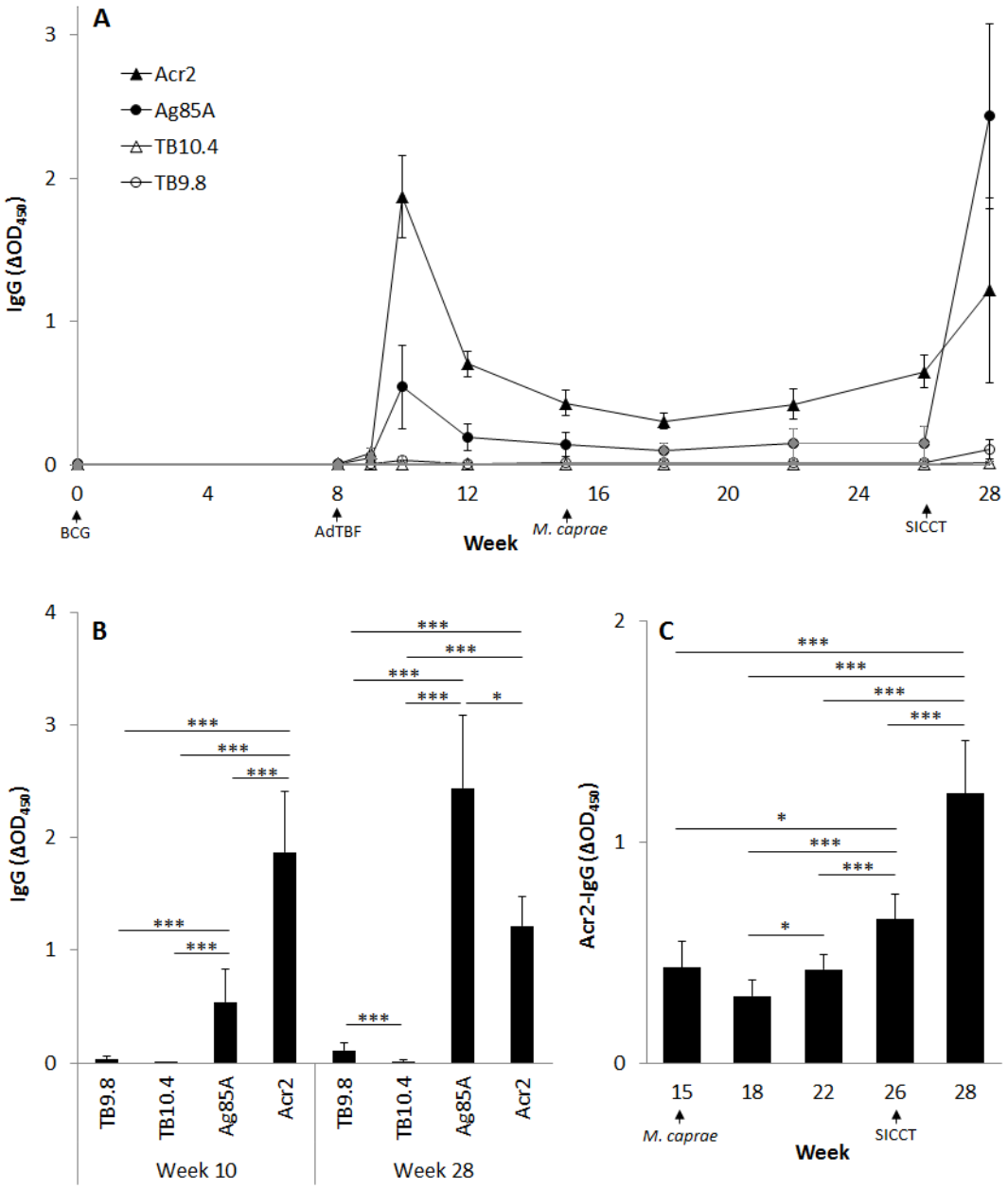


Figure 33. Vaccine and challenge-induced antibody responses. (A) Kinetics of the IgG specific responses in the 11 goats primed with 5×10^5 CFU BCG (week 0), boosted with 10^9 PFU AdTBF (week 8) and challenged wit 1500 CFU *M. caprae* (week 15), to the four AdTBF antigens (Acr2, Ag85A, TB10.4 and TB9.8). SICCT: single intradermal comparative cervical tuberculin test (week 26). (B) Comparison of IgG responses at 2 weeks after AdTBF immunization (week 10) and SICCT test (week 28). (C) Acr2-specific IgG responses postchallenge. *$p < 0.05$, ***$p < 0.001$, Kruskal-Wallis/Wilcoxon *post hoc* test.

The antibody responses to *M. caprae* were measured as MPB83-specific IgG. At week 20, a peak of IgG levels was found in the control group (mean ΔOD 1.2, 95% CI: 0.2-2.1), whereas IgG levels in vaccinated animals were practically undetectable in the two vaccinated groups (both with mean ΔOD: 0.01, 95% CI: 0-0.03), and thus were significantly lower compared with the control group ($p < 0.001$). All animals presented higher IgG levels at week 28 (after intradermal tuberculin boost effect) and statistically significant differences were not found among the three groups (Control group mean ΔOD: 2.9, 95% CI: 2.1-3.7; BCG group mean ΔOD: 2.4, 95% CI: 1.6-3.2; BCG-AdTBF group mean ΔOD: 2, 95% CI: 1.1-2.8).

3.6. Assessment of biomarkers

Once the critical time points where the immunological responses were significantly different among groups were determined, individual responses with postmortem parameters were compared. Immunological biomarkers were identified as predictors of vaccine efficacy or correlates of infection (assessed prior and after challenge respectively) as previously defined (Vordermeier et al., 2012).

Results of the IFN-γ cultured ELISPOT performed prior to challenge (week 15) were evaluated to identify predictors of vaccine efficacy. Fig. 34 shows the correlations between IFN-γ antigen-specific SFC and the total volume of gross lesions in 10 vaccinated goats (5 from BCG group and 5 from BCG-AdTBF group). Only Ag85A-specific SFC showed significant inverse correlation with gross lesions (r = -0.557, $p < 0.05$). Even though without statistical significance, slightly inverse and direct correlations of SFC specific to TB9.8 and PPD-B with the volume of gross lesions were also obtained (r = -0.276 and r = 0.445, respectively). No evident correlation was found between cultured ELISPOT results and bacterial counts (data not shown).

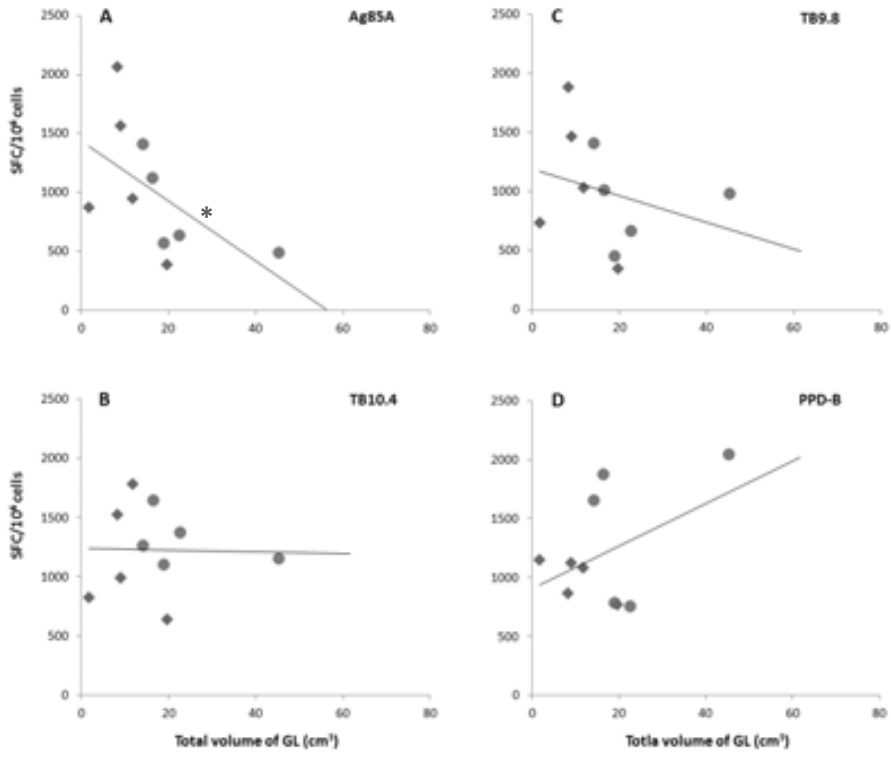


Figure 34. Vaccine-induced memory T cells correlated with infection outcome. IFN-γ cultured ELISPOT responses to bovine PPD (PPD-B) and 3 AdTBF antigens (Ag85A, TB9.8 and TB10.4) performed prior to *M. caprae*-challenge (week 15) in 10 vaccinated goats (5 BCG [●] and 5 BCG-AdTBF [◆]). Figure shows the correlations of Spot Forming Cells (SFC) with the total volume of gross lesions (GL) measured postmortem. *$p < 0.05$, Pearson's correlation.

Whole blood IFN-γ responses to E/C and Rv365c, as well as antibody responses to MPB83 were identified as correlates of disease progression. At week 20 of the experiment (when significant differences among groups were more pronounced), IFN-γ responses to both E/C and Rv3615c, as well as IgG responses to MPB83, correlated positively with the total volume of gross lesions (Spearman's ρ: 0.388, $p < 0.05$; 0.519, $p < 0.01$; 0.524, $p < 0.01$; respectively) and with the bacterial load (Spearman's ρ: 0.559, $p < 0.001$; 0.4, $p < 0.05$; 0.481, $p < 0.01$; respectively).

Specific IgG responses to AdTBF antigens did not show significant correlation with postmortem parameters neither after AdTBF vaccination (week 10) nor prior to the end point of the study (week 28).

4. Discussion

Recently, our research group, in collaboration with other partners involved in human and animal TB vaccine development, proposed domestic goats as a new experimental TB model to be used in trials with the advantage of its simple husbandry and relatively low economical costs (Pérez de Val et al., 2011). This model has been used herein to assess the efficacy of AdTBF, a new vaccine booster candidate against TB containing four MTBC antigens, in combination with BCG, following a heterologous prime-boost procedure (BCG priming and AdTBF boosting 8 weeks after). In BCG-AdTBF vaccinated animals, a protection improvement was shown compared to the protection observed in animals vaccinated only with BCG, namely a reduction of the bacterial load and the severity of the pathology. A previous study using BCG and an Ag85A-monovalent-booster vaccine with the same vaccination strategy yielded similar results in terms of protection (Pérez de Val et al., 2012).

In the present study, unvaccinated and experimentally infected goats progressed to active disease, while vaccinated goats (particularly BCG-AdTBF treated animals), showed mainly small, well-delimitated lesions, with microscopic features similar to granulomas found in human LTBI. Goat and human lungs share essential morphologic characteristics, particularly in their intralobulillar septation by connective tissue (see Fig. 30C). These structures may facilitate granuloma encapsulation and contention of mycobacterial spreading (Gil et al., 2010). On the contrary, the small laboratory animal models often used in TB vaccine trials, such as mice, guinea pigs, rabbits and even macaques, do not show this lung compartmentalization (Plopper and Harkema, 2005).

Furthermore, goats have the advantage of reproducing the main features of human active TB more accurately than other large animals. Even though cattle infected with *M. bovis* show similar lung granulomatous reaction and immune response to TB as humans do (Hewinson et al., 2003), the macroscopic lesions do not usually show evident cavitations as those observed in human patients with active TB (Buddle et al., 2005b). By contrast, lung cavitary lesions are frequently found in naturally infected goats (Domingo et al., 2009) and are also induced upon experimental infection (Pérez de Val et al., 2011).

With the aim of establishing a relationship between the protective effect of vaccination and the lesion pattern, we adopted, for the goat lung lesions, the four stage classification of LN granulomas developed by Wangoo et al. for *M. bovis* experimentally infected calves (Wangoo et al., 2005). Then we focused our histopathological study in the morphological features of

small granulomas (SG, lesions with < 0.5 cm diameter). Our aim was to determine if qualitative differences existed in a comparable lesion which could be found in the three groups of animals. After a comprehensive microscopic analysis, differences in structural features of SG were found between vaccinated and unvaccinated goats. Unvaccinated goats showed higher proportion of SG at initial developmental stages, while most SG from vaccinated animals showed characteristics of older, more evolved lesions, namely high degree of necrosis, mineralitzation and partial or total encapsulation by connective tissue.

Poorly organized SG (found mainly in unvaccinated goats), without evident arrangement in layers of lymphocytes and macrophages, suggest a lower capacity for cellular activation and mycobacteria elimination (Kaufmann. 2001). It could be speculated that these SG would easily progress to bigger granulomatous lesions. Indeed, all unvaccinated goats presented a variable number of LG, many of them liquefacted (shown at Fig. 29).

At the microscopic level, we have introduced an additional parameter to evaluate lesion containment, namely the formation of small satellite granulomas (SatG) surrounding a central lesion. Histological examination suggests that SatG surrounding the main lesion will likely progress to new SG, ultimately generating confluent multifocal larger lesions. The positive correlation between the extent of the pathology (volume of gross lesions and number of affected lung lobes) and the mean number of SatG (per animal) also supports this speculation.

SG in stages III and IV resemble lesions found in human LTBI (Canetti. 1955). Rapid granuloma encapsulation, avoiding its liquefaction, cavitation and bacillary drainage towards airways, has been hypothesized as a key step for containing TB infection, and as an explanation for the induction of a human-like LTBI in a minipig model of TB (Gil et al., 2010).

Also, the absence of big granulomatous lesions in BCG-AdTBF vaccinated animals (60% only showed a few SG) is in agreement with a lower Th1 pro-inflammatory response. Moreover, in some vaccinated individuals the gross lesion volume was proportionally more reduced than was the bacterial load found in the draining LN. This is consistent with the lower specific IFN-γ response against growth-related antigens (i.e. ESAT-6, CFP-10 and Rv3615c) in vaccinated animals, suggesting that the bacterial load found might be mainly composed of a non-replicating bacilli subpopulation (Guirado et al., 2008), similarly to what occurs in human LTBI. These observations suggest that vaccine-dependent pathogenesis of granuloma formation and development might exist in the goat TB model. This could contribute to the knowledge of LTBI inducing mechanisms and its progression to active TB in humans.

Induction of a specific protective immune response seems crucial to determine the pathological outcome. In this sense, the cultured IFN-γ ELISPOT is an innovative method for measuring memory T-cell immunity, which has been successfully used as predictor of vaccine efficacy against infections caused by intracellular pathogens such as malaria (Keating et al., 2005) or TB (Vordermeier et al., 2009). Accordingly, we found a negative correlation between Ag85A-specifc SFC and the total volume of gross lesions (lungs and LN). These results endorse the measurement of specific IFN-γ-producing memory cells as a predictor of TB vaccine efficacy. Unfortunately, recombinant Acr2 protein was not ready to use as PBMC-stimuli when the cultured ELISPOT was performed and Acr2-specific SFC data are missing.

Notwithstanding, the goats produced high levels of Acr2-specific antibodies after AdTBF-vaccination, indicating that the antigen was effectively expressed. These animals also showed a continuous increase of Acr2-specific antibody levels after challenge. Similarly, a progressive increase of Acr2-specific IFN-γ responses has been observed after challenging calves with *M. bovis* (Dean et al., 2008). The Acr2 is a heat shock protein belonging to the α-crystalin (Acr) family, involved in the survival of *M. tuberculosis* under stress conditions in a non-replicating state (Stewart et al., 2005; Pang and Howard, 2007). It has certain aminoacidic homology with the latency-associated protein Acr1 (16kDa), and is also an early immunodominant target for T-cells to MTBC infection (Wilkinson et al., 2005). Thus, the Acr2-induced CMI deserves further investigation of its potential use as immunological biomarker.

Concerning the immune correlates of infection assessed in the present study, the positive correlation of the specific IFN-γ levels to E/C and Rv3615c, as well as the specific antibody levels to the surface protein MPB83, with the severity of the pathology, are in agreement with other studies performed in calf and goats experimentally challenged with *M. bovis* and *M. caprae* respectively (Vordermeier et al., 2002; Lyashchenko et al., 2004; Pérez de Val et al., 2011; Pérez de Val et al., 2012).

Recently, the modified Vaccinia Ankara virus expressing antigen 85A (MVA85A), the first vaccine candidate after BCG, failed to confer statistically significant protection against TB disease or infection in 2794 south African infants (Tameris et al., 2013). Despite this discouraging result, several other viral-delivered vaccines in clinical trials have shown improved protection in a number of animal models (Dietrich et al., 2006; Vordermeier et al., 2009; Xing et al., 2009; Lin et al., 2012). Indeed, the results presented herein show enhanced protection of BCG-primed and AdTBF-boosted goats after the *M. caprae* challenge.

However, our findings are far away from the goal of obtaining a vaccine that prevents mycobacterial infection. In the last decade, the mucosal immunity in the respiratory airways has been receiving major attention in inducing protection against certain mucosal infectious diseases (Kyd et al., 2001). The availability of mucosal antibodies at the alveolar space might prevent that bacilli reach the lung and establish the initial infection (Ottenhoff and Kaufmann, 2012). A future generation of vaccines could be focused on inducing pre-existing antibodies at the site of infection to eradicate the mycobacteria before they can hide inside the target cells (Kaufmann. 2010). In this regard, mucosal vaccination studies have already showed encouraging results in terms of protection against *M. tuberculosis* in small animal models (Santosuosso et al., 2006; Xing et al., 2009). Future trials using the experimental goat TB model could be directed towards: a) the use of *M. tuberculosis* challenge instead of *M. caprae* and b) the use mucosal administration routes of TB vaccine.

Following the “One health” approach, promoted by the WHO and OIE, the present study addresses the public and animal health challenge of TB control and eradication. Using goats as a large animal model of TB, we have shown an improvement of protection, when BCG vaccinated goats are boosted with AdTBF. These results support further evaluation of adenoviral-based multi-antigenic TB vaccines in clinical trials.

5. Acknowledgements

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STUDY IV

Effects of vaccination against paratuberculosis on tuberculosis in goats: diagnostic interferences and cross-protection

Abstract

Most countries carrying out campaigns of bovine tuberculosis (TB) eradication impose a ban on the use of mycobacterial vaccines in cattle. However, vaccination against paratuberculosis (PTB) in goats is often allowed even when its effect on TB diagnosis has not been fully evaluated. To address this issue, goat kids previously vaccinated against PTB were experimentally infected with TB.

Evaluation of interferon-γ (IFN-γ) secretion induced by avian and bovine tuberculins (PPD) showed a predominant avian PPD-biased response in the vaccinated group from week 4 post¬vaccination onward. Although 60% of the animals were bovine reactors at week 14, avian PPD-biased responses returned at week 16. After challenge with *M. caprae*, the IFN-γ responses radically changed to show predominant bovine PPD-biased responses from week 18 onward. In addition, cross-reactions with bovine PPD that had been observed in the vaccinated group at week 14 were reduced when using the M. tuberculosis complex-specific antigens ESAT-6/CFP-10 and Rv3615c as new DIVA (differentiation of infected and vaccinated animals) reagents, which further maintained sensitivity post-challenge. Ninety percent of the animals reacted positively to the tuberculin cervical comparative intradermal test performed at 12 weeks post-infection. In addition, post-mortem analysis showed reductions in tuberculous lesions and bacterial burden in some vaccinated animals, particularly expressed in terms of the degree of extrapulmonary dissemination of TB infection.

Our results suggest a degree of interference of PTB vaccination with current TB diagnostics that can be fully mitigated when using new DIVA reagents. A partial protective effect associated with vaccination was also observed in some vaccinated animals.

1. Introduction

Caprine tuberculosis (TB), caused either by *Mycobacterium bovis* or *M. caprae*, and paratuberculosis (PTB), caused by *M. avium* subsp. *paratuberculosis* (*Map*), are endemic diseases in goat herds of the Iberian Peninsula (de Juan et al., 2006; Duarte et al., 2008; Rodriguez et al., 2011). Both infections may have an impact in terms of economic loss. Moreover, *M. caprae* and *Map* can be transmitted between domestic hosts and wildlife species (de Juan et al., 2006; Stevenson et al., 2009). In addition, *M. caprae* is a zoonotic agent (Kubica et al., 2003; Prodinger et al., 2005; Cvetnic et al., 2007) and *Map* has been associated with Crohn's disease (Grant. 2005; Juste et al., 2009a).

Control of PTB in small ruminants can be facilitated by vaccination because the bacterial burden is greatly reduced, containing the spread of the disease and preventing clinical expression (Uzonna et al., 2003). Furthermore, vaccination against PTB with *Map*-killed formulations has been demonstrated to be an economically efficient strategy to achieve control of the disease in small ruminants (Juste and Casal, 1993; Reddacliff et al., 2006; Singh et al., 2007) and cattle (Juste et al., 2009b).

On the other hand, it has been shown that exposure to *M. avium* can interfere with the diagnosis of *M. tuberculosis* complex organisms such as *M. bovis* or *M. caprae* (Amadori et al.,

2002; Howard et al., 2002; Hope et al., 2005). Similarly, there are some reports showing that natural infection with *Map* compromises TB diagnosis in cattle [(Muskens et al., 2002; Álvarez et al., 2009)(Álvarez et al., 2008) and goats (Álvarez et al., 2008). Therefore, PTB could also affect the specificity of diagnostic tests used in TB control programs. The Spanish eradication program of bovine TB expressly forbids the use of *Map*-based vaccines in cattle because of this potential interference with TB diagnostics. In contrast, vaccination against PTB in goats is permitted with the exception of a minority of herds that are subjected to TB control in some regions. To date, the potential effect of *Map*-based vaccines on the diagnosis of TB has only been investigated post hoc under field conditions (Bezos et al., 2012; Chartier et al., 2012).

It has been reported that TB diagnostic tests currently used in cattle, such as the skin test or the interferon-gamma (IFN-γ) assay, can be used for diagnosis of TB in goats (Gutiérrez et al., 1998; Liebana et al., 1998; Pérez de Val et al., 2011). Moreover, in recent years, new IFN-γ assays based on antigens secreted by active growing bacilli, such as the peptide cocktail ESAT-6/CFP-10 (E/C) or Rv3615c (Vordermeier et al., 2001; Sidders et al., 2008), have been developed as alternatives to bovine and avian tuberculins. These antigens are not present in either *M. bovis* BCG or *Map* and can be viewed as novel DIVA (differentiation of infected and vaccinated animals) regents able to distinguish TB-infected from TB-or PTB-vaccinated animals.

The aim of the present work was to assess how vaccination against *Map* affects standard and novel diagnostic tests in goats. Thus, we evaluated the interference of a commercial *Map*-killed vaccine on TB diagnosis in goats vaccinated and subsequently challenged with *M. caprae*.

The objectives of the present work were:

a) To investigate the possible interference of vaccination before and after *M. caprae* infection on TB diagnostic tests (single and comparative skin tests and IFN-γ assay).

b) To assess the usefulness of DIVA-peptide candidates E/C and Rv3615c.

c) To evaluate immunological and post-mortem indicators of the effects of PTB vaccination on *M. caprae* infection.

2. Materials and methods

2.1. Experimental animals

The experiment was carried out in 20 Murciano-Granadina female goats between 2 and 3 months of age from a herd free of TB and PTB in the Region of Murcia (southwest Spain). Before the experiment, goats were submitted to standard tests for diagnosis of TB: the single intradermal comparative cervical tuberculin (SICCT) test and IFN-γ assay (Bovigam™; Prionics AG, Schlieren, Switzerland). Experimental animals were confirmed to be negative for both tests. In addition, all goats had negative results for PTB (Paratub.Serum-S; Institut Pourquier, Montpellier, France).

2.2. Vaccination and infection

A group of 10 goats was subcutaneously inoculated with a single dose of 1 ml (2.5 mg/ml) of Silirum® (CZ Veterinaria, Porriño, Pontevedra, Spain), a commercial heat-inactivated and oil-

adjuvanted *Map* vaccine. Another group of 10 goats was maintained as unvaccinated controls. After 14 weeks, all animals were housed and acclimatized in two experimental boxes (level 3 biocontainment) for 1 week prior to experimental infection.

The field strain of *M. caprae* SB0416 (www.Mbovis.org) was used as the inoculum and was prepared as previously described (Pérez de Val et al., 2011). Goats were anesthetized by intravenous administration of 4 to 6 mg/kg of propofol (Propofol Lipuro®) and 0.2 mg/kg of midazolam (Dormicum®), and subsequently inoculated with approximately 1.5×10^3 cfu of *M. caprae* suspended in 0.5 ml of phosphate-buffered saline (PBS) by the endobronchial route (Pérez de Val et al., 2011).

All experimental procedures were approved by the Animal Welfare Committee of the Universitat Autònoma de Barcelona in agreement with the European Union Laws for protection of experimental animals.

2.3. Sampling and clinical observations

Animals were observed twice daily at feeding time. Prior to vaccination and infection and every 2 weeks throughout the experiment, animals were weighed and their rectal temperature was taken and blood samples were collected from the jugular vein into heparinized blood tubes for immunological studies.

2.4. Diagnostic tests

2.4.1. Skin tests

Skin tests were carried out in all goats at 26 wpv and 12 wpi. Four goats were also tested at 14 wpv (the remaining goats were not tested to minimize the potential effect of the intradermal tuberculin inoculation on the IFN-γ assay). Tests were performed by inoculating 0.1 ml (2500 IU) of bovine (PPD-B) and avian (PPD-A) tuberculins (Porriño, Pontevedra, Spain) on the left and the right side of the neck, respectively. The skin-fold thickness was recorded just before inoculation and after 72 h. Results were interpreted either by considering only the increase in thickness for PPD-B (as in a single intradermal tuberculin [SIT] test) or for both PPD-B and PPD-A (SICCT test). The results were read with the strict interpretation used in bovine TB eradication programs. Goats were considered positive to SIT test if the increase in skin-fold thickness at the PPD-B site was ≥2 mm. For the SICCT test interpretation, goats were considered positive if the increase in skin-fold thickness at the PPD-B site was ≥2 mm and higher than the increase at the PPD-A site.

2.4.2. IFN-γ assay

Whole-blood cultures were performed every 2 weeks in 96-well cell culture plates. Heparinized blood (1 ml per well) was incubated for 24 h at 37°C and 5% CO2 with either PPD-B or PPD-A at a final concentration of 10 μg/ml. Phytohemagglutinin (PHA, Sigma-Aldrich, Steinheim, Germany) was used as positive control at 10 μg/ml; PBS was used as a negative control. In addition, at 0, 2, 6, 10, and 14 wpi, the peptide cocktail ESAT-6/CFP-10 (E/C) and Rv3615c (Animal Health and Veterinary Laboratories Agency, Weybridge, UK) were used at a final concentration of 5 μg/ml. Plasma supernatants were collected after centrifugation and

transferred to a 96-well plate. The IFN-γ enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions (Bovigam™). Optical density was measured at 450 nm (OD_{450}) using a microplate reader (PowerWave XS; BioTek, Winooski, VT). Results were expressed as ΔOD_{450} (OD_{450} of antigen-stimulated sample minus OD450 of non-stimulated sample). A result was positive when $\Delta OD_{450} > 0.05$, whereas in the case of the standard test (using PPD-A and PPD-B as stimuli), a result was positive when ΔOD_{450} of PPD-B > 0.05 and OD_{450} of PPD-B > OD_{450} of PPD-A.

2.4.3. *Map* IgG ELISA

Plasma samples were analyzed in duplicate for antibodies to *Map* with Paratub.Serum-S™ ELISA before vaccination (week 0), before infection (week 14), and before the end of the experiment (week 28). The assay was performed according to the manufacturer's instructions. The ELISA reaction was also measured at OD_{450}. The results were expressed as S/P (%), calculated as (mean of sample OD_{450} -mean of negative control OD450)/(mean of positive control OD_{450} - mean of negative control OD_{450}) × 100. According to the criterion described by the manufacturer, a sample with an S/P of <45% was considered to be negative, of <55% and ≥45% to be doubtful, and of ≥55% to be positive.

2.5. Post-mortem examination

All goats were euthanized at 14 wpi (28 wpv) with an overdose of sodium pentobarbital administered intravenously. They were immediately necropsied to assess the presence and volume of tuberculous lesions in the lungs and pulmonary LN.

2.5.1. Lungs

Ten percent-buffered formalin was poured into the trachea, which then was tied, and the lungs were subsequently immersed in a container with formalin. After fixation, the lungs were sliced at 4-to 5-mm intervals, and each slice was photographed. Gross lesions were analyzed using image analyzer software (ImageJ 1.43u; National Institutes of Health, USA). The volume of gross lesions in each slice was calculated by multiplying the affected area and slice thickness. The total volume of gross lesions was calculated by adding the partial volumes of gross lesions obtained for each slice.

2.5.2. Lymph nodes

The number of gross lesions and their diameter were recorded for each LN at the time of necropsy. Data were recorded by the same pathologist to ensure measurement consistency. LN pathology scoring was calculated by the approximated total volume of granulomas per sample, calculated using the sphere formula ($4/3 \times \pi \times r^3$). After pathological evaluation, the whole LN was processed for bacterial culture to calculate the bacterial load.

2.6. Bacterial count

Cranial and caudal mediastinal, tracheobronchial, and retropharyngeal LN were individually weighed and homogenized with 10 ml of sterile distilled water using a tissue homogenizer (Masticator; IUL Instruments, Barcelona, Catalonia, Spain). Homogenates were

decontaminated with a final concentration of 0.35% w/v hexadecylpyridinium chloride (Corner and Trajstman, 1988) for 15 min with orbital shaking. Decontaminated homogenates were centrifuged at 2471 × g for 30 min, and pellets were resuspended in 10 ml of PBS containing 0.05% Tween 80. Aliquots of 0.1 ml of a 10-fold serial dilution of each homogenate were plated on Middlebrook 7H11 agar (BD Diagnostics, Sparks, MD). Plates were incubated at 37°C for 28 days, and colonies were then counted and the bacterial load (cfu/g) for each sample was calculated.

2.7. Statistical analysis

Student's unpaired two-sample t-test was used for comparisons between the groups in terms of differences in thickness increases in the SICCT test, S/P values of *Map* IgG ELISA, number of affected lung lobes, logarithm-transformed data of volumes of gross lesions (log_{10} mm^3), and bacterial loads in the LN (log_{10} cfu/g). Correlations between pathology and bacterial burden were assessed using linear regression analysis, whereas the non-parametric Spearman rank test was used to analyze correlations between IFN-γ responses (ΔOD_{450}) and post-mortem data. After cross-sectional analysis, IFN-γ responses and post-mortem parameters in the resulting groups were compared by applying the non-parametric Kruskal-Wallis test with Dunn's *post hoc* multiple comparison test. The normality of the data and the homogeneity of variances between treatment groups were assessed using the Shapiro-Wilk and Levene tests, respectively. Statistical analysis of the data was performed using SPSS Statistical Package version 17.0 (IBM Inc., Chicago, IL).

3. Results

3.1. Assessment of diagnostic tests

The effect of *Map* vaccination and subsequent infection with *M. caprae* on TB diagnostic assays based on cell-mediated immunity was assessed during this study. Four vaccinated goats were subjected to the SICCT test at week 14, and all were negative for TB and correctly classified as avian reactors. However, the four goats were classified as positive for TB if only the bovine tuberculin (PPD-B) result (as SIT test) was considered (data not shown), which indicated that the specificity of the SIT test was severely compromised by PTB vaccination.

The skin test was repeated in all goats at week 26 (12 weeks post-infection [wpi] with *M. caprae*). In the vaccinated group, 9 of 10 goats were bovine reactors (positive for TB) using the SICCT test (1 goat was an avian reactor), but all goats were classified as TB reactors by applying the SIT test interpretation. All unvaccinated goats were positive for TB with both tests (Table 16).

Differences between groups in the mean specific thickness increase for each tuberculin were statistically significant only for ΔPPD-A (avian tuberculin), with a mean increase of 14.4 mm (11.9–17, 95% CI) in the vaccinated group versus a mean increase of 10.8 mm (8.8–12.9, 95% CI) in the control group ($p < 0.05$, Table 16). Differences were also found in the mean value of ΔPPD-B minus ΔPPD-A. This value was significantly lower in the vaccinated group (5.2 mm, 2.9–7.5, 95% CI) than in the unvaccinated group (10.3 mm, 8.8–11.9, 95% CI, $p < 0.005$, Table 16).

Table 16. Increases in skin-fold thickness and corresponding results of tuberculin skin tests

Group	Goat	ΔAv [a]	ΔBov [b]	ΔBov-ΔAv	SICCTT[c]	SITT[d]
Vaccinated	1	14.2	22.6	8.4	+	+
	2	15.2	26.0	10.8	+	+
	3	17.6	20.9	3.3	+	+
	4	10.6	19.5	8.9	+	+
	5	16.2	18.1	1.9	+	+
	6	14.5	16.5	2.0	+	+
	7	6.2	14.2	8.0	+	+
	8	14.6	17.4	2.8	+	+
	9	13.2	19.4	6.2	+	+
	10	22.0	21.7	-0.3	- [e]	+
	Mean (95% CI)	14.4 (11.9-17)*	19.6 (17.5-21.7)	5.2 (2.9-7.5)**		
unvaccinated	11	11.3	19.7	8.4	+	+
	12	15.0	24.1	9.1	+	+
	13	3.3	14.5	11.2	+	+
	14	9.8	18.3	8.5	+	+
	15	11.3	21.2	9.9	+	+
	16	8.7	24.6	16.0	+	+
	17	11.4	19.1	7.7	+	+
	18	10.1	22.5	12.4	+	+
	19	14.6	25.8	11.2	+	+
	20	12.7	21.6	8.9	+	+
	Mean (95% CI)	10.8 (8.8-12.9)	21.1 (19-23.2)	10.3 (8.8-11.9)		

Test results are scored as (−) negative or (+) positive. [a] ΔAv, increase in skin-fold thickness (mm) 72 h after PPD-A application; [b] ΔBov, increase in skin-fold thickness (mm) 72 h after PPD-B application; [c] SICCTT, Single intradermal comparative cervical tuberculin test; [d] SITT, Single intradermal tuberculin test; [e] Avian reactor; *$p < 0.05$; **$p < 0.005$ (unpaired two-sample Student's t-test).

The tuberculin-based IFN-γ test was performed every 2 weeks throughout the experiment. The kinetics of IFN-γ responses of the vaccinated group showed a PPD-A-biased response, which switched to a PPD-B-biased mean response 4 weeks after *M. caprae* infection (at week 18 of the experiment, Fig. 35).

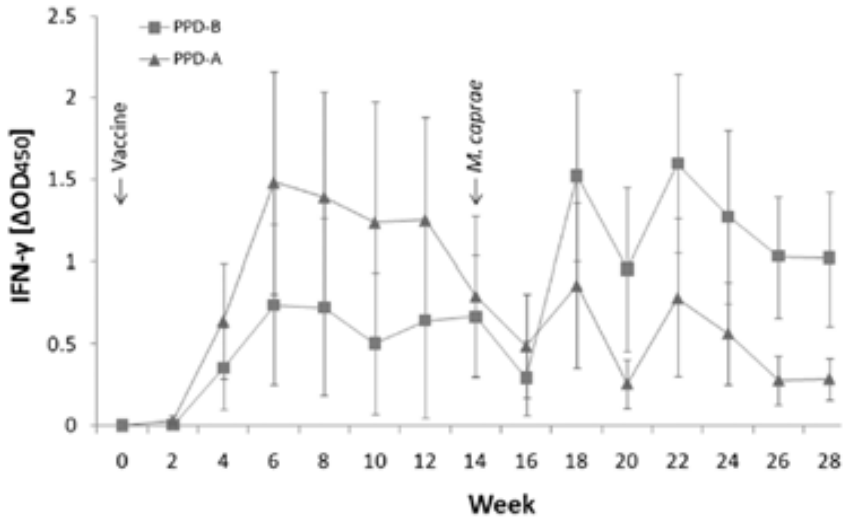

Figure 35. Kinetics of IFN-γ released after stimulation of whole blood with tuberculins in the vaccinated group. Avian tuberculin (PPD-A, ▲), bovine tuberculin (PPD-B, ■). Results are expressed as mean ΔOD450 (mean of OD450 of antigen-stimulated values with unstimulated values subtracted) ± 95% confidence interval.

In the unvaccinated control group, a PPD-B-biased response was obtained 4 weeks after *M. caprae* infection, whereas no response was observed before this time point in any of the goats (data not shown).

When the results were analyzed individually (Table 17), *Map*-vaccinated goats began showing responses to PPD-A ("avian reactors") at week 2 after vaccination (2 of 10 goats), and all of them subsequently became avian reactors between weeks 4 and 10. However, 1 of 10 goats became a "bovine reactor" (positive to PPD-B) at week 12 of the experiment (12 weeks post-vaccination [wpv]), and this figure rose to 6 of 10 at week 14, in the blood samples taken just before infection with *M. caprae*. Interestingly, in the next blood sampling at 2 wpi (week 16), again 8 of 10 goats showed a stronger reaction to PPD-A than to PPD-B, and the 2 other vaccinated goats were negative to both tuberculins. By contrast, at week 18 (4 weeks after *M. caprae* challenge), 9 of 10 (90%) of the vaccinated goats were bovine reactors. At week 20, all goats were positive to PPD-B (bovine reactors), and these results were maintained during the rest of the trial with the exception of one goat that tested bovine-negative at week 24 and another that was an avian reactor at week 28 (see Table 17). In the unvaccinated group, all goats were bovine reactors at week 18 and remained so until the end of the experiment, with the exception of two goats that were negative at week 24 but returned to positivity at weeks 26 and 28 (data not shown).

Table 17. Results of the standard IFN-γ assay (Bovigam®).

	Week														
Goat	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28
1	N	N	Av	Av	Av	Av	Av	Av	Av	Bov	Bov	Bov	Bov	Bov	Bov
2	N	Av	Av	Av	Av	Av	Av	Av	Av	Bov	Bov	Bov	Bov	Bov	Bov
3	N	N	Av	Av	Av	Av	Av	Bov	Av	Bov	Bov	Bov	Bov	Bov	Bov
4	N	N	Av	Av	Av	Av	Bov	Bov	Av	Bov	Bov	Bov	N	Bov	Bov
5	N	N	Av	Av	Av	Av	Av	Bov	N	Bov	Bov	Bov	Bov	Bov	Bov
6	N	N	Av	Av	Av	Av	Av	Av	Av	Bov	Bov	Bov	Bov	Bov	Bov
7	N	N	Av	Av	Av	Av	Av	Bov	N	Bov	Bov	Bov	Bov	Bov	Av
8	N	N	Av	Av	Av	Av	Av	Av	Av	Av	Bov	Bov	Bov	Bov	Bov
9	N	Av	Av	Av	Av	Av	Av	Bov	Av	Bov	Bov	Bov	Bov	Bov	Bov
10	N	N	Av	Av	Av	Av	Av	Bov	Av	Bov	Bov	Bov	Bov	Bov	Bov

Test results are classified as: Av, positive response to PPD-A; Bov, positive response to PPD-B; or N, neg(Pérez de Val et al., 2011)ative.

Next, we assessed the interference of *Map* vaccination with the IFN-γ assay by employing the defined antigen reagents E/C and Rv3615c. Responses were assessed at the same five time points described in the Materials and methods section. At weeks 14 (0 wpi) and 16, all goats were defined as TB-negative ($\Delta OD_{450} \leq 0.05$) according to the test results. At weeks 20, 24, and 28, 90%, 100%, and 70% of vaccinated goats were positive with the E/C cocktail and 100%, 90%, and 80% were positive with Rv3615c, respectively (Table 18). However, these proportions increased to 100%, 100%, and 90% at weeks 20, 24, and 28, respectively, when the E/C and Rv3615c results were considered together. Very similar results were obtained in the unvaccinated group, in which 100%, 80%, and 90% of goats were positive to E/C at weeks 20, 24, and 28, respectively; 100%, 80%, and 80% of goats were positive to Rv3615c; and 100%,

80%, and 90% of goats were positive again when combining the results for both antigens at the same time points (data not shown).

Table 18. Results of the IFN-γ assay using two DIVA reagents: ESAT-6/CFP10 (E/C) and Rv3615c

	Week									
	14		16		20		24		28	
Goat	E/C	Rv3615c	E/C	Rv3615c	E/C	Rv3615c	E/C	Rv3615c	E/C	Rv3615c
1	-	-	-	-	+	+	+	-	+	-
2	-	-	-	-	+	+	+	+	+	+
3	-	-	-	-	+	+	+	+	+	+
4	-	-	-	-	+	+	+	+	+	+
5	-	-	-	-	+	+	+	+	+	+
6	-	-	-	-	-	+	+	+	-	+
7	-	-	-	-	+	+	+	+	-	+
8	-	-	-	-	+	+	+	+	-	-
9	-	-	-	-	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	+	+

Test results are classified as (−) negative or (+) positive.

Seropositivity to *Map* was assessed using the Paratub.Serum-S ELISA kit (Institut Pourquier, Montpellier, France) applying the cut-off defined above. All goats (n = 20) were negative at week 0. Unvaccinated goats also remained negative during the experiment (data not shown). In contrast, 1 of 10 (10%) and 5 of 10 (50%) vaccinated goats were positive or doubtful at weeks 14 (0 wpi) and 28 (14 wpi), respectively, with different degrees of intensity (Table 19). The mean intensity of antibody response (%S/P) increased moderately, but was statistically significant ($p < 0.05$), from 20% (4%–36%, 95% CI) at week 14 to 55% (19%–91%, 95% CI) at week 28 (2 weeks after SICCT test).

Table 19. Seroreactivity to *Map* (Paratub.Serum-S™ ELISA).

	Week					
	0		14		28[a]	
Goat	Result	S/P	Result	S/P	Result	S/P
1	-	0%	-	30%	+	130%
2	-	0%	-	0%	-	0%
3	-	1%	-	8%	d	51%
4	-	0%	-	3%	+	113%
5	-	0%	-	36%	+	56%
6	-	0%	-	16%	-	19%
7	-	0%	-	6%	-	4%
8	-	0%	+	86%	+	157%
9	-	0%	-	4%	-	9%
10	-	0%	-	11%	-	11%

Test results are classified as (−) negative, (+) positive, or (d) doubtful.
[a] Two weeks after SICCT test.
[b] S/P, Seroreactivity rates.

3.2. Pathology and bacteriology

At necropsy on week 28 (14 wpi), visible pathology typical of TB was observed in the lungs and pulmonary lymph nodes (LN) of all animals irrespective of their vaccination status. Of the seven lung lobes evaluated, the vaccinated group presented an average of three lobes with gross lesions (2–4, 95% CI), slightly lower than the mean of four lobes (3–5, 95% CI) found in the unvaccinated group, but this difference was not statistically significant (p = 0.324).

The mean log10-transformed volume of gross lesions in lungs was similar in the unvaccinated

(1.3 $\log_{10}$ cm^3; 1–1.7, 95% CI) and vaccinated (1.1 log10 cm^3; 0.5–1.6, 95% CI) groups (p = 0.470), but values were spread over a wider range in the vaccinated group. By contrast, statistically significant differences between vaccinated and control goats were found after assessing the gross pathology in LN: The unvaccinated animals presented with a mean log10¬transformed volume of visibly affected tissue of 3.9 $\log_{10}$ mm^3 (3.6–4.3, 95% CI) compared with 3.3 $\log_{10}$ mm^3 (2.7–3.8, 95% CI) obtained in the vaccinated group ($p < 0.05$). The individual pathological parameters are represented in Fig. 36A and B.

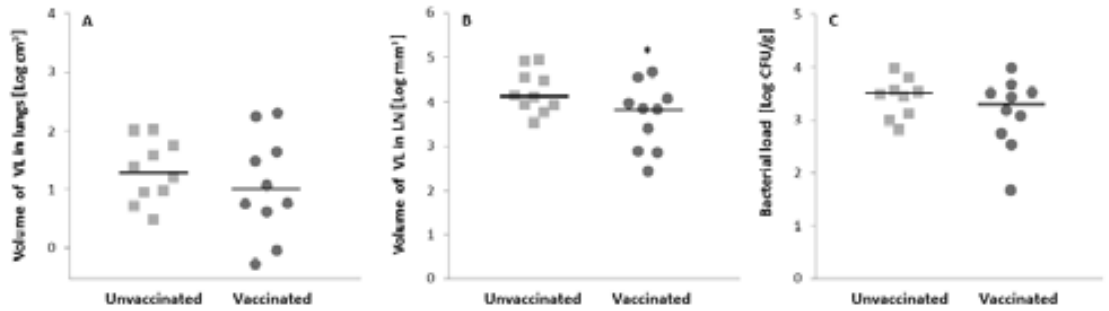

Figure 36. Post-mortem analysis as measured by gross pathology and bacterial burden. Results are plotted for individual goats. (A) Total volume of visible lesions (VL) in lungs as $\log_{10}$ cm^3. (B) Total volume of VL in respiratory lymph nodes (LN) as $\log_{10}$ mm^3. (C) Bacterial load as $\log_{10}$ cfu/g. (●) vaccinated goats, (■) unvaccinated goats. Horizontal lines indicate median values. Significance determined by unpaired t-test: *$p < 0.05$.

In addition, none of the vaccinated goats showed extrapulmonary gross lesions, whereas dissemination was observed in 4 of 10 unvaccinated goats that presented with gross lesions beyond the thoracic area (2 goats with lesions in retropharyngeal LN, 2 goats with lesions in mesenteric LN, and 1 goat with lesions in the spleen). These lesions were histopathologically confirmed to be tuberculous.

In three animals, focal lesions were also found in the LN draining the *Map* vaccine inoculation point (axillary, prescapular, or subcutaneous LN). These lesions were attributed to the vaccine inoculation because of their localization and histological characteristics: lesions predominantly necrotizing rather than granulomatous, without Langerhans cells, but surrounded by dispersed polymorphonuclear cells and abundant fibrosis.

Finally, the mycobacterial load in LN was calculated as $\log_{10}$ cfu/g. The bacterial burden in the pulmonary and retropharyngeal LN of all animals (n = 20) ranged from 2 to 4 $\log_{10}$ cfu/g (Fig. 36C). The total bacterial load in the unvaccinated group was 3.5 $\log_{10}$ cfu/g (3.2–3.7, 95% CI),

slightly higher than that in the vaccinated group (3.1 $\log_{10}$ cfu/g, 2.7–3.6, 95% CI). However, this difference was not statistically significant ($p = 0.203$).

3.3. Cross-sectional analysis

To evaluate the vaccine effect in terms of heterologous protection, post-mortem parameters were also assessed in combination. A mild positive correlation between bacteriological and pathological findings was found by correlating individual logarithmic values of bacterial load and total volume of visible lesions (r = 0.386, $p < 0.05$). However, a higher dispersion of individual values was observed in the vaccinated group compared with the unvaccinated group (Fig. 37A), and a stronger positive correlation was found when only vaccinated goats were assessed (r = 0.523, $p < 0.05$), whereas results obtained from unvaccinated goats did not show significant correlations (r = -0.274, $p > 0.05$).

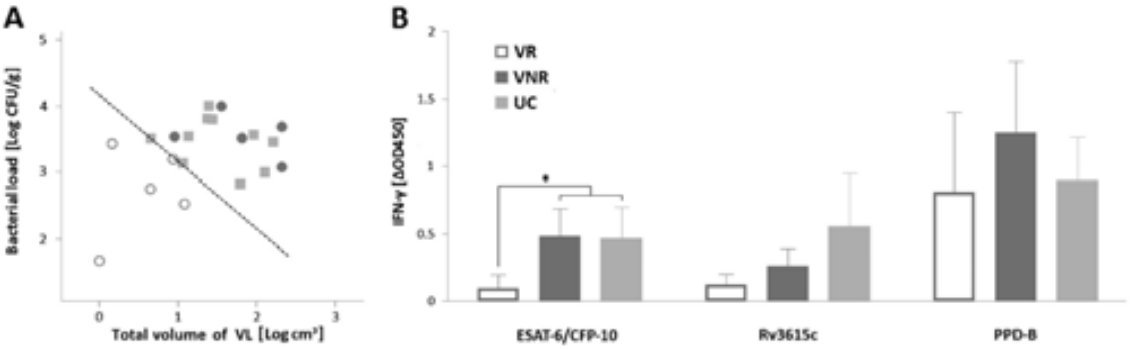

Figure 37. Protective efficacy measured by post-mortem results, and cross-sectional comparison of IFN-γ results with vaccination outcome. (A) Correlation between pathological and bacteriological parameters of all goats (n = 20). Dashed line (y = −x + 4.16) indicates the threshold defined to divide vaccine responders (VR) and vaccine non-responders (VNR). Symbols represent vaccine cross-protection outcome: (■) unvaccinated controls (UC), (○) VR goats, (●) VNR goats. (B) Specific IFN-γ responses at week 28 of the experiment as determined by ELISA after stimulation of whole blood with PPD-B, ESAT-6/CFP-10, or Rv3615c. Results are represented in relation to vaccine cross-protective outcome and are expressed as mean ΔOD_{450} ± 95% confidence interval. Significances determined by non-parametric Kruskal-Wallis test: *$p < 0.05$.

To further analyze the reductions in post-mortem parameters associated with vaccination, a cross-sectional study was performed on the basis of the criteria described in a previous TB vaccine efficacy trial (Vordermeier et al., 2009). The vaccinated animals were divided into two subgroups according to the severity of the pathology and the bacterial burden. This division was established by combining the data of the total volume of gross lesions and the bacterial load in LN. The mean between the volume of lesions ($\log_{10}$ cm^3) and the bacterial load ($\log_{10}$ cfu) was thereby calculated for each animal, and the cut-off point to classify the animals into categories of distinct vaccination outcomes was defined as the minimum value obtained among the unvaccinated control animals (Fig. 37A). Subsequently, a “vaccine responders” (VR) subgroup of five goats and another “vaccine non-responders” (VNR) subgroup of five goats were defined (Fig. 37A, open and closed circles).

Once the subgroup division was applied as described in the previous paragraph, the assumed pathological and bacteriological differences between the new subgroups were checked. The total volume of visible lesions and the bacterial load were significantly lower in VR compared with VNR and unvaccinated control (UC) animals ($p < 0.05$).

The release of IFN-γ specific to E/C, Rv3615c, and PPD-B at week 28 (performed just before sacrifice of the animals) was also compared between the VR, VNR, and UC groups (Figure 37B). A significantly lower IFN-γ to E/C response was found in VR animals than in VNR and UC animals ($p < 0.05$). The relationship between E/C-specific IFN-γ response and disease severity was confirmed by finding a positive correlation of IFN-γ-specific secretion to E/C at week 28 with the total volume of gross lesions (Spearman rank = 0.546, $p < 0.01$) and the bacterial load (but at the statistical limit of significance [Spearman rank = 0.379, $p = 0.050$], data not shown).

4. Discussion

The present experimental trial in goats was designed with the aim to determine the effects of *Map* vaccination on current TB diagnostic tests and assess the effects of PTB vaccination on TB infection.

All experimental goats became infected irrespective of vaccination status. However, a reduction in pulmonary pathology was observed in some vaccinated individuals compared with the unvaccinated group. Another remarkable finding was that all vaccinated goats showed only TB lesions at the site of infection (i.e., lungs and associated LN) in contrast to the increased dissemination frequency in non-vaccinated animals. This finding is analogous to the results of subcutaneous vaccination against PTB with the *Map*-killed vaccine in cattle subsequently challenged with *Map*. The vaccine in those studies induced systemic immunity, preventing bacteremia and, consequently, dissemination of mycobacteria from the primary infection site. However, they did not prevent the establishment of the initial infection (Uzonna et al., 2003)(Bezos et al., 2010). Similar results were obtained in another experimental infection with *M. caprae* in which three of six (50%) infected goats showed gross TB lesions in mesenteric LN (Bezos et al., 2010)(Daniel et al., 2009; Domingo et al., 2009; Quintas et al., 2010). This outcome is also consistent with findings observed in field cases of TB in goats (Daniel et al., 2009; Domingo et al., 2009; Quintas et al., 2010).

In this sense, the present results indicate a certain degree of containment of dissemination of the infection from the primary complex, even when a significant reduction in the bacterial load in pulmonary drainage LN has not been observed. Nevertheless, this containment may not be sufficient to effectively prevent excretion of mycobacteria and horizontal transmission within a herd.

The role of the immunological status of vaccinated animals, especially the T-cell response, can be critical in terms of control or spread of the infection from the primary respiratory focus. Similarly, Hope *et al*. found few lesions at necropsy and smaller bacterial loads in LN in calves inoculated with *M. avium* and subsequently challenged with *M. bovis* (Hope et al., 2005). The authors suggested that T-cell responses resulting from *M. avium* infection enhanced a protective secondary response after challenge with *M. bovis*.

After conducting cross-sectional analysis, we found positive correlations of the IFN-γ response to E/C with pathology severity, and more weakly with bacterial load. A similar result was obtained with the single ESAT-6-specific IFN-γ response compared with pathology scores in goats (Domingo et al., 2009) and calves (Vordermeier et al., 2002). Furthermore, a positive correlation between the E/C-specific IFN-γ response and bacterial load was also previously

described in goats (Pérez de Val et al., 2011) and cattle (Lyashchenko et al., 2004). Moreover, after analyzing post-mortem data, clear differences in the pathological and bacteriological results were found within the vaccinated group. The IFN-γ responses of vaccinated goats with low and high post-mortem scores (VR and VNR, respectively) were compared. The significantly lower E/C-specific IFN-γ responses obtained in VR animals demonstrated the capacity of this immunological biomarker to predict the severity of the disease and, by default, to predict the vaccine outcome.

In terms of assessment of TB diagnostic tests, we have adapted the diagnostic tests routinely used in cattle in bovine TB eradication programs. We have also introduced new DIVA reagents to perform the IFN-γ assay. Sensitivities obtained in all tests were higher than those obtained in previous studies in naturally infected goats (Gutiérrez et al., 1998; Álvarez et al., 2008). Notwithstanding this, the enhanced sensitivity described in these works when combining skin tests and IFN-γ tests was also confirmed in the present experimental trial.

The sensitivities of skin tests performed in vaccinated animals (100% and 90% for SIT and SICCT tests, respectively), were higher than those obtained in another study carried out with goat herds naturally co-infected with TB and PTB (71% and 42.7%, respectively) (Álvarez et al., 2008). In this work, the authors reported many animals with false-negative SICCT test results that showed higher skin-fold thickness to PPD-A than to PPD-B. It is important to note that the sensitivity of SICCT test in the present study was achieved using strict interpretation of the test. With the standard interpretation (ΔBov-ΔAv ≥ 3 mm), the sensitivity would be reduced to 60%.

By contrast, in our trial, only a slightly higher sensitivity was found when applying the SIT test in comparison with the SICCT test. However, the results showed serious compromise of the specificity of the SIT test in vaccinated animals prior to challenge (4 of 4 false-positive reactors), which completely disappeared when using the SICCT test. These results are in accordance with those found in another study performed in a PTB-vaccinated dairy goat herd free of TB (Chartier et al., 2012). Similarly, compromise of the specificity of the SIT test in PTB-vaccinated deer has also been reported (Mackintosh et al., 2005; Stringer et al., 2011).

Compared with the SIT test, the standard IFN-γ assay seemed to be more robust in terms of specificity. False-positive results were concentrated at the interval between weeks 12 and 14, and then disappeared at week 16 when there was not yet a response to *M. caprae* infection. After 12 wpv, the IFN-γ responses to PPD-A decreased faster than did those to PPD-B (see Fig. 35). As a consequence, 60% of the animals were false-positive “bovine reactors” at week 14. More long-term trials must be performed to study the kinetics of the IFN-γ response to *Map* vaccination.

Encouragingly, the sensitivity obtained for the standard IFN-γ test after *M. caprae* challenge was very high considering that elevated whole-blood IFN-γ responses to PPD-A were previously observed in *Map*-vaccinated cattle (Platt et al., 2010). In this sense, from weeks 18 to 28 (4–14 wpi), we only detected a masking due to PPD-A in two vaccinated animals. However, the high IFN-γ responses observed in our study (measured shortly after experimental infection) may decrease with the progression of natural TB infection under field conditions.

In the last decade, much effort has been focused on the development of novel antigens for bovine TB diagnosis that are more sensitive and specific than the avian and bovine tuberculins. This research has already resulted in the identification of several antigens, such as ESAT-6, CFP-10, and Rv3615c, that reduce cross-reactive immune responses to different mycobacterial infections or vaccinations in cattle (Vordermeier et al., 2001; Cockle et al., 2006; Sidders et al., 2008) and goats (Bezos et al., 2011; Pérez de Val et al., 2012). In the present study, we showed the capacity of both E/C and Rv3615c to distinguish PTB-vaccinated and TB-infected goats. Furthermore, no differences in sensitivity were observed between the experimental groups. Importantly, when combining the positive results of E/C and Rv3615c IFN-γ assays, the sensitivity was identical to that obtained by the tuberculin-based IFN-γ assay, which is currently being employed as an ancillary test to the skin test in some eradication campaigns. A similar pattern was previously described in cattle experimentally infected with *M. bovis*, in which the sensitivity increased from 77.9% to 91% when considering the results of Rv3615c and E/C IFN-γ assay together (Sidders et al., 2008).

By contrast, serological responses to the *Map* vaccine were moderate; only one and four vaccinated goats were seropositive to PTB at weeks 14 and 28, respectively. These data are not in agreement with the 50% positivity previously obtained in PTB-infected goats (Gumber et al., 2006). Interestingly, a boost effect on the *Map* ELISA due to SICCT test might be found at week 28 (2 weeks after tuberculin testing) when comparing the results with the test performed at week 14. The boost effect on the IgG response due to the skin test was previously described in M. bovis-infected cattle (Lyashchenko et al., 2004)(Pérez de Val et al., 2011) and *M. caprae*-infected goats (Pérez de Val et al., 2011).

Vaccination against *Map* represents an important advance in controlling PTB and improving the economic balance of affected farms. Therefore, the pros and cons of its application must be exhaustively evaluated. An attractive speculation is that the partial protection to TB infection observed in some PTB-vaccinated animals could indirectly facilitate the control of TB, although long-term field studies are required to confirm this perspective. Moreover, we have demonstrated that to some degree, PTB vaccination interfered (after *M. caprae* infection) with the sensitivity of tuberculin-based TB diagnostic tests. However, beyond the first 2 weeks post-infection, only 5% false-negative results were obtained in the IFN-γ assay. In addition, these animals reacted positively in more than 80% of the remaining post-challenge tests. Thus, considering the collective basis on which TB tests are usually applied, it is unlikely that an infected herd could be undiagnosed upon extrapolation to a larger population. On the other hand, our results confirm that the interference with the specificity can be fully overcome by using defined DIVA reagents. Thus, developing and subsequently introducing these reagents into routine diagnostics could represent an improvement in both strategies: control of PTB by vaccination and control of TB by rapid detection of infected animals.

5. Acknowledgements

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DISCUSIÓN GENERAL

Dentro de las enfermedades infecciosas, la TB es la principal causa de mortalidad y morbilidad en personas a nivel mundial (Lienhardt et al., 2012). Asimismo, la TB animal ocasiona graves pérdidas en la producción ganadera (Bennett and Cooke, 2006; Boland et al., 2010). Además, las micobacterias del MTBC tienen la capacidad de infectar a un amplio rango de hospedadores incluyendo al ser humano. Precisamente por la capacidad de transmisión de un animal infectado al ser humano, la TB animal se clasifica como zoonosis y por ello tiene también implicaciones en la salud pública (Grange and Yates, 1994; Cosivi et al., 1995; Thoen et al., 2006; Rodríguez et al., 2009; Torres-Gonzalez et al., 2013). Por lo tanto, la TB ofrece una interfaz común entre animales y personas que encaja en el concepto de "una sola salud" promovido por la OMS y la OIE.

El objetivo principal de la presente tesis era, siguiendo este enfoque integral, contribuir al conocimiento y a la mejora del conocimiento en 2 campos fundamentales de la investigación en TB: la vacunación y el diagnóstico. Los estudios fueron dirigidos a evaluar nuevas vacunas y reactivos de diagnóstico diferencial asociados, utilizando para ello el modelo caprino de TB. La detección precoz y la prevención son elementos cruciales para evitar la diseminación en un rebaño o entre rebaños y, en el caso de las personas, para prevenir tanto la infección como la progresión de infección a enfermedad. En este sentido, nuestros resultados pueden tener una aplicación directa en los programas de control y erradicación en animales domésticos, y en el desarrollo de nuevas vacunas antituberculosas.

a) Programas de control y erradicación en animales domésticos

Los resultados del primer estudio pueden ser de ayuda para mejorar las pruebas de diagnóstico que se utilizan actualmente en los programas de erradicación y calificación sanitaria frente a la TB, incrementando su especificidad sin disminuir su sensibilidad. La eventual vacunación de animales domésticos puede contribuir al control de la TB pero introduce un mayor grado de complejidad en el diagnóstico. El segundo y tercer estudio evaluaron tanto la eficacia de vacunas contra la TB, como la utilidad de nuevos reactivos de diagnóstico asociados a la vacunación. Finalmente, en el cuarto estudio se aportan datos sobre el efecto que pueden tener los programas de vacunación contra paratuberculosis (PTB) en el diagnóstico de TB.

Los resultados obtenidos en el segundo y tercer estudio sugieren que la vacunación es una opción eficaz para el control de la TB en el ganado caprino, ofreciendo una alternativa a la estrategia de prueba y sacrificio. Su uso en una primera fase para limitar la transmisión de MTBC y lograr reducción de la prevalencia, puede ser fundamental para la posterior erradicación, tal y como se ha descrito para controlar la TB en el bovino y sus reservorios silvestres (Waters et al., 2012a).

La vacuna BCG en solitario y, en mayor medida, el régimen de vacunación *prime-boost* heterólogo con BCG seguido de vacunas basadas en adenovirus recombinantes, confirieron protección en las cabras frente al desafío con *M. caprae*, en términos de reducción de la patología y de la carga bacteriana, así como evitando la diseminación extrapulmonar de la infección. La eficacia de BCG ya había sido previamente descrita en otras especies animales susceptibles a la infección natural por MTBC como el ganado vacuno, el oposum, el ciervo de cola blanca, el jabalí o el tejón (Buddle et al., 1995; Wedlock et al., 2005; Palmer et al., 2007;

Ballesteros et al., 2009; Chambers et al., 2011), mientras que recientemente se ha demostrado que el régimen *prime-boost* heterólogo con BCG y AdAg85A incrementa la protección de BCG en terneros desafiados experimentalmente con *M. bovis* (Vordermeier et al., 2009), en consistencia con los resultados obtenidos en el segundo y tercer estudio de la presente tesis, donde también se observa una mejora de la protección cuando se emplea este régimen de vacunación.

Los estudios realizados también han aportado información sobre el diagnóstico de la TB en la especie caprina, que complementa la obtenida anteriormente en estudios de campo. Todas las cabras no vacunadas resultaron positivas a la IDTB comparada (realizada a las 11 o 12 semanas post-infección, según el estudio) y a la prueba del IFN-γ (a partir de las 3 o 4 semanas post-infección, según el estudio). Aunque estas pruebas han sido optimizadas para ser usadas en el ganado bovino, su aplicación en caprinos naturalmente infectados ya había sido descrita, presentando valores de sensibilidad y especificidad similares a los del ganado bovino (Gutiérrez et al., 1998; Liebana et al., 1998; Bezos et al., 2012). Adicionalmente, en el primer estudio de la presente tesis se pudo demostrar que la respuesta humoral frente al antígeno estructural MPB83 puede ser detectable pocas semanas después de la infección experimental como sucede en bovinos infectados con *M. bovis* (Waters et al., 2006). Además, tras la realización de la IDTB se incrementó la sensibilidad de detección de anticuerpos frente a MBP83 y PPD-B (por la técnica ELISA), tal y como ya se había demostrado anteriormente en bovinos (Lyashchenko et al., 2004).

En nuestros cuatro estudios se realizó la prueba del IFN-γ basada en tuberculinas (con PPD-B y PPD-A) y con dos reactivos consistentes en antígenos seleccionados para diferenciar individuos infectados y vacunados (estrategia DIVA): a) el cóctel peptídico ESAT-6/CFP-10, formado por los 2 antígenos más utilizados para este propósito en bovinos (Vordermeier et al., 2001; Cockle et al., 2006) y personas (Brock et al., 2004; Pai et al., 2008); y b) el antígeno Rv3615c, recientemente utilizado para el mismo propósito en bovinos (Sidders et al., 2008). Así pues, mientras que algunos de los animales vacunados reaccionaron positivamente a la prueba basada en tuberculinas después de la vacunación con BCG, todos resultaron negativos cuando se utilizaron estos reactivos, confirmando así su utilidad en una estrategia DIVA. Además, agregando los resultados positivos a ESAT-6/CFP-10 y Rv3615c se obtuvieron unos valores de sensibilidad parecidos a los de la prueba basada en tuberculinas (Figura 38A-D). Estudios de campo en granjas de caprino infectadas naturalmente con *M. caprae* ya habían referido valores de sensibilidad equivalentes en la prueba del IFN-γ utilizando PPD-B y ESAT-6/CFP-10 (Bezos et al., 2011).

La infección o sensibilización por otras micobacterias distintas a MTBC es también un factor que puede afectar a las pruebas de diagnóstico inmunológico de la TB (IDTB y prueba del IFN-γ) en rumiantes domésticos. Particularmente, la coinfección de MTBC y *Mycobacterium avium* subsp. *paratuberculosis* (MAP) produce interferencia en estas pruebas en el ganado bovino (Álvarez et al., 2009) y en caprino (Álvarez et al., 2008; Bezos et al., 2010).

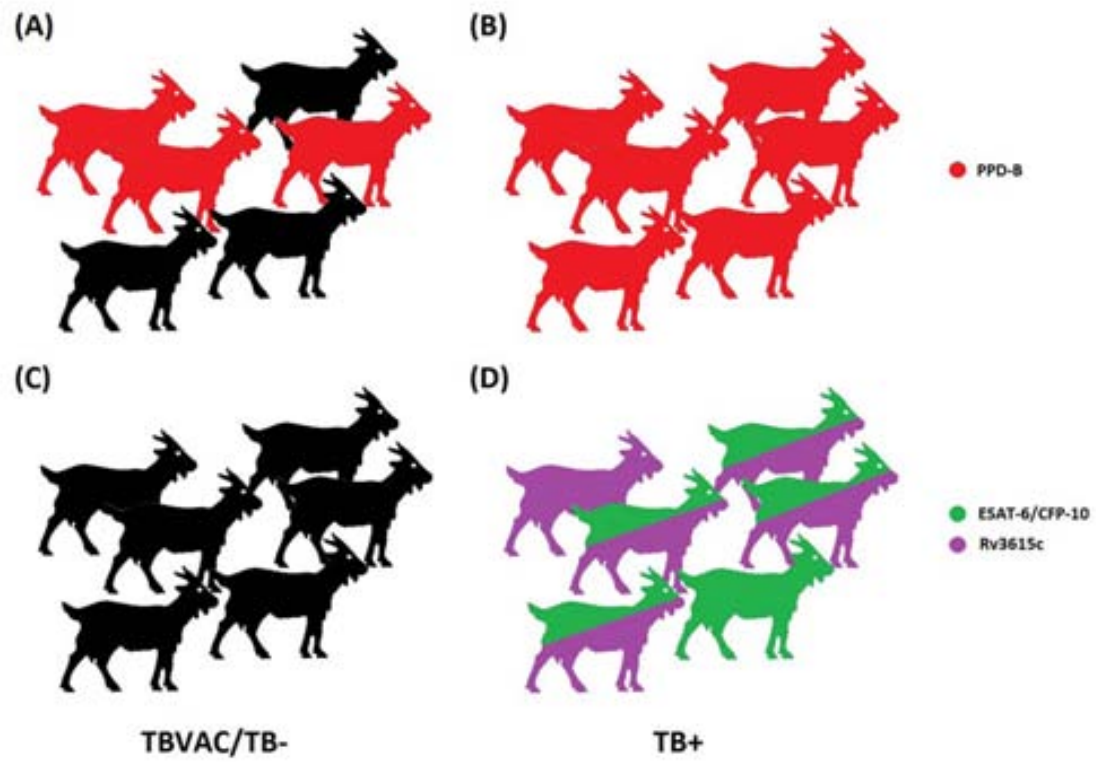

Figura 38. Representación esquemática de los resultados de la prueba del IFN-γ en los estudios de vacunación contra TB. Las figuras A y B hacen referencia la prueba basada en tuberculinas y las figuras C y D utilizando los reactivos DIVA. Algunas cabras vacunadas y no infectadas dan resultado positivo a la prueba basada en tuberculinas (A) mientras que todas ellas son negativas cuando se utilizan los reactivos DIVA (C). Todas las cabras infectadas son positivas a la prueba basada en tuberculinas (B) y también cuando se suman los resultados de la prueba con los 2 reactivos DIVA (D). TBVAC, animales vacunados (con BCG o BCG-*booster*); TB-, animales no infectados; TB+ animales infectados. PPD-B, tuberculina bovina utilizada en la prueba basada en tuberculinas; ESAT-6/CFP-10 y Rv3615c, reactivos DIVA.

La vacunación frente a PTB puede afectar a la especificidad del diagnóstico de TB (Chartier et al., 2012). La vacunación contra PTB está contribuyendo a controlar la enfermedad en pequeños rumiantes y a evitar la pérdida de productividad derivada (Reddacliff et al., 2006; Singh et al., 2007), pero la TB en el ganado caprino (Crawshaw et al., 2008; Daniel et al., 2009; Domingo et al., 2009), como también en el ovino (Munoz Mendoza et al., 2012; van der Burgt et al., 2013) es un enfermedad infecciosa emergente en muchos de los países que aplican campañas de vacunación contra PTB. Por lo tanto la posible interferencia de la vacuna en el actual diagnóstico de TB resulta crucial para evaluar los efectos de combinar ambas estrategias.

En nuestro cuarto estudio observamos que la IDTB simple es ligeramente más sensible que la IDTB comparada para detectar la infección por *M. caprae* en caprinos previamente vacunados contra PTB, pero la IDTB comparada es mucho más específica que la simple en caprinos vacunados y no infectados, en consistencia con lo que ya se había descrito en estudios de campo (Chartier et al., 2012). La prueba del IFN-γ también fue ligeramente menos específica y sensible que la IDTB simple en algunos puntos del estudio (Figura 39A-B). Aunque en baja proporción, la presencia de posibles falsos positivos a la prueba del IFN-γ (DO PPD-B > DO PPD-A) también se ha observado en bovinos infectados naturalmente por PTB (Vazquez et al.,

2013) o en caprinos en granjas donde se practica la vacunación contra PTB (Bezos et al., 2012), poniendo de relieve los problemas de especificidad que conlleva el uso de las tuberculinas en el diagnóstico de ambas enfermedades debido a la generación de reacciones cruzadas.

Por otro lado, nuestros resultados confirman que la sensibilidad se mantiene y las interferencias que afectan a la especificidad de la prueba del IFN-γ se eliminan completamente cuando se utilizan los reactivos DIVA ESAT-6/CFP-10 y Rv3615c (Figura 39C-D). Estos reactivos también ha sido desarrollados para el diagnóstico de la TB en bovinos mediante IDTB (Whelan et al., 2010), aunque su aplicación en caprinos y la evaluación de la interferencia de la vacunación contra PTB aún es objeto pendiente de estudio.

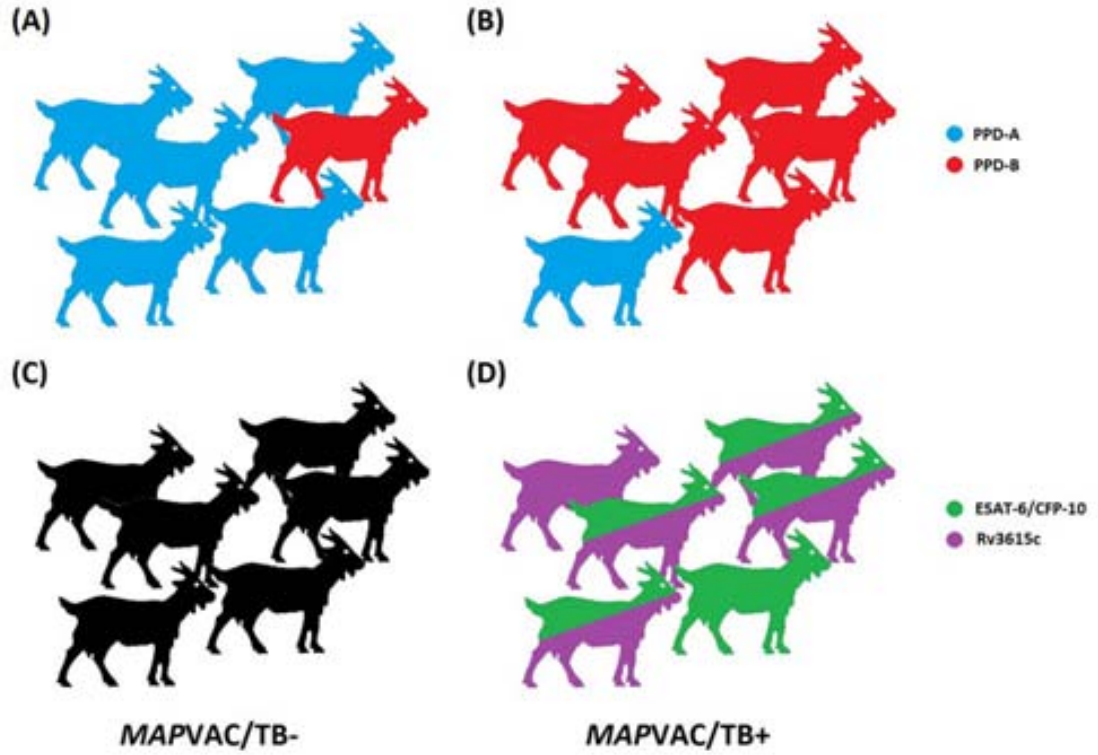

Figura 39. Representación esquemática de los resultados de la prueba del IFN-γ en los estudios de vacunación contra MAP. Las figuras A y B hacen referencia la prueba basada en tuberculinas y las figuras C y D utilizando los reactivos DIVA. (A), Algunas cabras vacunadas y no infectadas dan resultado positivo a la prueba basada en tuberculinas (PPD-B > PPD-A). (B), Como resultado del efecto de la vacunación contra MAP algunas cabras continúan siendo negativas a la prueba basada en tuberculinas después del desafío con *M. caprae* (PPD-A > PPD-B). Las cabras vacunadas contra MAP son negativas antes del desafío cuando se utilizan los reactivos DIVA (C) y la mayoría de ellas son positivas después del desafío cuando se suman los resultados de la prueba con los 2 reactivos DIVA (D). MAPVAC, animales vacunados (con Silirum®); TB-, animales no infectados; TB+ animales infectados. PPD-B y PPD-A, tuberculinas bovina y aviar utilizadas en la prueba basada en tuberculinas; ESAT-6/CFP-10 y Rv3615c, reactivos DIVA.

El desarrollo y progresiva incorporación de estos reactivos en el diagnóstico de rutina de la TB puede representar un impulso para las estrategias de control tanto de la TB como de la PTB a través de la vacunación, sin perjudicar la detección y rápida eliminación de los animales infectados.

b) Desarrollo de nuevas vacunas antituberculosas

La TB supone un reto tanto para la sanidad animal como para la salud pública que requiere colaboración médica y veterinaria en la búsqueda de soluciones integrales, entre las cuales se encuentra el hito de lograr una vacuna universal que pueda: a) prevenir la enfermedad y evitar la transmisión de la infección, b) ser utilizada en personas y/o animales en regiones endémicas con elevada prevalencia, o c) ser dirigida específicamente a poblaciones diana de mayor riesgo en las regiones con baja incidencia.

La BCG, la única vacuna actualmente disponible contra la TB, cumple con el requisito de ser una vacuna universal, pero ha presentado limitaciones importantes en eficacia e inmunogenicidad, tanto en humanos (Fine et al., 1995), como en animales (Thom et al., 2012). Los estudios de vacunación de esta tesis seguían la aproximación de la vacuna universal y para ello se utilizó un tándem patógeno-hospedador natural que sirviera para evaluar comparativamente la seguridad y eficacia de la BCG y de nuevos candidatos vacunales en la cabra doméstica, en tanto que hospedador final de la TB, así como modelo animal para ensayos cínicos de vacunas para humanos.

El modelo de infección experimental desarrollado y evaluado en la presente tesis presentó diversos elementos que refuerzan su idoneidad para ensayos clínicos de nuevos candidatos a vacunas antituberculosas. Se definió el modelo exhaustivamente en cuanto a la caracterización clínica, patológica y bacteriológica para poder evaluar la protección, así como la respuesta inmune post-vacunación y post-desafío para monitorizar la progresión de la infección e identificar biomarcadores de predicción de la eficacia vacunal o correlacionados con la infección tal y como se había descrito anteriormente para el modelo bovino de TB (Vordermeier et al., 2002; Vordermeier et al., 2009).

En el primer estudio se logró establecer un modelo de infección experimental eficiente, inoculando una dosis baja de bacilos tuberculosos directamente en el pulmón vía endobronquial, de manera que todos los cabritos reprodujeron lesiones tuberculosas similares a las descritas en casos naturales de cabras infectadas con *M. caprae* (Domingo et al., 2009; Sanchez et al., 2011), mostrando una rápida progresión de la infección después del desafío experimental. La inducción de una fuerte respuesta inmune tras la infección, basada en la producción de IFN-γ frente a antígenos de MTBC, similar a las respuestas inducidas en pacientes con TB, la rapidez en el desarrollo de la patología característica de TB, así como la similitud entre las lesiones inducidas por la infección experimental en caprinos y las generadas por la TB activa en humanos, son aspectos a favor del uso del modelo caprino para el estudio de la TB en humanos.

En este mismo estudio se realizó una validación del análisis cuantitativo de lesiones tuberculosas mediante la tomografía computarizada (CT), comparándola con el método tradicionalmente utilizado, basado en la medida directa de las lesiones por inspección visual. Este método, complementado por otras aproximaciones cualitativas (como la valoración de la distribución de lesiones), puede suponer una mejora sustancial a la valoración de la protección frente a la infección por MTBC mediante métodos semicuantitativos de puntuación basados en la inspección directa de lesiones (Vordermeier et al., 2002; Lyashchenko et al., 2004; Domingo et al., 2009; Lin et al., 2009).

Recientemente se ha extendido el uso de nuevas metodologías cuantitativas más objetivas de valoración de lesiones macroscópicas en estudios de eficacia de vacunas contra la TB, como la morfometría radiográfica (Maue et al., 2004) o la imagen por resonancia magnética (Sharpe et al., 2010). En los estudios de eficacia de vacunas antituberculosas de la presente tesis se utilizó por primera vez la CT para valorar cuantitativamente la reducción de la patología pulmonar en el punto final del estudio de los grupos vacunados respecto al grupo control. Este método demostró tener un alto grado de acuerdo con el método de inspección visual directa, pero con mayor sensibilidad para medir los volúmenes reales de las lesiones. Adicionalmente, se pudo establecer una relación individual entre el volumen de pulmón afectado y el volumen de pulmón total.

Además, en el tercer estudio se incluyó un análisis histopatológico cualitativo para evaluar las principales características microscópicas de las lesiones granulomatosas de pequeño tamaño. Anteriormente se habían utilizado criterios histopatológicos en bovinos infectados con *M. bovis* para clasificar las lesiones granulomatosas según su estadio de evolución (Wangoo et al., 2005). Posteriormente esta clasificación se ha aplicado en estudios de vacunas frente a *M. bovis* en terneros. Aplicado al total de lesiones encontradas, los animales no vacunados presentaban un mayor número de granulomas y una mayor proporción de ellos en fase avanzada de desarrollo en comparación con los animales vacunados (Dean et al., 2012). En cambio, en nuestro estudio se evaluaron solamente los granulomas más pequeñas (< 0,5 cm de diámetro), debido a que gran parte de los animales vacunados solamente presentaba este tipo de lesiones. Contrariamente a lo esperado, siguiendo el mismo criterio de clasificación que Dean y col., en los animales vacunados se hallaron lesiones pequeñas en estadios de desarrollo más avanzados que en los vacunados. Este hallazgo nos permitió especular sobre la diferente progresión de la infección entre los animales vacunados e infectados. De modo que mientras que todas las cabras no vacunadas acaban presentando lesiones caseosas-necrotizantes extensas y la formación de cavernas, la mayoría de las cabras vacunadas (y más en particular las vacunadas con las estrategia *prime-boost*) logran contener la diseminación de la infección generando lesiones similares a las observadas en casos de infección tuberculosa latente (LTBI) en personas.

En relación a la eficacia de las nuevas vacunas antituberculosas, en nuestros estudios se evaluaron dos candidatos basados en Adenovirus humano serotipo 5 (Ad5) deficiente en la replicación: monovalente (AdAg85A) y tetravalente (AdTBF). En ambos casos se pudo demostrar que el refuerzo de BCG con los antígenos expresados por el AdHu5 incrementa la protección conferida por BCG en solitario, medida por la reducción de la extensión y la diseminación de las lesiones tuberculosas, así como por la reducción de la carga bacteriana en los linfonodos respiratorios. La mejora de la protección de BCG tras el refuerzo con AdAg85A (actualmente en fase I de ensayos clínicos en la especie humana) ha sido demostrada previamente en pequeños animales de laboratorio (Santosuosso et al., 2006; Xing et al., 2009) y en bovinos (Vordermeier et al., 2009).

La estrategia de refuerzo de BCG con antígenos expresados en vectores virales es una de las más extendidas entre las vacunas antituberculosas que se encuentran actualmente en fase de estudios clínicos (ver Tabla 7 en el capítulo de Introducción general). El primero de estos candidatos en entrar en fase de ensayos clínicos fue la vacuna MVA85A (McShane et al., 2005).

Desafortunadamente, pese a superar exitosamente la primeras fases de eficacia en distintos modelos animales (Williams et al., 2005; Verreck et al., 2009), y de seguridad e inmunogenicidad en personas adultas (Hawkridge et al., 2008; Sander et al., 2009), no pudo demostrarse que confiriera un aumento estadísticamente significativo de la protección en comparación con BCG en niños (Tameris et al., 2013). No obstante, otros candidatos de refuerzo de la eficacia de BCG basados en virus recombinantes siguen en fases de ensayos clínicos con resultados prometedores que podrían mejorar la eficacia del MVA85A. Estos candidatos siguen fundamentalmente 2 aproximaciones:

i) *Vacunas multiantigénicas*

En la actualidad hay diversos candidatos vacunales que siguen la estrategia de reforzar la eficacia de BCG con vacunas subunidad que incluyen diferentes antígenos de MTBC. Algunos de estos candidatos vehiculan antígenos mediante vectores virales que los expresarían. Es el caso de las vacuna Aeras-402 que está basada en un Adenovirus humano serotipo 35 que expresa una proteína de fusión que incluye antígenos inducidos en la fase aguda de la infección: Ag85A, Ag85B y TB10.4 (Radošević et al., August 2007). De un modo parecido, la vacuna H1 consiste en una proteína de fusión que combina 2 antígenos de fase aguda: Ag85B y ESAT-6, que se administra directamente junto con un adyuvante sintético (Olsen et al., 2001; Dietrich et al., 2005; Langermans et al., 2005). Más recientemente se ha desarrollado la vacuna H56 que, además de los anteriormente mencionados, también expresa el antígeno Rv2660c (Aagaard et al., 2011), asociado al estrés nutricional (inducido por la micobacteria durante la fase de latencia). En este último caso, se ha desarrollado una vacuna trivalente concebida como vacuna "multifase" y se ha podido demostrar en macacos que refuerza la eficacia de BCG controlando la infección en fases tardías y conteniendo la LTBI (Lin et al., 2012).

En el tercer estudio de la presente tesis se ha evaluado una nueva vacuna multiantigénica (AdTBF) que expresa 4 antígenos de inducción temprana (ver Tabla 4 en el capítulo de Introducción), pero que se secretan en dos estados biológicos distintos de las micobacterias: estado replicativo (Ag85A, TB10.4 y TB9.8) y estado de estrés (Acr2). Los resultados de inmunogenicidad, protección y ganancia del peso corporal de los animales vacunados con el régimen BCG-AdTBF son prometedores en comparación con la BCG en solitario. A pesar de ello aún queda pendiente el estudio conjunto de las vacunas monovalente y polivalente que demuestre las posibles ventajas de esta última estrategia.

ii) *Vacunas de mucosas*

Diversas revisiones sobre el desarrollo de nuevas vacunación contra la TB señalan que la futura generación de vacunas deberá centrarse en generar inmunidad en el sitio de entrada de las Micobacterias (espacio interalveolar) para evitar la infección inicial de las células diana (Kaufmann. 2010; Ottenhoff and Kaufmann, 2012). Para generar inmunidad de mucosas la vía de inoculación de la vacuna resulta crítica. Estudios recientes en animales pequeños de laboratorio con la administración por vía mucosal de la vacuna AdAg85A han demostrado la inducción de inmunidad de mucosas (Santosuosso et al., 2005; Xing and Lichty, 2006), así como una mayor protección comparado con la vía de administración parenteral (Santosuosso et al., 2006; Xing et al., 2009).

En los estudios presentados en esta tesis se ha utilizado solamente la vía de inoculación intramuscular de los adenovirus recombinantes. En futuros estudios con el modelo caprino de TB, que den continuidad al trabajo iniciado en la presente tesis, merecería ser tenida en cuenta la vía mucosal (intranasal, intratraqueal, conjuntival, oral, etc.) de administración de estas vacunas.

En el cuarto estudio se pretendía evaluar de forma específica los efectos de la vacunación frente a MAP, actualmente autorizada en pequeños rumiantes en muchos países, sobre el diagnóstico de la TB y también en la respuesta de los animales vacunados frente a la propia infección tuberculosa. Los resultados mostraron una protección parcial en algunos animales, dando pie a especular sobre si la vacunación contra MAP puede indirectamente inducir cierta inmunidad cruzada que facilite el control de la infección por MTBC. De acuerdo con nuestras observaciones, un efecto de protección cruzada de intensidad similar se había observado previamente en terneros inoculados con una cepa viva de *M. avium* y posteriormente desafiados con *M. bovis* (Hope et al., 2005b). No obstante, los resultados obtenidos en la presente tesis no son conclusivos en términos de eficacia preventiva de la vacuna, debido a que los animales vacunados contra MAP no mostraron una reducción estadísticamente significativa de los principales parámetros relacionados con la protección (como el volumen de las lesiones en pulmón o la carga bacteriana en linfonodos respiratorios). Se requieren estudios adicionales, que incluyan un número mayor de animales, para confirmar que la vacuna contra MAP realmente produce un efecto protector en la transmisión horizontal de la TB en condiciones de campo.

Para la monitorización de nuevos candidatos a vacuna antituberculosa en estudios clínicos es necesario identificar biomarcadores que indiquen los efectos que causa el tratamiento o la infección en los individuos sujetos a estudio durante el transcurso de la fase experimental.

En los 4 estudios presentados en esta tesis se han evaluado algunos marcadores inmunológicos que se pueden clasificar en las siguientes categorías:

i) *Correlatos de progreso de la infección*.

Los niveles de expresión de determinados antígenos de MTBC durante el progreso de la infección tuberculosa se corresponden con los niveles de la respuesta inmunológica frente a estos antígenos tras la infección (Shi et al., 2004). La CMI frente a antígenos de secreción como ESAT-6, CFP-10 y Rv3615c está relacionada con la cantidad de bacilos tuberculosos en estado multiplicación (Pai et al., 2004; Andersen et al., 2007; Millington et al., 2011). En los diferentes estudios que forman parte de esta tesis se han observado correlaciones directas del IFN-γ liberado tras la estimulación de sangre periférica con ESAT-6/CFP-10 (E/C) y Rv3615c con la extensión de las lesiones tuberculosas al final de los experimentos, si bien en los estudios 1 y 4 esta correlación fue estadísticamente significativa solo con E/C, mientras que en los estudios 2 y 3 se pudo observar que la correlación era más significativa en la ventana comprendida entre las 5 y 6 semanas post-infección (cuando se detecta un pico en la respuesta de IFN-γ). La correlación entre el IFN-γ ESAT-6-específico y la extensión de las lesiones se había descrito anteriormente en infecciones experimentales con *M. bovis* en bovinos (Vordermeier et al., 2002; Lyashchenko et al., 2004), mientras que en granjas de caprino con infección natural por *M. caprae* no siempre se han descrito

resultados concordantes (Domingo et al., 2009; Bezos et al., 2011). Consecuentemente, si bien estos parámetros pueden ser muy útiles en estudios experimentales, en condiciones de campo deben ser valorados con precaución.

Por otro lado, en el punto final de la fase experimental de los estudios 1, 2 y 3 se detectó una correlación positiva de los niveles de anticuerpos frente el antígeno estructural MPB83 con la extensión de la patología y la carga bacteriana. Estos resultados son consistentes con los hallados previamente en bovinos infectados experimentalmente con *M. bovis* (Lyashchenko et al., 2004). En ambos casos los anticuerpos se midieron 2 semanas después de la realización de la IDTB, que actúa generando un efecto *booster* en la respuesta humoral (Lyashchenko et al., 2004; Waters et al., 2006).

ii) *Predictores de protección*.

La identificación de biomarcadores del hospedador (animal experimental) capaces de predecir la eficacia de una vacuna después de la vacunación pero antes del desafío experimental proporciona una nueva ventana de decisión en futuros estudios para el cribado inicial de vacunas (sin necesidad de desafío experimental) y la consecuente aceleración del desarrollo de nuevas vacunas. En el estudio número 3 de la presente tesis se demostró que el número de células de memoria central productoras de IFN-γ Ag85A-específico son proporcionales al grado de protección. La relación inversa entre este parámetro y la extensión de las lesiones o la carga bacteriana había sido descrito también en bovinos desafiados con *M. bovis* (Vordermeier et al., 2009).

iii) *Marcadores de inmunogenicidad vacunal*.

Más allá de su relación con la eficacia de vacunas, en la presente tesis se han evaluado parámetros inmunológicos para detectar la inducción de una respuesta inmune específica tras las vacunaciones. Es el caso de los anticuerpos frente a Ag85A 2 semanas después de la vacunación con AdAg85A y AdTBF (estudios 2 y 3 respectivamente) o frente a Acr2 tras la vacunación con AdTBF (estudio 3). La reactividad predominante frente a PPD-A en la IDTB comparada o en la prueba basada en tuberculinas del IFN-γ también sirvió para confirmar la inducción de inmunidad de base celular de la vacuna Silirum® en cabras en el cuarto estudio. Por su parte, la respuesta de IFN-γ frente a PPD-B también fue detectada en la mayoría de animales tras las vacunaciones con BCG (estudios 2 y 3) aunque esta respuesta no permite distinguir entre vacunación e infección por TB.

En conjunto, los dos estudios específicos de eficacia de vacunas antituberculosas en el modelo caprino que se presentan en esta tesis, los estudios recientemente realizados en bovinos (Hope et al., 2005a; Vordermeier et al., 2009; Dean et al., 2012; Thom et al., 2012) y los realizados en algunas especies de fauna silvestre (Nol et al., 2008; Ballesteros et al., 2009; Tompkins et al., 2009; Corner et al., 2010; Chambers et al., 2011; Garrido et al., 2011), suponen uno de los primeros ejemplos de la estrategia de "Una sola salud", con aplicaciones relevantes tanto para el campo veterinario como para la salud pública (Palmer et al., 2012; Waters et al., 2012a).

Toda la información obtenida con los trabajos incluidos en esta tesis puede contribuir a la mejora de la fase de ensayos clínicos de nuevas vacunas antituberculosas con nuevos y mejores reactivos de diagnóstico con el fin de obtener mejores instrumentos para combatir la TB en zonas endémicas, así como prevenir nuevos brotes o reemergencias con el objetivo último de erradicar la TB en personas y animales.

CONCLUSIONS

1. El model experimental de TB en cabres per mitjà de la inoculació de *M. caprae* per la via endobronquial, reprodueix de forma eficient la resposta immunològica i les característiques patològiques que s'observen en la infecció natural en cabres. Les similituds entre aquest model de TB i la infecció activa en persones, el converteixen en un model adequat per a ser utilitzat en assajos clínics de nous tractaments i vacunes.

2. La CT és un mètode més precís que l'examen visual directe, per quantificar l'extensió de les lesions tuberculoses al pulmó en cabres desafiades amb *M. caprae*, permetent avaluar més eficientment el grau de protecció en els estudis d'eficàcia vacunal.

3. La vacuna BCG protegeix a les cabres enfront al desafiament amb *M. caprae*, en termes de reducció de les lesions i de la càrrega bacteriana. El reforç de la BCG amb una dosi posterior de les vacunes AdAg85A o AdTBF, millora la protecció conferida per la BCG en solitari. Amb aquests resultats la vacunació contra la TB es pot plantejar com una alternativa eficient a l'estratègia de prova-sacrifici per tal de controlar la transmissió de la TB i reduir la prevalença en els ramats de cabrum infectats.

4. Les cabres vacunades amb BCG i BCG-AdTBF controlen més eficientment la infecció inicial, evitant l'extensió de les lesions i delimitant de forma ordenada el granulomes. El diferent estat de desenvolupament de les lesions granulomatoses amb diàmetre inferior a 5 mm entre les cabres vacunades i no vacunades, confirma una patogènesi vacuna-depenent.

5. L'anàlisi histopatològic dels granulomes permet identificar diferències qualitatives en l'extensió de la necrosi, la mineralització, el grau d'encapsulament i la formació de granulomes satèl·lits entre els grups vacunats (amb BCG i BCG-AdTBF) i no vacunat, suggerint una patogènia diferent deguda a l'efecte de la vacunació.

6. La vacunació contra la paratuberculosi amb Silirum® contribueix a reduir la multiplicació bacil·lar i la disseminació extrapulmonar de la infecció en cabres desafiades amb *M. caprae*, conferint un cert grau de protecció creuada.

7. La vacunació de cabres amb Silirum® interfereix el diagnòstic de la TB per mitjà de la IDTB simple, la IDTB comparada i la prova de l'IFN-γ amb PPD-B i PPD-A.

8. L'ús del còctel peptídic E/C i l'antigen Rv3615c en la prova de l'IFN-γ permet diferenciar cabres vacunades amb BCG, BCG-AdAg85A, BCG-AdTBF o Silirum® de cabres infectades amb *M. caprae*, sense perdre sensibilitat en comparació amb la prova de l'IFN-γ amb PPD-B i PPD-A.

9. Els nivells d'IFN-γ enfront a E/C o Rv3615c i els nivells d'anticossos IgG enfront a MPB83 2 setmanes després de la IDTB, són correlats de la progressió de la infecció tuberculosa en cabres desafiades amb *M. caprae*, mentre que el nombre de cèl·lules T de memòria productores d'IFN-γ Ag85A-específic són predictores de l'eficàcia vacunal en cabres vacunades amb BCG i 8 setmanes després amb AdTBF.

REFERÈNCIES

Aagaard, C., Govaerts, M., Meikle, V., Vallecillo, A.J., Gutierrez-Pabello, J.A., Suarez-Guemes, F., McNair, J., Cataldi, A., Espitia, C., Andersen, P., Pollock, J.M., 2006. Optimizing Antigen Cocktails for Detection of Mycobacterium Bovis in Herds with Different Prevalences of Bovine Tuberculosis: ESAT6-CFP10 Mixture shows Optimal Sensitivity and Specificity. J. Clin. Microbiol. 44, 4326-4335.

Aagaard, C., Hoang, T., Dietrich, J., Cardona, P.J., Izzo, A., Dolganov, G., Schoolnik, G.K., Cassidy, J.P., Billeskov, R., Andersen, P., 2011. A Multistage Tuberculosis Vaccine that Confers Efficient Protection Before and After Exposure. Nat. Med. 17, 189-194.

Aagaard, C., Brock, I., Olsen, A., Ottenhoff, T.H.M., Weldingh, K., Andersen, P., 2004. Mapping Immune Reactivity Toward Rv2653 and Rv2654: Two Novel Low-Molecular-Mass Antigens found Specifically in the Mycobacterium Tuberculosis Complex. J. Infect. Dis. 189, 812-819.

Abdallah, A.M., Gey van Pittius, N.C., Champion, P.A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C.M., Appelmelk, B.J., Bitter, W., 2007. Type VII Secretion-Mycobacteria show the Way. Nat. Rev. Microbiol. 5, 883-891.

Aimé, B., Lequen, L., Balageas, A., Haddad, N., Maugein, J., 2012. Infections à M. bovis Et M. caprae En Aquitaine : Étude Clinico-Épidémiologique De 15 cas. Pathologie Biologie 60, 156-159.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen Recognition and Innate Immunity. Cell 124, 783-801.

Alexander, K.A., Laver, P.N., Michel, A.L., Williams, M., van Helden, P.D., Warren, R.M., Gey van Pittius, N.C., 2010. Novel Mycobacterium Tuberculosis Complex Pathogen, M. Mungi. Emerg. Infect. Dis. 16, 1296-1299.

Allison, M.J., Mendoza, D., Pezzia, A., 1973. Documentation of a Case of Tuberculosis in Pre-Columbian America. Am. Rev. Respir. Dis. 107, 985-991.

Álvarez, J., de Juan, L., Bezos, J., Romero, B., Sáez, J.L., Gordejo, F.J.R., Briones, V., Moreno, M.Á., Mateos, A., Domínguez, L., Aranaz, A., 2008. Interference of Paratuberculosis with the Diagnosis of Tuberculosis in a Goat Flock with a Natural Mixed Infection. Vet. Microbiol. 128, 72-80.

Álvarez, J., de Juan, L., Bezos, J., Romero, B., Sáez, J.L., Marqués, S., Domínguez, C., Mínguez, O., Fernández-Mardomingo, B., Mateos, A., Domínguez, L., Aranaz, A., 2009. Effect of Paratuberculosis on the Diagnosis of Bovine Tuberculosis in a Cattle Herd with a Mixed Infection using Interferon-Gamma Detection Assay. Vet. Microbiol. 135, 389-393.

Amadori, M., Tagliabue, S., Lauzi, S., Finazzi, G., Lombardi, G., Telo, P., Pacciarini, L., Bonizzi, L., 2002. Diagnosis of Mycobacterium Bovis Infection in Calves Sensitized by Mycobacteria of the avium/intracellulare Group. J. Vet. Med. B Infect. Dis. Vet. Public Health 49, 89-96.

Amanfu, W., 2006. The Situation of Tuberculosis and Tuberculosis Control in Animals of Economic Interest. Tuberculosis (Edinb) 86, 330-335.

Andersen, P., Doherty, T.M., Pai, M., Weldingh, K., 2007. The Prognosis of Latent Tuberculosis: Can Disease be Predicted? Trends Mol. Med. 13, 175-182.

Aranaz, A., Liebana, E., Gomez-Mampaso, E., Galan, J., Cousins, D., Ortega, A., Blazquez, J., Baquero, F., Mateos, A., Suarez, G., Dominguez, L., 1999. Mycobacterium Tuberculosis Subsp. Caprae Subsp. Nov.: A Taxonomic Study of a New Member of the Mycobacterium Tuberculosis Complex Isolated from Goats in Spain. Int. J. Syst. Bacteriol. 49, 1263-1273.

Aranaz, A., Cousins, D., Mateos, A., Dominguez, L., 2003. Elevation of Mycobacterium Tuberculosis Subsp. Caprae Aranaz et al. 1999 to Species Rank as Mycobacterium Caprae Comb. Nov., Sp. Nov. Int. J Syst. Evol. Microbiol. 53, 1785-1789.

Aranday-Cortes, E., Bull, N.C., Villarreal-Ramos, B., Gough, J., Hicks, D., Ortiz-Pelaez, A., Vordermeier, H.M., Salguero, F.J., 2012a. Upregulation of IL-17A, CXCL9 and CXCL10 in Early-Stage Granulomas Induced by Mycobacterium Bovis in Cattle. Transbound. Emerg. Dis.

Aranday-Cortes, E., Hogarth, P.J., Kaveh, D.A., Whelan, A.O., Villarreal-Ramos, B., Lalvani, A., Vordermeier, H.M., 2012b. Transcriptional Profiling of Disease-Induced Host Responses in Bovine Tuberculosis and the Identification of Potential Diagnostic Biomarkers. PLoS One 7, e30626.

Armitige, L.Y., Jagannath, C., Wanger, A.R., Norris, S.J., 2000. Disruption of the Genes Encoding Antigen 85A and Antigen 85B ofMycobacterium Tuberculosis H37Rv: Effect on Growth in Culture and in Macrophages. Infect. Immun. 68, 767-778.

Ayele, W.Y., Neill, S.D., Zinsstag, J., Weiss, M.G., Pavlik, I., 2004a. Bovine Tuberculosis: An Old Disease but a New Threat to Africa. Int. J. Tuberc. Lung Dis. 8, 924-937.

Ballesteros, C., Garrido, J.M., Vicente, J., Romero, B., Galindo, R.C., Minguijón, E., Villar, M., Martín-Hernando, M.P., Sevilla, I., Juste, R., Aranaz, A., de la Fuente, J., Gortázar, C., 2009. First Data on Eurasian Wild Boar Response to Oral Immunization with BCG and Challenge with a Mycobacterium Bovis Field Strain. Vaccine 27, 6662-6668.

Balu, S., Reljic, R., Lewis, M.J., Pleass, R.J., McIntosh, R., van Kooten, C., van Egmond, M., Challacombe, S., Woof, J.M., Ivanyi, J., 2011. A Novel Human IgA Monoclonal Antibody Protects Against Tuberculosis. J. Immunol. 186, 3113-3119.

Bayraktar, B., Bulut, E., Barış, A.B., Toksoy, B., Dalgıc, N., Celikkan, C., Sevgi, D., 2011. Species Distribution of the Mycobacterium Tuberculosis Complex in Clinical Isolates from 2007 to 2010 in Turkey: A Prospective Study. J. Clin. Microbiol. 49, 3837-3841.

Behr, M.A., Sherman, D.R., 2007. Mycobacterial Virulence and Specialized Secretion: Same Story, Different Ending. Nat. Med. 13, 286-287.

Behr, M.A., Small, P.M., 1999. A Historical and Molecular Phylogeny of BCG Strains. Vaccine 17, 915-922.

Bennett, R.M., Cooke, R.J., 2006. Costs to Farmers of a Tuberculosis Breakdown. Vet. Rec. 158, 429-432.

Bezos, J., Alvarez, J., Romero, B., Aranaz, A., Juan, L., 2012. Tuberculosis in Goats: Assessment of Current in Vivo Cell-Mediated and Antibody-Based Diagnostic Assays. Vet. J. 191, 161-165.

Bezos, J., Álvarez, J., de Juan, L., Romero, B., Rodríguez, S., Fernández-de-Mera, I.G., Hewinson, R.G., Vordermeier, M., Mateos, A., Domínguez, L., Aranaz, A., 2011. Assessment of in Vivo and in Vitro Tuberculosis Diagnostic Tests in Mycobacterium Caprae Naturally Infected Caprine Flocks. Prev. Vet. Med. 100, 187-192.

Bezos, J., Álvarez, J., Mínguez, O., Marqués, S., Martín, O., Vigo, V., Pieltain, C., Romero, B., Rodríguez, S., Casal, C., Mateos, A., Domínguez, L., de Juan, L., 2012. Evaluation of Specificity of Tuberculosis Diagnostic Assays in Caprine Flocks Under Different Epidemiological Situations. Res. Vet. Sci. 93, 636-640.

Bezos, J., de Juan, L., Romero, B., Álvarez, J., Mazzucchelli, F., Mateos, A., Domínguez, L., Aranaz, A., 2010. Experimental Infection with Mycobacterium Caprae in Goats and Evaluation of Immunological Status in Tuberculosis and Paratuberculosis Co-Infected Animals. Vet. Immunol. Immunopathol. 133, 275.

Bhatt, K., Hickman, S.P., Salgame, P., 2004. Cutting Edge: A New Approach to Modeling Early Lung Immunity in Murine Tuberculosis. J. Immunol. 172, 2748-2751.

Bhuju, S., Aranday-Cortes, E., Villarreal-Ramos, B., Xing, Z., Singh, M., Vordermeier, H.M., 2012. Global Gene Transcriptome Analysis in Vaccinated Cattle Revealed a Dominant Role of IL-22 for Protection Against Bovine Tuberculosis. PLoS Pathog. 8, e1003077.

Bishop, C.P., Barnes, M.F., Reidy, J.B., 1934. Tuberculosis in a Goat. J. Am. Vet. Med. Assoc. 85, 457-458.

Bitter, W., Houben, E.N., Bottai, D., Brodin, P., Brown, E.J., Cox, J.S., Derbyshire, K., Fortune, S.M., Gao, L.Y., Liu, J., Gey van Pittius, N.C., Pym, A.S., Rubin, E.J., Sherman, D.R., Cole, S.T., Brosch, R., 2009. Systematic Genetic Nomenclature for Type VII Secretion Systems. PLoS Pathog. 5, e1000507.

Blanco, F.C., Nunez-Garcia, J., Garcia-Pelayo, C., Soria, M., Bianco, M.V., Zumarraga, M., Golby, P., Cataldi, A.A., Gordon, S.V., Bigi, F., 2009. Differential Transcriptome Profiles of Attenuated and Hypervirulent Strains of Mycobacterium Bovis. Microbes Infect. 11, 956-963.

Blanco, F.C., Bianco, M.V., Meikle, V., Garbaccio, S., Vagnoni, L., Forrellad, M., Klepp, L.I., Cataldi, A.A., Bigi, F., 2011. Increased IL-17 Expression is Associated with Pathology in a Bovine Model of Tuberculosis. Tuberculosis (Edinb) 91, 57-63.

Blumerman, S.L., Herzig, C.T., Baldwin, C.L., 2007. WC1+ Gammadelta T Cell Memory Population is Induced by Killed Bacterial Vaccine. Eur. J. Immunol. 37, 1204-1216.

Boland, F., Kelly, G.E., Good, M., More, S.J., 2010. Bovine Tuberculosis and Milk Production in Infected Dairy Herds in Ireland. Prev. Vet. Med. 93, 153-161.

Bolin, C.A., Whipple, D.L., Khanna, K.V., Risdahl, J.M., Peterson, P.K., Molitor, T.W., 1997. Infection of Swine with Mycobacterium Bovis as a Model of Human Tuberculosis. J. Infect. Dis. 176, 1559-1566.

Boniotti, M.B., Goria, M., Loda, D., Garrone, A., Benedetto, A., Mondo, A., Tisato, E., Zanoni, M., Zoppi, S., Dondo, A., Tagliabue, S., Bonora, S., Zanardi, G., Pacciarini, M.L., March 2009. Molecular Typing of Mycobacterium Bovis Strains Isolated in Italy from 2000 to 2006 and Evaluation of Variable-Number Tandem Repeats for Geographically Optimized Genotyping. J. Clin. Microbiol. 47, 636-644.

Bothamley, G.H., Grange, J.M., 1991. The Koch Phenomenon and Delayed Hypersensitivity: 1891-1991. Tubercle 72, 7-11.

Brewer, T.F., 2000. Preventing Tuberculosis with Bacillus Calmette-Guérin Vaccine: A Meta-Analysis of the Literature. Clin. Infect. Dis. 31, S64-S67.

Brock, L., Weldingh, K., Lillebaek, T., Follmann, F., Andersen, P., 2004. Comparison of Tuberculin Skin Test and New Specific Blood Test in Tuberculosis Contacts. Am. J. Respir. Crit. Care Med. 170, 65-69.

Brosch, R., Gordon, S.V., Garnier, T., Eiglmeier, K., Frigui, W., Valenti, P., Dos Santos, S., Duthoy, S., Lacroix, C., Garcia-Pelayo, C., Inwald, J.K., Golby, P., Garcia, J.N., Hewinson, R.G., Behr, M.A., Quail, M.A., Churcher, C., Barrell, B.G., Parkhill, J., Cole, S.T., 2007. Genome Plasticity of BCG and Impact on Vaccine Efficacy. Proc. Natl. Acad. Sci. U. S. A. 104, 5596-5601.

Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L.M., Pym, A.S., Samper, S., van Soolingen, D., Cole, S.T., 2002. A New Evolutionary Scenario for the Mycobacterium Tuberculosis Complex. Proc. Natl. Acad. Sci. U. S. A. 99, 3684-3689.

Buddle, B.M., Aldwell, F.E., Pfeffer, A., de Lisle, G.W., 1994. Experimental Mycobacterium Bovis Infection in the Brushtail Possum (Trichosurus Vulpecula): Pathology, Haematology and Lymphocyte Stimulation Responses. Vet. Microbiol. 38, 241-254.

Buddle, B.M., de Lisle, G.W., Pfeffer, A., Aldwell, F.E., 1995. Immunological Responses and Protection Against Mycobacterium Bovis in Calves Vaccinated with a Low Dose of BCG. Vaccine 13, 1123-1130.

Buddle, B.M., Parlane, N.A., Heiser, A., Wedlock, D.N., 2012. Vaccination Against Bovine Tuberculosis: A Promising Future Control Strategy. In: Proceedings of the first Mycobacterium bovis American Regional Conference, Merida (Mexico), August 19th-22nd, 72.

Buddle, B.M., Wedlock, D.N., Denis, M., 2006. Progress in the Development of Tuberculosis Vaccines for Cattle and Wildlife. Vet. Microbiol., 112, 191-200.

Buddle, B.M., Wedlock, D.N., Parlane, N.A., Corner, L.A.L., de Lisle, G.W., Skinner, M.A., 2003. Revaccination of Neonatal Calves with Mycobacterium Bovis BCG Reduces the Level of Protection Against Bovine Tuberculosis Induced by a Single Vaccination. Infect. Immun. 71, 6411-6419.

Buddle, B.M., Wedlock, D.N., Denis, M., Skinner, M.A., 2005a. Identification of Immune Response Correlates for Protection Against Bovine Tuberculosis. Vet. Immunol. Immunopathol. 108, 45-51.

Buddle, B., Skinner, M., NeilWedlock, D., deLisle, G., MartinVordermeier, H., GlynHewinson, R., 2005b. Cattle as a Model for Development of Vaccines Against Human Tuberculosis. Tuberculosis (Edinb) 85, 19-24.

Cadmus, S.I., Adesokan, H.K., Jenkins, A.O., van Soolingen, D., 2009. Mycobacterium Bovis and M. Tuberculosis in Goats, Nigeria. Emerg. Infect. Dis. 15, 2066-2067.

Calmette, A., 1927. Sur La Vaccination Préventive Des Enfants Nouveau-Nés Contra La Tuberculose Par Le BCG. Ann. Inst. Pasteur 41, 201-232.

Canetti, G., 1955. The Tubercle Bacillus in the Pulmonary Lesion of Man. Histobacteriology and its Bearing on the Therapy of Pulmonary Tuberculosis. New York, Springer Pub. Co.

Cardona, P., 2006. RUTI: A New Chance to Shorten the Treatment of Latent Tuberculosis Infection. Tuberculosis (Edinb) 86, 273-289.

Caro, M.R., Gallego, M.C., Buendía, A.J., Del Rio, L., Seva, J., Navarro, J.A., 2001. Differences in Lymphocyte Subpopulations from Peripheral Blood and Lymphoid Organs in Natural Caprine Tuberculosis Infection. J. Vet. Med. B Infect. Dis. Vet. Public Health 48, 81-88.

Casal, C., Bezos, J., Díez-Guerrier, A., Álvarez, J., Romero, B., de Juan, L., Rodriguez-Campos, S., Vordermeier, M., Whelan, A., Hewinson, R.G., Mateos, A., Domínguez, L., Aranaz, A., 2012. Evaluation of Two Cocktails Containing ESAT-6, CFP-10 and Rv-3615c in the Intradermal Test and the Interferon-γ Assay for Diagnosis of Bovine Tuberculosis. Prev. Vet. Med. 105, 149-154.

Cassidy, J.P., 2006. The Pathogenesis and Pathology of Bovine Tuberculosis with Insights from Studies of Tuberculosis in Humans and Laboratory Animal Models. Vet. Microbiol. 112, 151-161.

Castets, M., Boisvert, H., Grumbach, F., Brunel, M., Rist, N., 1968. Tuberculosis Bacilli of the African Type: Preliminary Note. Rev. Tuberc. Pneumol. (Paris) 32, 179-184.

Chackerian, A.A., Alt, J.M., Perera, T.V., Dascher, C.C., Behar, S.M., 2002. Dissemination of Mycobacterium Tuberculosis is Influenced by Host Factors and Precedes the Initiation of T-Cell Immunity. Infect. Immun. 70, 4501-4509.

Chambers, M.A., Rogers, F., Delahay, R.J., Lesellier, S., Ashford, R., Dalley, D., Gowtage, S., Dave, D., Palmer, S., Brewer, J., Crawshaw, T., Clifton-Hadley, R., Carter, S., Cheeseman, C., Hanks, C., Murray, A., Palphramand, K., Pietravalle, S., Smith, G.C., Tomlinson, A., Walker, N.J., Wilson, G.J., Corner, L.A., Rushton, S.P., Shirley, M.D., Gettinby, G., McDonald, R.A., Hewinson, R.G., 2011. Bacillus Calmette-Guerin Vaccination Reduces the Severity and Progression of Tuberculosis in Badgers. Proc. Biol. Sci. 278, 1913-1920.

Chartier, C., Mercier, P., Pellet, M., Vialard, J., 2012. Effect of an Inactivated Paratuberculosis Vaccine on the Intradermal Testing of Goats for Tuberculosis. Vet. J. 191(3), 360-363.

Cockle, P.J., Gordon, S.V., Hewinson, R.G., Vordermeier, H.A., 2006. Field Evaluation of a Novel Differential Diagnostic Reagent for Detection of Mycobacterium Bovis in Cattle. Clin. Vaccine Immunol. 13, 1119-1124.

Colditz, G.A., Berkey, C.S., Mosteller, F., Brewer, T.F., Wilson, M.E., Burdick, E., Fineberg, H.V., 1995. The Efficacy of Bacillus Calmette-Guerin Vaccination of Newborns and Infants in the Prevention of Tuberculosis: Meta-Analyses of the Published Literature. Pediatrics 96, 29-35.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., 1998. Deciphering the Biology of Mycobacterium Tuberculosis from the Complete Genome Sequence. Nature 393, 537-544.

Collins, D.M., 2001. Virulence Factors of Mycobacterium Bovis. Tuberculosis (Edinb) 81, 97-102.

Cooper, A.M., 2009. Cell-Mediated Immune Responses in Tuberculosis. Annu. Rev. Immunol. 27, 393-422.

Corner, L.A.L., Costello, E., Lesellier, S., O'Meara, D., Sleeman, D.P., Gormley, E., 2007. Experimental Tuberculosis in the European Badger (Meles Meles) After Endobronchial Inoculation of Mycobacterium Bovis: I. Pathology and Bacteriology. Res. Vet. Sci. 83, 53-62.

Corner, L.A.L., Trajstman, A.C., 1988. An Evaluation of 1-Hexadecylpyridinium Chloride as a Decontaminant in the Primary Isolation of Mycobacterium Bovis from Bovine Lesions. Vet. Microbiol. 18, 127-134.

Corner, L.A.L., Costello, E., Lesellier, S., O'Meara, D., Gormley, E., 2008. Vaccination of European Badgers (Meles Meles) with BCG by the Subcutaneous and Mucosal Routes Induces Protective Immunity Against Endobronchial Challenge with Mycobacterium Bovis. Tuberculosis (Edinb) 88, 601-609.

Corner, L.A.L., Costello, E., O'Meara, D., Lesellier, S., Aldwell, F.E., Singh, M., Hewinson, R.G., Chambers, M.A., Gormley, E., 2010. Oral Vaccination of Badgers (Meles Meles) with BCG and Protective Immunity Against Endobronchial Challenge with Mycobacterium Bovis. Vaccine 28, 6265-6272.

Cosivi, O., Grange, J.M., Daborn, C.J., Raviglione, M.C., Fujikura, T., Cousins, D., Robinson, R.A., Huchzermeyer, H.F., de Kantor, I., Meslin, F.X., 1998. Zoonotic Tuberculosis due to Mycobacterium Bovis in Developing Countries. Emerg. Infect. Dis. 4, 59-70.

Cosivi, O., Meslin, F.X., Daborn, C.J., Grange, J.M., 1995. Epidemiology of Mycobacterium Bovis Infection in Animals and Humans, with Particular Reference to Africa. Rev. Sci. Tech. 14, 733-746.

Cousins, D.V., Bastida, R., Cataldi, A., Quse, V., Redrobe, S., Dow, S., Duignan, P., Murray, A., Dupont, C., Ahmed, N., Collins, D.M., Butler, W.R., Dawson, D., Rodríguez, D., Loureiro, J., Romano, M.I., Alito, A., Zumarraga, M., Bernardelli, A., 2003. Tuberculosis in Seals Caused by a Novel Member of the Mycobacterium Tuberculosis Complex: Mycobacterium Pinnipedii Sp. Nov. Int. J. Syst. Bacteriol. 53, 1305-1314.

Crawshaw, T., Daniel, R., Clifton-Hadley, R., Clark, J., Evans, H., Rolfe, S., de la Rua-Domenech, R., 2008. TB in Goats Caused by Mycobacterium Bovis. Vet. Rec. 163, 127.

Cunha, M.V., Monteiro, M., Carvalho, P., Mendonca, P., Albuquerque, T., Botelho, A., 2011. Multihost Tuberculosis: Insights from the Portuguese Control Program. Vet. Med. Int. 2011, 795165.

Cvetnic, Z., Katalinic-Jankovic, V., Sostaric, B., Katalinic-Jankovic, V., Obrovac, M., Marjanovic, S., Benic, M., Kirin, B.K., Katalinic-Jankovic, V., 2007. Mycobacterium Caprae in Cattle and Humans in Croatia. Int. J. Tuberc. Lung Dis. 11, 658.

Daffé, M., Draper, P., 1998. The Envelope Layers of Mycobcateria with Reference to their Pathogenicity. Adv. Microb. Physiol. 39, 131-203.

Daniel, R., Evans, H., Rolfe, S., de la Rua-Domenech, R., Crawshaw, T., Higgins, R.J., Schock, A., Clifton-Hadley, R., 2009. Outbreak of Tuberculosis Caused by Mycobacterium Bovis in Golden Guernsey Goats in Great Britain. Vet. Rec. 165, 335-342.

Dannenberg, A.M., 1991. Delayed-Type Hypersensitivity and Cell-Mediated Immunity in the Pathogenesis of Tuberculosis. Immunol. Today 12, 228-233.

Dannenberg, A.M., 2001. Pathogenesis of Pulmonary Mycobacterium Bovis Infection: Basic Principles Established by the Rabbit Model. Tuberculosis (Edinb) 81, 87-96.

de Jonge, M.I., Pehau-Arnaudet, G., Fretz, M.M., Romain, F., Bottai, D., Brodin, P., Honore, N., Marchal, G., Jiskoot, W., England, P., Cole, S.T., Brosch, R., 2007. ESAT-6 from Mycobacterium Tuberculosis Dissociates from its Putative Chaperone CFP-10 Under Acidic Conditions and Exhibits Membrane-Lysing Activity. J. Bacteriol. 189, 6028-6034.

de Juan, L., Alvarez, J., Aranaz, A., Rodriguez, A., Romero, B., Bezos, J., Mateos, A., Dominguez, L., 2006. Molecular Epidemiology of Types I/III Strains of Mycobacterium Avium Subspecies Paratuberculosis Isolated from Goats and Cattle. Vet. Microbiol. 115, 102-110.

de Kantor, I.N., Ritacco, V., 2006. An Update on Bovine Tuberculosis Programmes in Latin American and Caribbean Countries. Vet. Microbiol. 112, 111-118.

de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H., Clifton-Hadley, R.S., 2006. Ante Mortem Diagnosis of Tuberculosis in Cattle: A Review of the Tuberculin Tests, γ-Interferon Assay and Other Ancillary Diagnostic Techniques. Res. Vet. Sci. 81, 190-210.

Dean, G.S., Rhodes, S.G., Coad, M., Whelan, A.O., Wheeler, P., Villareal-Ramos, B., Mead, E., Johnson, L., Clifford, D.J., Hewinson, R.G., Vordermeier, H.M., 2008. Isoniazid Treatment of Mycobacterium Bovis in Cattle as a Model for Human Tuberculosis. Tuberculosis (Edinb) 88, 586-594.

Dean, G.S., Whelan, A.O., Clifford, D., Salguero, J., Xing, Z., Gilbert, S., McShane, H., Vordermeier, H.M., Villareal-Ramos, B., 2012. Comparison of the Immunogenicity and Protection Against Infection with *Mycobacterium Bovis* conferred by Prime Boost Vaccination Regimens using BCG as Priming and Viral Vectors Or Adjuvants as Boosting

Agents. In: Proceedings of the first Mycobacterium bovis American Regional Conference, Merida (Mexico), August 19th-22nd, 76.

Dean, G.S., Rhodes, S.G., Coad, M., Whelan, A.O., Cockle, P.J., Clifford, D.J., Hewinson, R.G., Vordermeier, H.M., 2005. Minimum Infective Dose of Mycobacterium Bovis in Cattle. Infect. Immun. 73, 6467-6471.

Demangel, C., Bertolino, P., Britton, W.J., 2002. Autocrine IL-10 Impairs Dendritic Cell (DC)-Derived Immune Responses to Mycobacterial Infection by Suppressing DC Trafficking to Draining Lymph Nodes and Local IL-12 Production. Eur. J. Immunol. 32, 994-1002.

Diel, R., Nienhaus, A., Lange, C., Meywald-Walter, K., ForSZbohm, M., Schaberg, T., 2006. Tuberculosis Contact Investigation with a New, Specific Blood Test in a Low-Incidence Population Containing a High Proportion of BCG-Vaccinated Persons. Respir. Res. 7, 77.

Diel, R., Loddenkemper, R., Meywald-Walter, K., Niemann, S., Nienhaus, A., 2008. Predictive Value of a Whole Blood IFN-γ Assay for the Development of Active Tuberculosis Disease After Recent Infection with Mycobacterium Tuberculosis. Am. J. Respir. Crit. Care Med. 177, 1164-1170.

Dietrich, J., Andersen, C., Rappuoli, R., Doherty, T.M., Jensen, C.G., Andersen, P., 2006. Mucosal Administration of Ag85B-ESAT-6 Protects Against Infection with Mycobacterium Tuberculosis and Boosts Prior Bacillus Calmette-Guerin Immunity. J. Immunol. 177, 6353-6360.

Dietrich, J., Aagaard, C., Leah, R., Olsen, A.W., Stryhn, A., Doherty, T.M., Andersen, P., 2005. Exchanging ESAT6 with TB10.4 in an Ag85B Fusion Molecule-Based Tuberculosis Subunit Vaccine: Efficient Protection and ESAT6-Based Sensitive Monitoring of Vaccine Efficacy. J Immunol. 174, 6332-6339.

Domingo, M., Gil, O., Serrano, E., Guirado, E., Nofrarias, M., Grassa, M., Cáceres, N., Pérez, B., Vilaplana, C., Cardona, P.J., 2009. Effectiveness and Safety of a Treatment Regimen Based on Isoniazid Plus Vaccination with Mycobacterium Tuberculosis Cells' Fragments: Field-Study with Naturally Mycobacterium Caprae-Infected Goats. Scand. J. Immunol. 69, 500-507.

Domingo, M., Liebana, E., Carrera, J., Vilafranca, M., Casal, J., Aranaz, A., Altimira, J., Vidal, D., Marco, A., Planell, J.M., Mateos, A., Dominguez, L., 1995. Comparative Efficacity of the Skin Test and the γ-IFN Assay to Diagnose Bovine Tuberculosis in a Field Assay. Medicina Veterinaria 12, 307-317.

Domogalla, J., Prodinger, W.M., Blum, H., Krebs, S., Gellert, S., Müller, M., Neuendorf, E., Sedlmaier, F., Büttner, M., 2013. Region of Difference Four (RD4) in Alpine Mycobacterium Caprae Isolates Indicates Three Variants. J. Clin. Microbiol. .

Duarte, E.L., Domingos, M., Amado, A., Botelho, A., 2008. Spoligotype Diversity of Mycobacterium Bovis and Mycobacterium Caprae Animal Isolates. Vet. Microbiol. 130, 415-421.

EFSA Panel on Animal Health and Welfare (AHAW), 2012. Scientific Opinion on the use of a Gamma Interferon Test for the Diagnosis of Bovine Tuberculosis. EFSA J. 10(12), 2975.

Eisenach, K.D., Donald Cave, M., Bates, J.H., Crawford, J.T., 1990. Polymerase Chain Reaction Amplification of a Repetitive DNA Sequence Specific for Mycobacterium Tuberculosis. J. Infect. Dis. 161, 977-981.

Elamin, A.A., Stehr, M., Spallek, R., Rohde, M., Singh, M., 2011. The Mycobacterium Tuberculosis Ag85A is a Novel Diacylglycerol Acyltransferase Involved in Lipid Body Formation. Mol. Microbiol. 81, 1577-1592.

Endsley, J.J., Hogg, A., Shell, L.J., McAulay, M., Coffey, T., Howard, C., Capinos Scherer, C.F., Waters, W.R., Nonnecke, B., Estes, D.M., Villarreal-Ramos, B., 2007. Mycobacterium Bovis BCG Vaccination Induces Memory CD4+ T Cells Characterized by Effector Biomarker Expression and Anti-Mycobacterial Activity. Vaccine 25, 8384-8394.

Erler, W., Martin, G., Sachse, K., Naumann, L., Kahlau, D., Beer, J., Bartos, M., Nagy, G., Cvetnic, Z., Zolnir-Dovc, M., Pavlik, I., 2004. Molecular Fingerprinting of Mycobacterium Bovis Subsp. Caprae Isolates from Central Europe. J. Clin. Microbiol. 42, 2234-2238.

European Food Safety Authority, 2012. The European Union Summary Report on Trends and Sources of Zoonoses,Zoonotic Agents and Food-Borne Outbreaks in 2010. EFSA J. 10(3), 2597.

Ferrara, G., Losi, M., D'Amico, R., Roversi, P., Piro, R., Meacci, M., Meccugni, B., Dori, I.M., Andreani, A., Bergamini, B.M., Mussini, C., Rumpianesi, F., Fabbri, L.M., Richeldi, L., 2006. Use in Routine Clinical Practice of Two Commercial Blood Tests for Diagnosis of Infection with Mycobacterium Tuberculosis: A Prospective Study. Lancet 367, 1328-1334.

Fine, P.E., 1995. Variation in Protection by BCG: Implications of and for Heterologous Immunity. Lancet 346, 1339-1345.

Flynn, J., Chan, J., Triebold, K., Dalton, D., Stewart, T., Bloom, B., 1993. An Essential Role for Interferon Gamma in Resistance to Mycobacterium Tuberculosis Infection. J. Exp. Med. 178, 2249-2254.

Flynn, J.L., 2004. Immunology of Tuberculosis and Implications in Vaccine Development. Tuberculosis (Edinb) 84, 93-101.

Flynn, J.L., Chan, J., 2001. Immunology of Tuberculosis. Annu. Rev. Immunol. 19, 93-129.

Foulds, K.E., Wu, C.Y., Seder, R.A., 2006. Th1 Memory: Implications for Vaccine Development. Immunol. Rev. 211, 58-66.

Francis, J., 1958. Tuberculosis in Animals and Man. London: Cassell.

Ganguly, N., Siddiqui, I., Sharma, P., 2008. Role of M. Tuberculosis RD-1 Region Encoded Secretory Proteins in Protective Response and Virulence. Tuberculosis (Edinb) 88, 510-517.

García Marín, J.F., 2010. Tuberculosis Caprina: Diagnóstico. Pequeños rumiantes 11, 25-33.

Garcia Pelayo, M.C., Uplekar, S., Keniry, A., Mendoza Lopez, P., Garnier, T., Nunez Garcia, J., Boschiroli, L., Zhou, X., Parkhill, J., Smith, N., Hewinson, R.G., Cole, S.T., Gordon, S.V., May

2009. A Comprehensive Survey of Single Nucleotide Polymorphisms (SNPs) Across Mycobacterium Bovis Strains and M. Bovis BCG Vaccine Strains Refines the Genealogy and Defines a Minimal Set of SNPs that Separate Virulent M. Bovis Strains and M. Bovis BCG Strains. Infect. Immun. 77, 2230-2238.

Garcia-Jimenez, W.L., Benitez-Medina, J.M., Fernandez-Llario, P., Abecia, J.A., Garcia-Sanchez, A., Martinez, R., Risco, D., Ortiz-Pelaez, A., Salguero, F.J., Smith, N.H., Gomez, L., Hermoso de Mendoza, J., 2013. Comparative Pathology of the Natural Infections by Mycobacterium Bovis and by Mycobacterium Caprae in Wild Boar (Sus Scrofa). Transbound. Emerg. Dis. 60(2), 102-109.

Garnier, T., Eiglmeier, K., Camus, J.C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Doggett, J., Mayes, R., Keating, L., Wheeler, P.R., Parkhill, J., Barrell, B.G., Cole, S.T., Gordon, S.V., Hewinson, R.G., 2003. The Complete Genome Sequence of Mycobacterium Bovis. Proc. Natl. Acad. Sci. U. S. A. 100, 7877-7882.

Garrido, J.M., Sevilla, I.A., Beltran-Beck, B., Minguijon, E., Ballesteros, C., Galindo, R.C., Boadella, M., Lyashchenko, K.P., Romero, B., Geijo, M.V., Ruiz-Fons, F., Aranaz, A., Juste, R.A., Vicente, J., de la Fuente, J., Gortazar, C., 2011. Protection Against Tuberculosis in Eurasian Wild Boar Vaccinated with Heat-Inactivated Mycobacterium Bovis. PLoS One 6, e24905.

Geluk, A., Lin, M.Y., van Meijgaarden, K.E., Leyten, E.M.S., Franken, K.L.M.C., Ottenhoff, T.H.M., Klein, M.R., June 2007. T-Cell Recognition of the HspX Protein of Mycobacterium Tuberculosis Correlates with Latent M. Tuberculosis Infection but Not with M. Bovis BCG Vaccination. Infect. Immun. 75, 2914-2921.

Gey, v.P., Gamieldien, J., Hide, W., Brown, G., Siezen, R., Beyers, A., 2001. The ESAT-6 Gene Cluster of Mycobacterium Tuberculosis and Other High G+C Gram-Positive Bacteria. Genome Biol. 2, research0044.1-research0044.18.

Gil, O., Díaz, I., Vilaplana, C., Tapia, G., Díaz, J., Fort, M., Cáceres, N., Pinto, S., Caylà , J., Corner, L., Domingo, M., Cardona, P.J., 2010. Granuloma Encapsulation is a Key Factor for Containing Tuberculosis Infection in Minipigs. PLoS One 5, e10030.

Gil, O., Guirado, E., Gordillo, S., Díaz, J., Tapia, G., Vilaplana, C., Ariza, A., Ausina, V., Cardona, P., 2006. Intragranulomatous Necrosis in Lungs of Mice Infected by Aerosol with Mycobacterium Tuberculosis is Related to Bacterial Load rather than to any One Cytokine Or T Cell Type. Microbes Infect. 8, 628-636.

Gil, O., Vilaplana, C., Guirado, E., Diaz, J., Caceres, N., Singh, M., Cardona, P., 2008. Enhanced Gamma Interferon Responses of Mouse Spleen Cells Following Immunotherapy for Tuberculosis Relapse. Clin.Vaccine Immunol. 15, 1742-1744.

Good, M., Clegg, T., Sheridan, H., Yearsely, D., O'Brien, T., Egan, J., Mullowney, P., 2009. Prevalence and Distribution of Paratuberculosis (Johne's Disease) in Cattle Herds in Ireland. Ir. Vet. J. 62, 597-606.

Gordon, S.V., Bottai, D., Simeone, R., Stinear, T.P., Brosch, R., 2009. Pathogenicity in the Tubercle Bacillus: Molecular and Evolutionary Determinants. Bioessays 31, 378-388.

Grange, J.M., Yates, M.D., 1994. Zoonotic Aspects of Mycobacterium Bovis Infection. Vet. Microbiol. 40, 137-151.

Grant, I.R., 2005. Zoonotic Potential of Mycobacterium Avium Ssp. Paratuberculosis: The Current Position. J. Appl. Microbiol. 98, 1282-1293.

Guirado, E., Amat, I., Gil, O., Díaz, J., Arcos, V., Caceres, N., Ausina, V., Cardona, P., 2006. Passive Serum Therapy with Polyclonal Antibodies Against Mycobacterium Tuberculosis Protects Against Post-Chemotherapy Relapse of Tuberculosis Infection in SCID Mice. Microb. Infect. 8, 1252-1259.

Guirado, E., Gil, O., Cáceres, N., Singh, M., Vilaplana, C., Cardona, P., 2008. Induction of a Specific Strong Polyantigenic Cellular Immune Response After Short-Term Chemotherapy Controls Bacillary Reactivation in Murine and Guinea Pig Experimental Models of Tuberculosis. Clin. Vaccine Immunol. 15, 1229-1237.

Gumber, S., Eamens, G., Whittington, R.J., 2006. Evaluation of a Pourquier ELISA Kit in Relation to Agar Gel Immunodiffusion (AGID) Test for Assessment of the Humoral Immune Response in Sheep and Goats with and without Mycobacterium Paratuberculosis Infection. Vet. Microbiol. 115, 91-101.

Gutierrez, M.C., Brisse, S., Brosch, R., Fabre, M., Omais, B., Marmiesse, M., Supply, P., Vincent, V., 2005. Ancient Origin and Gene Mosaicism of the Progenitor of Mycobacterium Tuberculosis. PLoS Pathog. 1, e5.

Gutiérrez, M., Tellechea, J., García Marín, J.F., 1998. Evaluation of Cellular and Serological Diagnostic Tests for the Detection of Mycobacterium Bovis-Infected Goats. Vet. Microbiol. 62, 281-290.

Haddad, N., Ostyn, A., Karoui, C., Masselot, M., Thorel, M.F., Hughes, S.L., Inwald, J., Hewinson, R.G., Durand, B., 2001. Spoligotype Diversity of Mycobacterium Bovis Strains Isolated in France from 1979 to 2000. J. Clin. Microbiol. 39, 3623-3632.

Hansen, N., Seiler, C., Rumpf, J., Kraft, P., Dlaske, H., Abele-Horn, M., Muellges, W., 2012. Human Tuberculous Meningitis Caused by Mycobacterium Caprae. Case Rep. Neurol. 4, 54-60.

Hawkridge, T., Scriba, T.J., Gelderbloem, S., Smit, E., Tameris, M., Moyo, S., Lang, T., Veldsman, A., Hatherill, M., Merwe, L.v.d., Fletcher, H.A., Mahomed, H., Hill, A.V.S., Hanekom, W.A., Hussey, G.D., McShane, H., 2008. Safety and Immunogenicity of a New Tuberculosis Vaccine, MVA85A, in Healthy Adults in South Africa. J. Infect. Dis. 198, 544-552.

Hewinson, R.G., Vordermeier, H.M., Buddle, B.M., 2003. Use of the Bovine Model of Tuberculosis for the Development of Improved Vaccines and Diagnostics. Tuberculosis (Edinb) 83, 119-130.

Hiko, A., Agga, G.E., 2011. First-Time Detection of Mycobacterium Species from Goats in Ethiopia. Trop. Anim. Health Prod. 43, 133-139.

Hope, J.C., Thom, M.L., Villarreal-Ramos, B., Vordermeier, H.M., Hewinson, R.G., Howard, C.J., 2005a. Vaccination of Neonatal Calves with Mycobacterium Bovis BCG Induces Protection Against Intranasal Challenge with Virulent M. Bovis. Clin. Exp. Immunol- 139, 48-56.

Hope, J.C., Thom, M.L., Villarreal-Ramos, B., Vordermeier, H.M., Hewinson, R.G., Howard, C.J., 2005b. Exposure to Mycobacterium Avium Induces Low-Level Protection from Mycobacterium Bovis Infection but Compromises Diagnosis of Disease in Cattle. Clin. Exp. Immunol. 141, 432-439.

Hotter, G.S., Collins, D.M., 2011. Mycobacterium Bovis Lipids: Virulence and Vaccines. Vet. Microbiol. 151, 91-98.

Houben, D., Demangel, C., van Ingen, J., Perez, J., Baldeon, L., Abdallah, A.M., Caleechurn, L., Bottai, D., van Zon, M., de Punder, K., van der Laan, T., Kant, A., Bossers-de Vries, R., Willemsen, P., Bitter, W., van Soolingen, D., Brosch, R., van der Wel, N., Peters, P.J., 2012. ESX-1-Mediated Translocation to the Cytosol Controls Virulence of Mycobacteria. Cell. Microbiol. 14, 1287-1298.

Howard, C.J., Kwong, L.S., Villarreal-Ramos, B., Sopp, P., Hope, J.C., 2002. Exposure to Mycobacterium Avium Primes the Immune System of Calves for Vaccination with Mycobacterium Bovis BCG. Clin. Exp. Immunol. 130, 190-195.

Huard, R.C., Fabre, M., de Haas, P., Lazzarini, L.C., van Soolingen, D., Cousins, D., Ho, J.L., 2006. Novel Genetic Polymorphisms that further Delineate the Phylogeny of the Mycobacterium Tuberculosis Complex. J. Bacteriol. 188, 4271-4287.

Humblet, M.F., Boschiroli, M.L., Saegerman, C., 2009. Classification of Worldwide Bovine Tuberculosis Risk Factors in Cattle: A Stratified Approach. Vet. Res. 40, 50.

Ikonomomopoulos, J., Aranaz, A., Balaskas, C., Sechi, L., Gazouli, M., 2006. Outbreak of Acute Tuberculosis in a Goat Herd; the First Report of *Mycobacterium Caprae* Isolation in Greece. Online J. Vet. Res. 10, 108-115.

Inwald, J., Hinds, J., Dale, J., Palmer, S., Butcher, P., Hewinson, R.G., Gordon, S.V., 2002. Microarray-Based Comparative Genomics: Genome Plasticity in Mycobacterium Bovis. Comp. Funct. Genomics 3, 342-344.

Jassal, M., Nedeltchev, G., Osborne, J., Bishai, W., 2011. A Modified Scoring System to Describe Gross Pathology in the Rabbit Model of Tuberculosis. BMC Microbiol. 11, 49.

Jeon, B., Je, S., Park, J., Kim, Y., Lee, E.G., Lee, H., Seo, S., Cho, S.N., 2008. Variable Number Tandem Repeat Analysis of Mycobacterium Bovis Isolates from Gyeonggi-do, Korea. J. Vet. Sci. 9, 145-153.

Jones, G.J., Whelan, A., Clifford, D., Coad, M., Vordermeier, H.M., 2012. Improved Skin Test for Differential Diagnosis of Bovine Tuberculosis by the Addition of Rv3020c-Derived Peptides. Clin. Vaccine Immunol. 19, 620-622.

Juste, R.A., Casal, J., 1993. An Economic and Epidemiologic Simulation of Different Control Strategies for Ovine Paratuberculosis. Prev. Vet. Med. 15, 101-115.

Juste, R.A., Elguezabal, N., Pavón, A., Garrido, J.M., Geijo, M., Sevilla, I., Cabriada, J.L., Tejada, A., García-Campos, F., Casado, R., Ochotorena, I., Izeta, A., 2009a. Association between Mycobacterium Avium Subsp. Paratuberculosis DNA in Blood and Cellular and Humoral Immune Response in Inflammatory Bowel Disease Patients and Controls. International J. Infect. Dis. 13, 247-254.

Juste, R., Alonso-Hearn, M., Molina, E., Geijo, M., Vazquez, P., Sevilla, I., Garrido, J., 2009b. Significant Reduction in Bacterial Shedding and Improvement in Milk Production in Dairy Farms After the use of a New Inactivated Paratuberculosis Vaccine in a Field Trial. BMC Res. Notes 2, 233.

Kappelman, J., Alcicek, M.C., Kazanci, N., Schultz, M., Ozkul, M., Sen, S., 2008. First Homo Erectus from Turkey and Implications for Migrations into Temperate Eurasia. Am. J. Phys. Anthropol. 135, 110-116.

Karlson, A.G., Lessel, E.F., 1970. *Mycobacterium Bovis* Nom. Nov. Int. J. Syst. Bacteriol. 20, 273-282.

Kaufmann, S.H., 2010. Future Vaccination Strategies Against Tuberculosis: Thinking Outside the Box. Immunity 33, 567-577.

Kaufmann, S.H., 2008. Tuberculosis: Deadly Combination. Nature 453, 295-296.

Kaufmann, S.H., 2006. Envisioning Future Strategies for Vaccination Against Tuberculosis. Nat. Rev. Immunol. 6, 699-704.

Kaufmann, S.H.E., 2005. Recent Findings in Immunology Give Tuberculosis Vaccines a New Boost. Trends Immunol. 26, 660-667.

Kaufmann, S.H.E., 2001. How can Immunology Contribute to the Control of Tuberculosis?. Nat. Rev. Immunol. 1, 30.

Kaufmann, S.H., Hussey, G., Lambert, P., 2010. New Vaccines for Tuberculosis. Lancet 375, 2110-2119.

Keane, J., Balcewicz-Sablinska, M.K., Remold, H.G., Chupp, G.L., Meek, B.B., Fenton, M.J., Kornfeld, H., 1997. Infection by Mycobacterium Tuberculosis Promotes Human Alveolar Macrophage Apoptosis. Infect. Immun. 65, 298-304.

Keating, S.M., Bejon, P., Berthoud, T., Vuola, J.M., Todryk, S., Webster, D.P., Dunachie, S.J., Moorthy, V.S., McConkey, S.J., Gilbert, S.C., Hill, A.V.S., 2005. Durable Human Memory T Cells Quantifiable by Cultured Enzyme-Linked Immunospot Assays are Induced by Heterologous Prime Boost Immunization and Correlate with Protection Against Malaria. J. Immunol. 175, 5675-5680.

Khader, S.A., Bell, G.K., Pearl, J.E., Fountain, J.J., Rangel-Moreno, J., Cilley, G.E., Shen, F., Eaton, S.M., Gaffen, S.L., Swain, S.L., Locksley, R.M., Haynes, L., Randall, T.D., Cooper, A.M., 2007. IL-23 and IL-17 in the Establishment of Protective Pulmonary CD4+ T Cell Responses After Vaccination and during Mycobacterium Tuberculosis Challenge. Nat. Immunol. 8, 369-377.

Khader, S.A., Cooper, A.M., 2008. IL-23 and IL-17 in Tuberculosis. Cytokine 41, 79-83.

Koch, R., 1891. A further Communication on a Remedy for Tuberculosis. Br. Med. J. 1, 125-127.

Koch, R., 1882. Aetiologie Der Tuberculose. Berl. Klin. Wochenschr. 19, 221-230.

Krebs, J.R., The Independent Scientific Review Group, 1997. Bovine Tuberculosis in Cattle and Badgers. London, Ministry of Agriculture, Fisheries and Food Publications, London, UK .

Kubica, T., Rusch-Gerdes, S., Niemann, S., 2003. Mycobacterium Bovis Subsp. Caprae Caused One-Third of Human M. Bovis-Associated Tuberculosis Cases Reported in Germany between 1999 and 2001. J. Clin. Microbiol. 41, 3070-3077.

Kyd, J.M., Foxwell, A.R., Cripps, A.W., 2001. Mucosal Immunity in the Lung and Upper Airway. Vaccine 19, 2527-2533.

Langermans, J.A.M., Doherty, T.M., Vervenne, R.A.W., Laan, T.v.d., Lyashchenko, K., Greenwald, R., Agger, E.M., Aagaard, C., Weiler, H., Soolingen, D.v., Dalemans, W., Thomas, A.W., Andersen, P., 2005. Protection of Macaques Against Mycobacterium Tuberculosis Infection by a Subunit Vaccine Based on a Fusion Protein of Antigen 85B and ESAT-6. Vaccine 23, 2740-2750.

Lasaro, M.O., Ertl, H.C., 2009. New Insights on Adenovirus as Vaccine Vectors. Mol. Ther. 17, 1333-1339.

Lawn, S.D., Zumla, A.I., 2011. Tuberculosis. Lancet 378, 57-72.

Leal, I.S., Smedegard, B., Andersen, P., Appelberg, R., 2001. Failure to Induce Enhanced Protection Against Tuberculosis by Increasing T-Cell-Dependent Interferon-Gamma Generation. Immunology 104, 157-161.

Lesellier, S., Corner, L., Costello, E., Lyashchenko, K., Greenwald, R., Esfandiari, J., Singh, M., Hewinson, R.G., Chambers, M., Gormley, E., 2009. Immunological Responses and Protective Immunity in BCG Vaccinated Badgers Following Endobronchial Infection with Mycobacterium Bovis. Vaccine 27, 402-409.

Leyten, E.M.S., Lin, M.Y., Franken, K.L.M.C., Friggen, A.H., Prins, C., van Meijgaarden, K.E., Voskuil, M.I., Weldingh, K., Andersen, P., Schoolnik, G.K., Arend, S.M., Ottenhoff, T.H.M., Klein, M.R., 2006. Human T-Cell Responses to 25 Novel Antigens Encoded by Genes of the Dormancy Regulon of Mycobacterium Tuberculosis. Microb. Infect. 8, 2052-2060.

Liebana , E., Aranaz, A., Urquía, J.J., Mateos, A., Dominguez, L., 1998. Evaluation of the Gamma-Interferon Assay for Eradication of Tuberculosis in a Goat Herd. Aust. Vet. J. 76, 50-53.

Lienhardt, C., Glaziou, P., Uplekar, M., Lonnroth, K., Getahun, H., Raviglione, M., 2012. Global Tuberculosis Control: Lessons Learnt and Future Prospects. Nat. Rev. Microbiol. 10, 407-416.

Lightbody, K.A., Skuce, R.A., Neill, S.D., Pollock, J.M., 1998. Mycobacterial Antigen-Specific Antibody Responses in Bovine Tuberculosis: An ELISA with Potential to Confirm Disease Status. Vet. Rec. 142, 295-300.

Lin, P.L., Dietrich, J., Tan, E., Abalos, R.M., Burgos, J., Bigbee, C., Bigbee, M., Milk, L., Gideon, H.P., Rodgers, M., Cochran, C., Guinn, K.M., Sherman, D.R., Klein, E., Janssen, C., Flynn, J.L., Andersen, P., 2012. The Multistage Vaccine H56 Boosts the Effects of BCG to Protect Cynomolgus Macaques Against Active Tuberculosis and Reactivation of Latent Mycobacterium Tuberculosis Infection. J. Clin. Invest. 122, 303-314.

Lin, P.L., Rodgers, M., Smith, L., Bigbee, M., Myers, A., Bigbee, C., Chiosea, I., Capuano, S.V., Fuhrman, C., Klein, E., Flynn, J.L., 2009. Quantitative Comparison of Active and Latent Tuberculosis in the Cynomolgus Macaque Model. Infect. Immun. 77, 4631-4642.

Lindestam Arlehamn, C.S., Sidney, J., Henderson, R., Greenbaum, J.A., James, E.A., Moutaftsi, M., Coler, R., McKinney, D.M., Park, D., Taplitz, R., Kwok, W.W., Grey, H., Peters, B., Sette, A., 2012. Dissecting Mechanisms of Immunodominance to the Common Tuberculosis Antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ). J. Immunol. 188, 5020-5031.

Lyashchenko, K., Whelan, A.O., Greenwald, R., Pollock, J.M., Andersen, P., Hewinson, R.G., Vordermeier, H.M., 2004. Association of Tuberculin-Boosted Antibody Responses with Pathology and Cell-Mediated Immunity in Cattle Vaccinated with Mycobacterium Bovis BCG and Infected with M-Bovis. Infect. Immun. 72, 2462-2467.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Chambers, M.A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B.M., Jackson, R., O'Brien, D.J., Schmitt, S., Palmer, M.V., Delahay, R.J., Waters, W.R., 2008. Animal-Side Serologic Assay for Rapid Detection of Mycobacterium Bovis Infection in Multiple Species of Free-Ranging Wildlife. Vet. Microbiol. 132, 283-292.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Greenwald, D., Nacy, C.A., Gibson, S., Didier, P.J., Washington, M., Szczerba, P., Motzel, S., Handt, L., Pollock, J.M., McNair, J., Andersen, P., Langermans, J.A.M., Verreck, F., Ervin, S., Ervin, F., McCombs, C., 2007. PrimaTB STAT-PAK Assay, a Novel, Rapid Lateral-Flow Test for Tuberculosis in Nonhuman Primates. Clin. Vaccine Immunol. 14, 1158-1164.

Mackintosh, C.G., Labes, R.E., Griffin, J.F.T., 2005. The Effect of Johne's Vaccination on Tuberculin Testing in Farmed Red Deer (Cervus Elaphus). N. Z. Vet. J. 53, 216-222.

Mahairas, G.G., Sabo, P.J., Hickey, M.J., Singh, D.C., Stover, C.K., 1996. Molecular Analysis of Genetic Differences between Mycobacterium Bovis BCG and Virulent M. Bovis. J. Bacteriol. 178, 1274-1282.

Martin, C., Williams, A., Hernandez-Pando, R., Cardona, P.J., Gormley, E., Bordat, Y., Soto, C.Y., Clark, S.O., Hatch, G.J., Aguilar, D., Ausina, V., Gicquel, B., 2006. The Live Mycobacterium Tuberculosis phoP Mutant Strain is More Attenuated than BCG and Confers Protective Immunity Against Tuberculosis in Mice and Guinea Pigs. Vaccine 24, 3408-3419.

Martin-Hernando, M., Hofle, U., Vicente, J., Ruiz-Fons, F., Vidal, D., Barral, M., Garrido, J.M., de, l.F., Gortazar, C., 2007. Lesions Associated with Mycobacterium Tuberculosis Complex Infection in the European Wild Boar. Tuberculosis (Edinb) 87, 360-367.

Maue, A.C., Waters, W.R., Palmer, M.V., Whipple, D.L., Minion, F.C., Brown, W.C., Estes, D.M., 2004. CD80 and CD86, but Not CD154, Augment DNA Vaccine-Induced Protection in Experimental Bovine Tuberculosis. Vaccine 23, 769-779.

McCorry, T., Whelan, A.O., Welsh, M.D., McNair, J., Walton, E., Bryson, D.G., Hewinson, R.G., Vordermeier, H.M., Pollock, J.M., 2005. Shedding of Mycobacterium Bovis in the Nasal Mucus of Cattle Infected Experimentally with Tuberculosis by the Intranasal and Intratracheal Routes. Vet. Rec. 157, 613-618.

McLaughlin, B., Chon, J.S., MacGurn, J.A., Carlsson, F., Cheng, T.L., Cox, J.S., Brown, E.J., 2007. A Mycobacterium ESX-1-Secreted Virulence Factor with Unique Requirements for Export. PLoS Pathog. 3, e105.

McShane, H., Hill, A., 2005a. Prime-Boost Immunisation Strategies for Tuberculosis. Microb. Infect. 7, 962-967.

McShane, H., Pathan, A., Sander, C., Goonetilleke, N., Fletcher, H., Hill, A.S., 2005b. Boosting BCG with MVA85A: The First Candidate Subunit Vaccine for Tuberculosis in Clinical Trials. Tuberculosis (Edinb) 85, 47-52.

Michel, A.L., Hlokwe, T.M., Coetzee, M.L., Maré, L., Connoway, L., Rutten, V.P.M.G., Kremer, K., 2008. High Mycobacterium Bovis Genetic Diversity in a Low Prevalence Setting. Vet. Microbiol. 126, 151-159.

Millington, K.A., Fortune, S.M., Low, J., Garces, A., Hingley-Wilson, S.M., Wickremasinghe, M., Kon, O.M., Lalvani, A., 2011. Rv3615c is a Highly Immunodominant RD1 (Region of Difference 1)-Dependent Secreted Antigen Specific for Mycobacterium Tuberculosis Infection. Proc. Natl. Acad. Sci. U. S. A. 108, 5730-5735.

Mostowy, S., Cousins, D., Brinkman, J., Aranaz, A., Behr, M.A., 2002. Genomic Deletions Suggest a Phylogeny for the Mycobacterium Tuberculosis Complex. J. Infect. Dis. 186, 74-80.

Munoz Mendoza, M., Juan, L., Menendez, S., Ocampo, A., Mourelo, J., Saez, J.L., Dominguez, L., Gortazar, C., Garcia Marin, J.F., Balseiro, A., 2012. Tuberculosis due to Mycobacterium Bovis and Mycobacterium Caprae in Sheep. Vet. J. 191, 267-269.

Muskens, J., van Zijderveld, F., Eger, A., Bakker, D., 2002. Evaluation of the Long-Term Immune Response in Cattle After Vaccination Against Paratuberculosis in Two Dutch Dairy Herds. Vet. Microbiol. 86, 269-278.

Mustafa, A.S., Al-Attiyah, R., Hanif, S.N., Shaban, F.A., 2008. Efficient Testing of Large Pools of Mycobacterium Tuberculosis RD1 Peptides and Identification of Major Antigens and Immunodominant Peptides Recognized by Human Th1 Cells. Clin. Vaccine Immunol. 15, 916-924.

Mustafa, A.S., Skeiky, Y.A., Al-Attiyah, R., Alderson, M.R., Hewinson, R.G., Vordermeier, H.M., 2006. Immunogenicity of Mycobacterium Tuberculosis Antigens in Mycobacterium Bovis BCG-Vaccinated and M. Bovis-Infected Cattle. Infect. Immun. 74, 4566-4572.

Napp, S., Allepuz, A., Mercader, I., Nofrarías, M., López-Soria, S., Domingo, M., Romero, B., Bezos, J., Pérez de Val, B., 2013. Evidence of Goats Acting as Domestic Reservoirs of Bovine Tuberculosis. Vet. Rec. 172, 663-663.

Niu, H., Hu, L., Li, Q., Da, Z., Wang, B., Tang, K., Xin, Q., Yu, H., Zhang, Y., Wang, Y., Ma, X., Zhu, B., 2011. Construction and Evaluation of a Multistage Mycobacterium Tuberculosis Subunit Vaccine Candidate Mtb10.4–HspX. Vaccine 29, 9451-9458.

Nol, P., Palmer, M.V., Waters, W.R., Aldwell, F.E., Buddle, B.M., Triantis, J.M., Linke, L.M., Phillips, G.E., Thacker, T.C., Rhyan, J.C., Dunbar, M.R., Salman, M.D., 2008. Efficacy of Oral and Parenteral Routes of Mycobacterium Bovis Bacille Calmette-Guerin Vaccination Against Experimental Bovine Tuberculosis in White-Tailed Deer (Odocoileus Virginianus): A Feasibility Study. J. Wildl. Dis. 44, 247-259.

Oettinger, T., Andersen, A.B., 1994. Cloning and B-Cell-Epitope Mapping of MPT64 from Mycobacterium Tuberculosis H37Rv. Infect. Immun. 62, 2058-2064.

Olsen, A.W., van Pinxteren, L.A.H., Okkels, L.M., Rasmussen, P.B., Andersen, P., 2001. Protection of Mice with a Tuberculosis Subunit Vaccine Based on a Fusion Protein of Antigen 85B and ESAT-6. Infect. Immun. 69, 2773-2778.

Orme, I.M., 2005. Mouse and Guinea Pig Models for Testing New Tuberculosis Vaccines. Tuberculosis (Edinb) 85, 13-17.

Orme, I.M., 1998. The Immunopathogenesis of Tuberculosis: A New Working Hypothesis. Trends Microbiol. 6, 94-97.

Ottenhoff, T.H., Kaufmann, S.H., 2012. Vaccines Against Tuberculosis: Where are we and Where do we Need to Go? PLoS Pathog. 8, e1002607.

Pai, M., Zwerling, A., Menzies, D., 2008. Systematic Review: T-Cell-Based Assays for the Diagnosis of Latent Tuberculosis Infection: An Update. Ann. Intern. Med. 149, 177-184.

Pai, M., Kalantri, S., Dheda, K., 2006. New Tools and Emerging Technologies for the Diagnosis of Tuberculosis: Part II. Active Tuberculosis and Drug Resistance. Expert Rev. Mol. Diagn. 6, 423-432.

Pai, M., Riley, L.W., Colford, J.,John M., 2004. Interferon-γ Assays in the Immunodiagnosis of Tuberculosis: A Systematic Review. Lancet Infect. Dis. 4, 761-776.

Palmer, M.V., Thacker, T.C., Waters, W.R., 2007. Vaccination of White-Tailed Deer (Odocoileus Virginianus) with Mycobacterium Bovis Bacillus Calmette Guerín. Vaccine 25, 6589-6597.

Palmer, M.V., Thacker, T.C., Waters, W.R., Gortazar, C., Corner, L.A., 2012. Mycobacterium Bovis: A Model Pathogen at the Interface of Livestock, Wildlife, and Humans. Vet. Med. Int. 2012, 236205.

Palmer, M.V., Waters, W.R., 2006a. Advances in Bovine Tuberculosis Diagnosis and Pathogenesis: What Policy Makers Need to Know. Vet. Microbiol. 112, 181-190.

Palmer, M.V., Waters, W.R., Thacker, T.C., Greenwald, R., Esfandiari, J., Lyashchenk, K.P., 2006b. Effects of Different Tuberculin Skin-Testing Regimens on Gamma Interferon and Antibody Responses in Cattle Experimentally Infected with Mycobacterium Bovis. Clin. Vaccine Immunol. 13, 387-394.

Palmer, M.V., Waters, W.R., Whipple, D.L., 2004. Investigation of the Transmission of Mycobacterium Bovis from Deer to Cattle through Indirect Contact. Am. J. Vet. Res. 65, 1483-1489.

Pang, X., Howard, S.T., 2007. Regulation of the α-Crystallin Gene acr2 by the MprAB Two-Component System of Mycobacterium Tuberculosis. J. Bacteriol. 189, 6213-6221.

Pate, M., Svara, T., Gombac, M., Paller, T., Zolnir-Dovc, M., Emersic, I., Prodinger, W.M., Bartos, M., Zdovc, I., Krt, B., Pavlik, I., Cvetnic, Z., Pogacnik, M., Ocepek, M., 2006. Outbreak of Tuberculosis Caused by Mycobacterium Caprae in a Zoological Garden. J.Vet. Med. B Infect. Dis. Vet. Public Health. 53, 387-392.

Pavlik, I., 2006. The Experience of New European Union Member States Concerning the Control of Bovine Tuberculosis. Vet. Microbiol. 112, 221-230.

Pérez de Val, B., López-Soria, S., Nofrarías, M., Martín, M., Vordermeier, H.M., Villarreal-Ramos, B., Romera, N., Escobar, M., Solanes, D., Cardona, P.J., Domingo, M., 2011. Experimental Model of Tuberculosis in the Domestic Goat After Endobronchial Infection with Mycobacterium Caprae. Clin. Vaccine Immunol. 18, 1872-1881.

Pérez de Val, B., Villarreal-Ramos, B., Nofrarías, M., López-Soria, S., Romera, N., Singh, M., Abad, F.X., Xing, Z., Vordermeier, H.M., Domingo, M., 2012. Goats Primed with Mycobacterium Bovis BCG and Boosted with a Recombinant Adenovirus Expressing Ag85A show Enhanced Protection Against Tuberculosis. Clin. Vaccine Immunol. 19, 1339-1347.

Platt, R., Thoen, C.O., Stalberger, R.J., Chiang, Y., Roth, J.A., 2010. Evaluation of the Cell-Mediated Immune Response to Reduced Doses of Mycobacterium Avium Ssp. Paratuberculosis Vaccine in Cattle. Vet. Immunol. Immunopathol. 136, 122-126.

Plattner, B.L., Doyle, R.T., Hostetter, J.M., 2009. Gamma-Delta T Cell Subsets are Differentially Associated with Granuloma Development and Organization in a Bovine Model of Mycobacterial Disease. Int. J. Exp. Pathol. 90, 587-597.

Plikaytis, B.B., Eisenach, K.D., Crawford, J.T., Shinnick, T.M., 1991. Differentiation of Mycobacterium Tuberculosis and Mycobacterium Bovis BCG by a Polymerase Chain Reaction Assay. Mol. Cell. Probes 5, 215-219.

Plopper, C.G., Harkema, J.R., 2005. The Respiratory Sytem and its use in Research. The laboratory primate. Elsevier Academic Press, San Diego. 30, 503-526.

Pollock, J.M., Neill, S.D., 2002. Mycobacterium Bovis Infection and Tuberculosis in Cattle. Vet. J. 163, 115-127.

Pollock, J.M., Pollock, D.A., Campbell, D.G., Girvin, R.M., Crockard, A.D., Neill, S.D., Mackie, D.P., 1996. Dynamic Changes in Circulating and Antigen-Responsive T-Cell Subpopulations Post-Mycobacterium Bovis Infection in Cattle. Immunology 87, 236-241.

Pollock, J.M., Rodgers, J.D., Welsh, M.D., McNair, J., 2006. Pathogenesis of Bovine Tuberculosis: The Role of Experimental Models of Infection. Vet. Microbiol. 112, 141-150.

Pollock, J.M., Welsh, M.D., McNair, J., 2005. Immune Responses in Bovine Tuberculosis: Towards New Strategies for the Diagnosis and Control of Disease. Vet. Immunol. Immunopathol. 108, 37-43.

Pollock, J., Andersen, P., 1997. Predominant Recognition of the ESAT-6 Protein in the First Phase of Interferon with Mycobacterium Bovis in Cattle. Infect. Immun. 65, 2587-2592.

Prodinger, W.M., Brandstatter, A., Naumann, L., Pacciarini, M., Kubica, T., Boschiroli, M.L., Aranaz, A., Nagy, G., Cvetnic, Z., Ocepek, M., Skrypnyk, A., Erler, W., Niemann, S., Pavlik, I., Moser, I., 2005. Characterization of Mycobacterium Caprae Isolates from Europe by Mycobacterial Interspersed Repetitive Unit Genotyping. J. Clin. Microbiol. 43, 4984-4992.

Prodinger, W.M., Eigentler, A., Allerberger, F., Schonbauer, M., Glawischnig, W., 2002. Infection of Red Deer, Cattle, and Humans with Mycobacterium Bovis Subsp. Caprae in Western Austria. J. Clin. Microbiol. 40, 2270-2272.

Quintas, H., Reis, J., Pires, I., Alegria, N., 2010. Tuberculosis in Goats. Vet. Rec. 166, 437-a-438.

Radošević, K., Wieland, C.W., Rodriguez, A., Weverling, G.J., Mintardjo, R., Gillissen, G., Vogels, R., Skeiky, Y.A.W., Hone, D.M., Sadoff, J.C., van der Poll, T., Havenga, M., Goudsmit, J., August 2007. Protective Immune Responses to a Recombinant Adenovirus Type 35 Tuberculosis Vaccine in Two Mouse Strains: CD4 and CD8 T-Cell Epitope Mapping and Role of Gamma Interferon. Infect. Immun. 75, 4105-4115.

Ramirez-Villaescusa, A.M., Medley, G.F., Mason, S., Green, L.E., 2010. Risk Factors for Herd Breakdown with Bovine Tuberculosis in 148 Cattle Herds in the South West of England. Prev. Vet. Med. 95, 224-230.

Rastogi, N., Legrand, E., Sola, C., 2001. The Mycobacteria: An Introduction to Nomenclature and Pathogenesis. Rev. Sci. Tech. 20, 21-54.

Reddacliff, L., Eppleston, J., Windsor, P., Whittington, R., Jones, S., 2006. Efficacy of a Killed Vaccine for the Control of Paratuberculosis in Australian Sheep Flocks. Vet. Microbiol. 115, 77-90.

Reiley, W.W., Calayag, M.D., Wittmer, S.T., Huntington, J.L., Pearl, J.E., Fountain, J.J., Martino, C.A., Roberts, A.D., Cooper, A.M., Winslow, G.M., Woodland, D.L., 2008. ESAT-6-Specific CD4 T Cell Responses to Aerosol Mycobacterium Tuberculosis Infection are Initiated in the Mediastinal Lymph Nodes. Proc. Natl. Acad. Sci. U. S. A. 105, 10961-10966.

Renshaw, P.S., Lightbody, K.L., Veverka, V., Muskett, F.W., Kelly, G., Frenkiel, T.A., Gordon, S.V., Hewinson, R.G., Burke, B., Norman, J., Williamson, R.A., Carr, M.D., 2005. Structure and Function of the Complex Formed by the Tuberculosis Virulence Factors CFP-10 and ESAT-6. EMBO J. 24, 2491-2498.

Rhodes, S.G., Hewinson, R.G., Vordermeier, H.M., 2001. Antigen Recognition and Immunomodulation by γδ T Cells in Bovine Tuberculosis. J. Immunol. 166, 5604-5610.

Rizzi, C., Bianco, M.V., Blanco, F.C., Soria, M., Gravisaco, M.J., Montenegro, V., Vagnoni, L., Buddle, B., Garbaccio, S., Delgado, F., Leal, K.S., Cataldi, A.A., Dellagostin, O.A., Bigi, F., 2012. Vaccination with a BCG Strain Overexpressing Ag85B Protects Cattle Against Mycobacterium Bovis Challenge. PLoS One 7, e51396.

Rodrigues, L.C., Diwan, V.K., Wheeler, J.G., 1993. Protective Effect of BCG Against Tuberculous Meningitis and Miliary Tuberculosis: A Meta-Analysis. Int. J. Epidemiol. 22, 1154-1158.

Rodríguez, E., Sánchez, L.P., Pérez, S., Herrera, L., Jiménez, M.S., Samper, S., Iglesias, M.J., 2009. Human Tuberculosis due to Mycobacterium Bovis and M. Caprae in Spain, 20042007. Int. J. Tuberc. Lung Dis. 13, 1536-1541.

Rodriguez, S., Bezos, J., Romero, B., de Juan, L., Alvarez, J., Castellanos, E., Moya, N., Lozano, F., Javed, M.T., Saez-Llorente, J.L., Liebana, E., Mateos, A., Dominguez, L., Aranaz, A., The Spanish Network on Surveillance and Monitoring of Animal Tuberculosis, 2011. Mycobacterium Caprae Infection in Livestock and Wildlife, Spain. Emerg. Infect. Dis. 17, 532-535.

Rodriguez-Campos, S., Gonzalez, S., de Juan, L., Romero, B., Bezos, J., Casal, C., Alvarez, J., Fernandez-de-Mera, I.G., Castellanos, E., Mateos, A., Saez-Llorente, J.L., Dominguez, L., Aranaz, A., Spanish Network on Surveillance Monitoring of Animal Tuberculosis, 2012. A Database for Animal Tuberculosis (mycoDB.Es) within the Context of the Spanish National Programme for Eradication of Bovine Tuberculosis. Infect. Genet. Evol. 12, 877-882.

Rothel, J.S., Jones, S.L., Corner, L.A., Cox, J.C., Wood, P.R., 1990. A Sandwich Enzyme Immunoassay for Bovine Interferon-Gamma and its use for the Detection of Tuberculosis in Cattle. Aust. Vet. J. 67, 134-137.

Rothschild, B.M., Martin, L.D., Lev, G., Bercovier, H., Bar-Gal, G.K., Greenblatt, C., Donoghue, H., Spigelman, M., Brittain, D., 2001. Mycobacterium Tuberculosis Complex DNA from an Extinct Bison Dated 17,000 Years before the Present. Clin. Infect. Dis. 33, 305-311.

Ruhwald, M., Dominguez, J., Latorre, I., Losi, M., Richeldi, L., Pasticci, M.B., Mazzolla, R., Goletti, D., Butera, O., Bruchfeld, J., Gaines, H., Gerogianni, I., Tuuminen, T., Ferrara, G., Eugen-Olsen, J., Ravn, P., 2011. A Multicentre Evaluation of the Accuracy and Performance of IP-10 for the Diagnosis of Infection with M. Tuberculosis. Tuberculosis (Edinb) 91, 260-267.

Russell, D.G., 2007. Who Puts the Tubercle in Tuberculosis? Nat. Rev. Microbiol. 5, 39-47.

Russell, M.S., Dudani, R., Krishnan, L., Sad, S., 2009. IFN-γ Expressed by T Cells Regulates the Persistence of Antigen Presentation by Limiting the Survival of Dendritic Cells. J. Immunol. 183, 7710-7718.

Sanchez, J., Tomás, L., Ortega, N., Buendía, A.J., del Rio, L., Salinas, J., Bezos, J., Caro, M.R., Navarro, J.A., 2011. Microscopical and Immunological Features of Tuberculoid Granulomata and Cavitary Pulmonary Tuberculosis in Naturally Infected Goats. J. Comp. Pathol. 145, 107-117.

Sander, C.R., Pathan, A.A., Beveridge, N.E., Poulton, I., Minassian, A., Alder, N., Van Wijgerden, J., Hill, A.V., Gleeson, F.V., Davies, R.J., Pasvol, G., McShane, H., 2009. Safety and Immunogenicity of a New Tuberculosis Vaccine, MVA85A, in Mycobacterium Tuberculosis-Infected Individuals. Am. J. Respir. Crit. Care Med. 179, 724-733.

Santosuosso, M., McCormick, S., Zhang, X., Zganiacz, A., Xing, Z., 2006. Intranasal Boosting with an Adenovirus-Vectored Vaccine Markedly Enhances Protection by Parenteral Mycobacterium Bovis BCG Immunization Against Pulmonary Tuberculosis. Infect. Immun. 74, 4634-4643.

Santosuosso, M., Zhang, X., McCormick, S., Wang, J., Hitt, M., Xing, Z., 2005. Mechanisms of Mucosal and Parenteral Tuberculosis Vaccinations: Adenoviral-Based Mucosal Immunization Preferentially Elicits Sustained Accumulation of Immune Protective CD4 and CD8 T Cells within the Airway Lumen. J. Immunol. 174, 7986-7994.

Saunders, B.M., Cooper, A.M., 2000. Restraining Mycobacteria: Role of Granulomas in Mycobacterial Infections. Immunol. Cell Biol. 78, 334-341.

Schaible, U.E., Sturgill-Koszycki, S., Schlesinger, P.H., Russell, D.G., 1998. Cytokine Activation Leads to Acidification and Increases Maturation of Mycobacterium Avium-Containing Phagosomes in Murine Macrophages. J. Immunol. 160, 1290-1296.

Schuck, S.D., Mueller, H., Kunitz, F., Neher, A., Hoffmann, H., Franken, K.L.C.M., Repsilber, D., Ottenhoff, T.H.M., Kaufmann, S.H.E., Jacobsen, M., 2009. Identification of T-Cell Antigens Specific for Latent Mycobacterium Tuberculosis Infection. PLoS One 4, e5590.

Schwabacher, H., 1934. A Case of Spontaneous Tuberculosis in a Goat. J. Comp. Pathol. Ther. 47, 214-218.

Scriba, T.J., Kalsdorf, B., Abrahams, D., Isaacs, F., Hofmeister, J., Black, G., Hassan, H.Y., Wilkinson, R.J., Walzl, G., Gelderbloem, S.J., Mahomed, H., Hussey, G.D., Hanekom, W.A., 2008. Distinct, Specific IL-17- and IL-22-Producing CD4+ T Cell Subsets Contribute to the Human Anti-Mycobacterial Immune Response. J. Immunol. 180, 1962-1970.

Sharpe, A.E., Brady, C.P., Johnson, A., Byrne, W., Kenny, K., Costello, E., 2010. Concurrent Outbreak of Tuberculosis and Caseous Lymphadenitis in a Goat Herd. Vet. Rec. 166, 591-592.

Sharpe, S.A., Eschelbach, E., Basaraba, R.J., Gleeson, F., Hall, G.A., McIntyre, A., Williams, A., Kraft, S.L., Clark, S., Gooch, K., Hatch, G., Orme, I.M., Marsh, P.D., Dennis, M.J., 2009. Determination of Lesion Volume by MRI and Stereology in a Macaque Model of Tuberculosis. Tuberculosis (Edinb) 89, 405-416.

Sharpe, S.A., McShane, H., Dennis, M.J., Basaraba, R.J., Gleeson, F., Hall, G., McIntyre, A., Gooch, K., Clark, S., Beveridge, N.E.R., Nuth, E., White, A., Marriott, A., Dowall, S., Hill, A.V.S., Williams, A., Marsh, P.D., 2010. Establishment of an Aerosol Challenge Model of Tuberculosis in Rhesus Macaques and an Evaluation of Endpoints for Vaccine Testing. Clin. Vaccine Immunol. 17, 1170-1182.

Shi, L., North, R., Gennaro, M.L., 2004. Effect of Growth State on Transcription Levels of Genes Encoding Major Secreted Antigens of Mycobacterium Tuberculosis in the Mouse Lung. Infect. Immun. 72, 2420-2424.

Shuralev, E., Quinn, P., Doyle, M., Duignan, A., Kwok, H.F., Bezos, J., Olwill, S.A., Gormley, E., Aranaz, A., Good, M., Davis, W.C., Clarke, J., Whelan, C., 2012. Application of the Enfer Chemiluminescent Multiplex ELISA System for the Detection of Mycobacterium Bovis Infection in Goats. Vet. Microbiol. 154, 292-297.

Sidders, B., Pirson, C., Hogarth, P.J., Hewinson, R.G., Stoker, N.G., Vordermeier, H.M., Ewer, K., 2008. Screening of Highly Expressed Mycobacterial Genes Identifies Rv3615c as a Useful Differential Diagnostic Antigen for the Mycobacterium Tuberculosis Complex. Infect. Immun. 76, 3932-3939.

Singh, S.V., Singh, P.K., Singh, A.V., Sohal, J.S., Gupta, V.K., Vihan, V.S., 2007. Comparative Efficacy of an Indigenous 'inactivated Vaccine' using Highly Pathogenic Field Strain of Mycobacterium Avium Subspecies Paratuberculosis 'Bison Type' with a Commercial Vaccine for the Control of Capri-Paratuberculosis in India. Vaccine, 25, 7102-7110.

Skjøt, R.L.V., Brock, I., Arend, S.M., Munk, M.E., Theisen, M., Ottenhoff, T.H.M., Andersen, P., 2002. Epitope Mapping of the Immunodominant Antigen TB10.4 and the Two Homologous Proteins TB10.3 and TB12.9, which Constitute a Subfamily of the Esat-6 Gene Family. Infect. Immun. 70, 5446-5453.

Smith, N.H., Gordon, S.V., de la Rua-Domenech, R., Clifton-Hadley, R.S., Hewinson, R.G., 2006a. Bottlenecks and Broomsticks: The Molecular Evolution of Mycobacterium Bovis. Nat. Rev. Microbiol. 4, 670-681.

Smith, N.H., Kremer, K., Inwald, J., Dale, J., Driscoll, J.R., Gordon, S.V., van Soolingen, D., Glyn Hewinson, R., Maynard Smith, J., 2006b. Ecotypes of the Mycobacterium Tuberculosis Complex. J. Theor. Biol. 239, 220-225.

Sonnenberg, G.F., Fouser, L.A., Artis, D., 2011. Border Patrol: Regulation of Immunity, Inflammation and Tissue Homeostasis at Barrier Surfaces by IL-22. Nat. Immunol. 12, 383-390.

Stamm, L.M., Morisaki, J.H., Gao, L.Y., Jeng, R.L., McDonald, K.L., Roth, R., Takeshita, S., Heuser, J., Welch, M.D., Brown, E.J., 2003. Mycobacterium Marinum Escapes from Phagosomes and is Propelled by Actin-Based Motility. J. Exp. Med. 198, 1361-1368.

Standford, J.L., 1983. Immunologically Important Constituents of Mycobacteria Antigens. A: The Biology of Mycobacteria (Ratledge C. i Standford J., Eds.). Academic Press, Londres 2, 85-127.

Stead, W.W., Eisenach, K.D., Cave, M.D., Beggs, M.L., Templeton, G.L., Thoen, C.O., Bates, J.H., 1995. When did Mycobacterium Tuberculosis Infection First Occur in the New World? an Important Question with Public Health Implications. Am. J. Respir. Crit. Care Med. 151, 1267-1268.

Stenger, S., Hanson, D.A., Teitelbaum, R., Dewan, P., Niazi, K.R., Froelich, C.J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S.A., Bloom, B.R., Krensky, A.M., Modlin, R.L.,

1998. An Antimicrobial Activity of Cytolytic T Cells Mediated by Granulysin. Science 282, 121-125.

Stevenson, K., Alvarez, J., Bakker, D., Biet, F., de Juan, L., Denham, S., Dimareli, Z., Dohmann, K., Gerlach, G.F., Heron, I., Kopecna, M., May, L., Pavlik, I., Sharp, J.M., Thibault, V.C., Willemsen, P., Zadoks, R.N., Greig, A., 2009. Occurrence of Mycobacterium Avium Subspecies Paratuberculosis Across Host Species and European Countries with Evidence for Transmission between Wildlife and Domestic Ruminants. BMC Microbiol. 9, 212.

Stewart, G.R., Newton, S.M., Wilkinson, K.A., Humphreys, I.R., Murphy, H.N., Robertson, B.D., Wilkinson, R.J., Young, D.B., 2005. The Stress-Responsive Chaperone Alpha-Crystallin 2 is Required for Pathogenesis of Mycobacterium Tuberculosis. Mol. Microbiol. 55, 1127-1137.

Stringer, L.A., Wilson, P.R., Heuer, C., Hunnam, J.C., Mackintosh, C.G., 2011. Effect of Vaccination and Natural Infection with Mycobacterium Avium Subsp. Paratuberculosis on Specificity of Diagnostic Tests for Bovine Tuberculosis in Farmed Red Deer (Cervus Elaphus). N. Z. Vet. J. 59, 218-224.

Supply, P., Marceau, M., Mangenot, S., Roche, D., Rouanet, C., Khanna, V., Majlessi, L., Criscuolo, A., Tap, J., Pawlik, A., Fiette, L., Orgeur, M., Fabre, M., Parmentier, C., Frigui, W., Simeone, R., Boritsch, E.C., Debrie, A.S., Willery, E., Walker, D., Quail, M.A., Ma, L., Bouchier, C., Salvignol, G., Sayes, F., Cascioferro, A., Seemann, T., Barbe, V., Locht, C., Gutierrez, M.C., Leclerc, C., Bentley, S.D., Stinear, T.P., Brisse, S., Medigue, C., Parkhill, J., Cruveiller, S., Brosch, R., 2013. Genomic Analysis of Smooth Tubercle Bacilli Provides Insights into Ancestry and Pathoadaptation of Mycobacterium Tuberculosis. Nat. Genet. 45, 172-179.

Tadayon, K., Mosavari, N., Sadeghi, F., Forbes, K.J., 2008. Mycobacterium Bovis Infection in Holstein Friesian Cattle, Iran. Emerg. Infect. Dis. 14, 1919-1921.

Tameris, M.D., Hatherill, M., Landry, B.S., Scriba, T.J., Snowden, M.A., Lockhart, S., Shea, J.E., McClain, J.B., Hussey, G.D., Hanekom, W.A., Mahomed, H., McShane, H., 2013. Safety and Efficacy of MVA85A, a New Tuberculosis Vaccine, in Infants Previously Vaccinated with BCG: A Randomised, Placebo-Controlled Phase 2b Trial. Lancet. In press.

Thoen, C., LoBue, P., de Kantor, I., 2006a. The Importance of Mycobacterium Bovis as a Zoonosis. Vet. Microbiol. 112, 339-345.

Thom, M.L., McAulay, M., Vordermeier, H.M., Clifford, D., Hewinson, R.G., Villarreal-Ramos, B., Hope, J.C., 2012. Duration of Immunity Against Mycobacterium Bovis Following Neonatal Vaccination with Bacillus Calmette-Guérin Danish: Significant Protection Against Infection at 12, but Not 24, Months. Clin. Vaccine Immunol. 19, 1254-1260.

Tompkins, D.M., Ramsey, D.S., Cross, M.L., Aldwell, F.E., de Lisle, G.W., Buddle, B.M., 2009. Oral Vaccination Reduces the Incidence of Tuberculosis in Free-Living Brushtail Possums. Proc. Biol. Sci. 276, 2987-2995.

Torres-Gonzalez, P., Soberanis-Ramos, O., Martinez-Gamboa, A., Chavez-Mazari, B., Barrios-Herrera, M.T., Torres-Rojas, M., Cruz-Hervert, L.P., Garcia-Garcia, L., Singh, M., Gonzalez-Aguirre, A., Ponce de Leon-Garduno, A., Sifuentes-Osornio, J., Bobadilla-Del-Valle, M.,

2013. Prevalence of Latent and Active Tuberculosis among Dairy Farm Workers Exposed to Cattle Infected by Mycobacterium Bovis. PLoS Negl. Trop. Dis. 7, e2177.

Turner, O.C., Basaraba, R.J., Orme, I.M., 2003. Immunopathogenesis of Pulmonary Granulomas in the Guinea Pig After Infection with Mycobacterium Tuberculosis. Infect. Immun. 71, 864-871.

Ulrichs, T., Kaufmann, S.H., 2006. New Insights into the Function of Granulomas in Human Tuberculosis. J. Pathol. 208, 261-269.

Uzonna, J.E., Chilton, P., Whitlock, R.H., Habecker, P.L., Scott, P., Sweeney, R.W., 2003. Efficacy of Commercial and Field-Strain Mycobacterium Paratuberculosis Vaccinations with Recombinant IL-12 in a Bovine Experimental Infection Model. Vaccine, 21, 3101-3109.

van der Burgt, G.M., Drummond, F., Crawshaw, T., Morris, S., 2013. An Outbreak of Tuberculosis in Lleyn Sheep in the UK Associated with Clinical Signs. Vet. Rec. 172, 69-69.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., Peters, P.J., 2007. M. Tuberculosis and M. Leprae Translocate from the Phagolysosome to the Cytosol in Myeloid Cells. Cell 129, 1287-1298.

van Dissel, J.T., Arend, S.M., Prins, C., Bang, P., Tingskov, P.N., Lingnau, K., Nouta, J., Klein, M.R., Rosenkrands, I., Ottenhoff, T.H., Kromann, I., Doherty, T.M., Andersen, P., 2010. Ag85B-ESAT-6 Adjuvanted with IC31 Promotes Strong and Long-Lived Mycobacterium Tuberculosis Specific T Cell Responses in Naive Human Volunteers. Vaccine 28, 3571-3581.

van Soolingen, D., Hoogenboezem, T., de Haas, P.E., Hermans, P.W., Koedam, M.A., Teppema, K.S., Brennan, P.J., Besra, G.S., Portaels, F., Top, J., Schouls, L.M., van Embden, J.D., 1997. A Novel Pathogenic Taxon of the Mycobacterium Tuberculosis Complex, Canetti: Characterization of an Exceptional Isolate from Africa. Int. J. Syst. Bacteriol. 47, 1236-1245.

Vazquez, P., Garrido, J.M., Juste, R.A., 2013. Specific Antibody and Interferon-Gamma Responses Associated with Immunopathological Forms of Bovine Paratuberculosis in Slaughtered Friesian Cattle. PLoS One 8, e64568.

Verreck, F.A., Vervenne, R.A., Kondova, I., van Kralingen, K.W., Remarque, E.J., Braskamp, G., van der Werff, N.M., Kersbergen, A., Ottenhoff, T.H., Heidt, P.J., Gilbert, S.C., Gicquel, B., Hill, A.V., Martin, C., McShane, H., Thomas, A.W., 2009. MVA.85A Boosting of BCG and an Attenuated, phoP Deficient M. Tuberculosis Vaccine both show Protective Efficacy Against Tuberculosis in Rhesus Macaques. PLoS One 4, e5264.

Vesosky, B., Turner, O.C., Turner, J., Orme, I.M., 2004. Gamma Interferon Production by Bovine {Gamma}{Delta} T Cells Following Stimulation with Mycobacterial Mycolylarabinogalactan Peptidoglycan. Infect. Immun. 72, 4612-4618.

Vincent Lévy-Frébault, V., Portaels, F., 1992. Proposed Minimal Standards for the Genus Mycobacterium and for Description of New Slowly Growing Mycobacterium Species†. Int. J. Syst. Bacteriol. 42, 315-323.

Von Eschen, K., Morrison, R., Braun, M., Ofori-Anyinam, O., De Kock, E., Pavithran, P., Koutsoukos, M., Moris, P., Cain, D., Dubois, M.C., Cohen, J., Ballou, W.R., 2009. The Candidate Tuberculosis Vaccine Mtb72F/AS02A: Tolerability and Immunogenicity in Humans. Hum. Vaccine 5, 475-482.

Vordermeier, H.M., Chambers, M.A., Cockle, P.J., Whelan, A.O., Simmons, J., Hewinson, R.G., 2002. Correlation of ESAT-6-Specific Gamma Interferon Production with Pathology in Cattle Following Mycobacterium Bovis BCG Vaccination Against Experimental Bovine Tuberculosis. Infect. Immun. 70, 3026-3032.

Vordermeier, H.M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N., Hewinson, R.G., 2001. Use of Synthetic Peptides Derived from the Antigens ESAT-6 and CFP-10 for Differential Diagnosis of Bovine Tuberculosis in Cattle. Clin. Diagn. Lab. Immunol. 8, 571-578.

Vordermeier, H.M., Aranday-Cortes, E., Villareal-Ramos, B., Hogarth, P.J., Whelan, A.O., 2012. Development of TB Vaccines for Cattle: Biomarkers Predicting Vaccine Success Or Failure. In: Proceedings of the first Mycobacterium bovis American Regional Conference, Merida (Mexico), August 19th-22nd, 71.

Vordermeier, H.M., Huygen, K., Singh, M., Hewinson, R.G., Xing, Z., 2006. Immune Responses Induced in Cattle by Vaccination with a Recombinant Adenovirus Expressing Mycobacterial Antigen 85A and Mycobacterium Bovis BCG. Infect. Immun. 74, 1416-1418.

Vordermeier, H.M., Villarreal-Ramos, B., Cockle, P.J., McAulay, M., Rhodes, S.G., Thacker, T., Gilbert, S.C., McShane, H., Hill, A.V.S., Xing, Z., Hewinson, R.G., 2009. Viral Booster Vaccines Improve Mycobacterium Bovis BCG-Induced Protection Against Bovine Tuberculosis. Infect. Immun. 77, 3364-3373.

Vordermeier, M., Goodchild, A., Clifton-Hadley, R., de la Rua, R., 2004. The Interferon-Gamma Field Trial: Background, Principles and Progress. Vet. Rec. 155, 37-38.

Vordermeier, M., Whelan, A.O., 2012. ELISPOT Assays to Enumerate Bovine IFN-Gamma-Secreting Cells for the Development of Novel Vaccines Against Bovine Tuberculosis. Methods Mol. Biol. 792, 219-227.

Waddington, F.G., Ellwood, D.C., 1972. An Experiment to Challenge the Resistance to Tuberculosis in B.C.G. Vaccinated Cattle in Malawi. Br. Vet. J. 128, 541-552.

Wang, J., Thorson, L., Stokes, R.W., Santosuosso, M., Huygen, K., Zganiacz, A., Hitt, M., Xing, Z., 2004. Single Mucosal, but Not Parenteral, Immunization with Recombinant Adenoviral-Based Vaccine Provides Potent Protection from Pulmonary Tuberculosis. J. Immunol. 173, 6357-6365.

Wangoo, A., Johnson, L., Gough, J., Ackbar, R., Inglut, S., Hicks, D., Spencer, Y., Hewinson, G., Vordermeier, M., 2005. Advanced Granulomatous Lesions in Mycobacterium Bovis-Infected Cattle are Associated with Increased Expression of Type I Procollagen, γδ (WC1+) T Cells and CD 68+ Cells. J. Comp. Pathol. 133, 223-234.

Waters, W.R., Nonnecke, B.J., Palmer, M.V., Robbe-Austermann, S., Bannantine, J.P., Stabel, J.R., Whipple, D.L., Payeur, J.B., Estes, D.M., Pitzer, J.E., Minion, F.C., 2004. Use of Recombinant ESAT-6:CFP-10 Fusion Protein for Differentiation of Infections of Cattle by

Mycobacterium Bovis and by M. Avium Subsp. Avium and M. Avium Subsp. Paratuberculosis. Clin. Diagn. Lab. Immunol. 11, 729-735.

Waters, W.R., Palmer, M.V., Buddle, B.M., Vordermeier, H.M., 2012a. Bovine Tuberculosis Vaccine Research: Historical Perspectives and Recent Advances. Vaccine 30, 2611-2622.

Waters, W.R., Palmer, M.V., Thacker, T.C., Bannantine, J.P., Vordermeier, H.M., Hewinson, R.G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J.M., Andersen, P., Lyashchenko, K.P., 2006. Early Antibody Responses to Experimental Mycobacterium Bovis Infection of Cattle. Clin. Vaccine Immunol. 13, 648-654.

Waters, W.R., Thacker, T.C., Nonnecke, B.J., Palmer, M.V., Schiller, I., Oesch, B., Vordermeier, H.M., Silva, E., Estes, D.M., 2012b. Evaluation of Gamma Interferon (IFN-Gamma)-Induced Protein 10 Responses for Detection of Cattle Infected with Mycobacterium Bovis: Comparisons to IFN-Gamma Responses. Clin. Vaccine Immunol. 19, 346-351.

Wedlock, D.N., Aldwell, F.E., Keen, D., Skinner, M.A., Buddle, B.M., 2005. Oral Vaccination of Brushtail Possums (Tichosurus Vulpecula) with BCG: Immune Responses, Persistence of BCG in Lymphoid Organs and Excretion in Faeces. N. Z. Vet. J. 53, 301-306.

Wedlock, D.N., Denis, M., Vordermeier, H.M., Hewinson, R.G., Buddle, B.M., 2007. Vaccination of Cattle with Danish and Pasteur Strains of Mycobacterium Bovis BCG Induce Different Levels of IFN Gamma Post-Vaccination, but Induce Similar Levels of Protection Against Bovine Tuberculosis. Vet. Immunol. Immunopathol. 118, 50-58.

Weldingh, K., Andersen, P., 2008. ESAT-6/CFP10 Skin Test Predicts Disease in *M. Tuberculosis*-Infected Guinea Pigs. PLoS One 3, e1978.

Wells, A.Q., Oxon, D.M., 1937. Tuberculosis in Wild Voles. Lancet 1, 1221.

Welsh, M.D., Kennedy, H.E., Smyth, A.J., Girvin, R.M., Andersen, P., Pollock, J.M., 2002. Responses of Bovine WC1(+) Gamma Delta T Cells to Protein and Nonprotein Antigens of Mycobacterium Bovis. Infect. Immun. 70, 6114-6120.

Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G., Pollock, J.M., 2005. Influence of Pathological Progression on the Balance between Cellular and Humoral Immune Responses in Bovine Tuberculosis. Immunology 114, 101-111.

Whelan, A.O., Clifford, D., Upadhyay, B., Breadon, E.L., McNair, J., Hewinson, G.R., Vordermeier, M.H., 2010. Development of a Skin Test for Bovine Tuberculosis for Differentiating Infected from Vaccinated Animals. J. Clin. Microbiol. 48, 3176-3181.

Wijngaard, P.L., Metzelaar, M.J., MacHugh, N.D., Morrison, W.I., Clevers, H.C., 1992. Molecular Characterization of the WC1 Antigen Expressed Specifically on Bovine CD4-CD8- Gamma Delta T Lymphocytes. J. Immunol. 149, 3273-3277.

Wilkinson, K.A., Stewart, G.R., Newton, S.M., Vordermeier, H.M., Wain, J.R., Murphy, H.N., Horner, K., Young, D.B., Wilkinson, R.J., 2005. Infection Biology of a Novel α-Crystallin of Mycobacterium Tuberculosis: Acr2. J. Immunol. 174, 4237-4243.

Wilkinson, Wilkinson, De Smet, Haslov, Pasvol, Singh, Svarcova, Ivanyi, 1998. Human T- and B-Cell Reactivity to the 16 kDa ?-Crystallin Protein of Mycobacterium Tuberculosis. Scand. J. Immunol. 48, 403-409.

Williams, A., Goonetilleke, N.P., McShane, H., Clark, S.O., Hatch, G., Gilbert, S.C., Hill, A.V., 2005. Boosting with Poxviruses Enhances Mycobacterium Bovis BCG Efficacy Against Tuberculosis in Guinea Pigs. Infect. Immun. 73, 3814-3816.

Wolf, A.J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K., Ernst, J.D., 2008. Initiation of the Adaptive Immune Response to Mycobacterium Tuberculosis Depends on Antigen Production in the Local Lymph Node, Not the Lungs. J. Exp. Med. 205, 105-115.

Wood, P.R., Corner, L.A., Rothel, J.S., Ripper, J.L., Fifis, T., McCormick, B.S., Francis, B., Melville, L., Small, K., de Witte, K., 1992. A Field Evaluation of Serological and Cellular Diagnostic Tests for Bovine Tuberculosis. Vet. Microbiol. 31, 71-79.

Wood, P.R., Rothel, J.S., 1994. In Vitro Immunodiagnostic Assays for Bovine Tuberculosis. Vet. Microbiol. 40, 125-135.

World Health Organization, 2010a. Global Tuberculosis Control. Geneva, WHO.

World Health Organization, 2010b. Multidrug and Extensively Drug-Resistant TB (M/XDR-TB): 2010 Global Report on Surveillance and Response. Geneva, WHO.

World Health Organization, 2009. Global Tuberculosis Control: A Short Update to the 2009 Report. Geneva, WHO.

World Health Organization, 1994. Tuberculosis Notification Update. Tuberculosis Programme. Geneva, WHO.

Xing, Z., McFarland, C.T., Sallenave, J.M., Izzo, A., Wang, J., McMurray, D.N., 2009. Intranasal Mucosal Boosting with an Adenovirus-Vectored Vaccine Markedly Enhances the Protection of BCG-Primed Guinea Pigs Against Pulmonary Tuberculosis. PLoS One 4, e5856.

Xing, Z., Charters, T.J., 2007. Heterologous Boost Vaccines for Bacillus Calmette-Guerin Prime Immunization Against Tuberculosis. Expert Rev. Vaccines 6, 539-546.

Xing, Z., Lichty, B.D., 2006. Use of Recombinant Virus-Vectored Tuberculosis Vaccines for Respiratory Mucosal Immunization. Tuberculosis (Edinb) 86, 211-217.

Young, D., 2009. Animal Models of Tuberculosis. Eur. J. Immunol. 39, 2011-2014.

Young, D., Dye, C., 2006. The Development and Impact of Tuberculosis Vaccines. Cell 124, 683-687.

Yuan, Y., Crane, D.D., Simpson, R.M., Zhu, Y., Hickey, M.J., Sherman, D.R., Barry, C.E., 1998. The 16-kDa α-Crystallin (Acr) Protein of Mycobacterium Tuberculosis is Required for Growth in Macrophages. Proc. Natl. Acad. Sci. U. S. A. 95, 9578-9583.

Zanardi, G., Boniotti, M.B., Gaffuri, A., Casto, B., Zanoni, M., Pacciarini, M.L., 2013. Tuberculosis Transmission by Mycobacterium Bovis in a Mixed Cattle and Goat Herd. Res. Vet. Sci. In press.

ABREVIACIONS

Acr	*Alfa-crystalin*
AdAg85A	*Adenovirus* Ag85A
AdHu5	Adenovirus humà serotip 5
AdTBF	*Adenovirus* TB *fusion*
Ag85	*Antigen* 85 *complex*
AHVLA	*Animal Health and Veterinary Laboratories Agency*
ANOVA	*Analysis of variance*
APC	*Antigen Presenting Cells*
BCG	*bacillus Calmette-Guerin*
CFP-10	*Culture filtrate protein* 10
CFU	*Colony forming units*
CI	Confidence interval
CMI	*Cell-mediated immunity*
CReSA	Centre de Recerca en Sanitat Animal
CT	Computed Tomography
DC	Dendritic cell
DIVA	*Differentiation of infected from vaccinated animals*
DTH	*Delayed-Type Hypersensitivity*
E/C	ESAT-6/CFP-10
ELISA	*Enzyme Linked Immunosorbent Assay*
ESAT-6	6 kDa *early secretory antigenic target*
EspC	Esx-1 *substrate protein* C
Esx	ESAT-6 *secretion complex* 1
FAO	*Food and Agriculture Organization*
FcR	*Fragment crystallizable (region) receptor*
GL	*Gross lesions*
HE	*Hematoxylin and eosin*
IDTB	Itradermotuberculinització
Ig	Immunoglobulina
IGRA	*Interferon-Gamma Release Assay*
IL	Interleuquina
INF	Interferó
IP-10	*Interferon gamma-induced protein* 10
LG	*Large granuloma*
LN	*Lymph node*
LTBI	*Latent* TB *infection*
Map	*Mycobacterium avium* subespecies *paratuberculosis*
MDCT	*Multi-Detector* CT
MDR	*Multi-drug-resistant*
MG	*Medium granuloma*
MHC	*Major histocompatibility complex*
MNGC	*Multi-nucleated giant cells*
MRI	*Magnetic resonance imaging*

MTBC	*Mycobacterium tuberculosis complex*
MVA	*Modified vaccinia* (virus) *Ankara*
NF-kB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
OD	*Optical Density*
OIE	Organització Internacional d'Epizooties
OMS	Organització Mundial de la Salut
PAMP	*Pathogen-associated molecular pattern*
PBS	*Phosphate buffered saline*
PBMC	*Peripheral blood mononuclear cell*
PHA	*Phytohaemagglutinin*
PNG	*Polymorphonuclear granulocytes*
PPD-A	*Avian purified protein derivative*
PPD-B	*Bovine purified protein derivative*
PRR	*Pattern recognition receptor*
PTB	Paratuberculosi
QFT	QuantiFERON
RD	*Regions of differences*
SatG	*Satellite granulomas*
SD	*Standard deviation*
SFC	*Spot forming cells*
SG	*Small granuloma*
SICCT	*Single intradermal comparative cervical tuberculin*
SIT	*Single intradermal tuberculin*
SNK	Student-Newman-Keuls
TB	Tuberculosis
TCR	*T-cell receptor*
Th	*T helper*
TLR	*Toll-like receptors*
TNF	*Tumor necrosis factor*
UFC	Unitats formadores de colònia
VL	*Visible lesions*
WC1	*Workshop cluster* 1
XDR	*Extensively drug-resistant*

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El món canviarà. Tot va tan de pressa..., aquest paper desapareixerà, el seu contingut romandrà en bytes i després jo què se! potser esdevindrà coneixement col·lectiu en xarxa. Sigui com sigui, que consti una darrera paraula que és un nom: Laia

CReSA