GENETIC RESISTANCE TO HUMAN PULMONARY TUBERCULOSIS: THE RESULT OF ALLELIC AND NON-ALLELIC INTERACTIONS.

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I inspired by the
love of a wonderful woman and the
tenacity of an untiring man,
with all my heart and devotion
To my loving parents

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Summary

La tubercolosi (TB) è una malattia di origine infettiva causata dal bacillo *Mycobacterium tuberculosis* (MTB), chiamato anche Bacillo di Koch. Essa figura al secondo posto nelle cause di morte per malattie infettive in tutto il mondo dopo lo Human Immunodeficiency Virus (HIV). Nel 1993, la TB è stata classificata come emergenza mondiale dalla World Health Organisation (WHO) che ha stimato circa 9 milioni di nuovi casi nel 2011 e 1.4 milioni di decessi dovuti alla TB in 84 Paesi. La stessa WHO considera l'Italia uno dei paesi occidentali in cui si sarebbe verificato, tra il 1988 ed il 1990, il maggior incremento del numero di casi di TB. Nonostante la disponibilità di trattamenti efficaci e poco costosi, la tubercolosi risulta più diffusa nella popolazione povera che tra quella ricca, sia nelle regioni industrializzate che nelle regioni in via di sviluppo. Le regioni nelle quali si riscontra il 60% dei casi totali di TB sono: Pakistan, sud dell'Africa, China e India. Il più alto tasso di incidenza della tubercolosi si riscontra nell'Africa Sub-Sahariana, soprattutto associata all'infezione da (HIV). Oltre all'infezione da HIV, l'incremento nel tasso di incidenza della tubercolosi è provocato dalla comparsa di ceppi resistenti alla maggior parte dei farmaci anti-TB. Altri fattori che incidono sulla diffusione della TB sono: l'aumento della popolazione; la difficoltà a rilevare i casi tra le masse povere; il ridotto tasso di cure in paesi in via di sviluppo; la trasmissione dell'infezione in ospedali e prigioni sovraffollati e l'immigrazione di individui da paesi dove la TB è endemica (WHO 2012).
La principale fonte di contagio è rappresentata dalle persone affette da TB, anche se soltanto il 10% degli individui mostra i segni di tubercolosi attiva entro i 18 mesi dall'avvenuta infezione. Nei restanti casi, la risposta immunitaria conseguente all'infezione riesce ad arrestare la crescita del MTB. Ciò nonostante, il patogeno è completamente eradicato soltanto nel 10% dei casi mentre, nel restante 90%, il MTB riesce ad eludere i meccanismi antibatterici delle cellule del sistema immunitario restando in uno stato non-replicativo (dormiente o latente). Questo processo è chiamato Latent Tuberculosis Infection (LTI) e colpisce circa due miliardi di persone al mondo (Koul, Herget et al. 2004).

Mycobacterium tuberculosis

Il *Mycobacterium tuberculosis* è un membro della specie *Mycobacterium tuberculosis* complex (MTBC). Ad esso appartengono altre 6 specie correlate geneticamente: *M. bovis*, *M. africanum*, *M. microti, M. pinnipedii, M. caprae e M. capretti*. Tutte possono provocare tubercolosi, ma hanno un diverso host range (Cole, Brosch et al. 1998).
Il M. tuberculosis ha una struttura unica che lo rende diverso da tutti gli altri batteri. Partendo dall'interno verso l'esterno troviamo la membrana cellulare rivestita da diversi strati di peptidoglicano (PG), arabinogalattani (AG), lipomannani (LM), mannose-lipoarabinomannani (ManLAM) e acidi micolici (MA) legati covalentemente tra di loro a formare una struttura molto complessa. Il peptidoglicano costituisce lo scheletro di questa struttura. Esso è costituito da lunghe catene di N-acetilglucosammina (NAG) legate a catene di acido muramico (NAM) attraverso dei residui di L-alanyl-D-iso-glutaminyl-meso-diaminopimelic acid (DAP) (Velayati, Farnia et al. 2012). I polisaccaridi a lunga catena (LM, AG e LAM) formano un ponte tra il peptidoglicano e gli acidi micolici che sono acidi grassi a lunga catena che rappresentano i componenti principali della parete del micobatterio.

Riconoscimento *del Mycobacterium tuberculosis*

I Pathogen-associated molecular patterns (PAMP) del MTB vengono riconosciuti da specifici Pathogen recognition receptors (PRRs) posti sulla superficie delle cellule del sistema immunitario dell'ospite. Questi recettori, tra cui i Toll-like receptors (TLRs) sono essenziali per coordinare la risposte immunitaria innata dell'ospite (Jo 2008). L'interazione tra i PAMPs del MTB ed i TLRs innesca una cascata di trasduzione del segnale che culmina in una risposta pro-infiammatoria da parte delle sistema immunitario dell'ospite (Harding and Boom 2010). Tuttavia, il micobatterio ha sviluppato efficaci strategie atte a modulare o addirittura inibire tale risposta. I più importanti ligandi posti sulla superficie del micobatterio i quali interagiscono con i TLRs sono: 19 kDa lipoprotein, il lipomannano (LM) ed il mannose-lipoarabinomannano (Jo, Yang et al. 2007). L'interazione di questi ligandi con i TLRs porta all'attivazione del nuclear trascription factor B (NF-kB) e la conseguente produzione di citochine pro-infiammatorie come tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) e anche dell'ossido nitrico attraverso via myeloid differentiation primary response protein 88 (MyD88)-dipendenti che indipendenti (Yamamoto, Sato et al. 2003, Jo 2008). Uno studio ha mostrato che, almeno nel topo, il contatto prolungato con la 19 kDa del MTB da parte dei macrofagi alveolari attenua la processazione dell'antigene che a sua volta attenua l'espressione del major histocompatibility complex (MHC)-II smorzando così l'attivazione delle T cells (Fulton, Reba et al. 2004). In questo modo, una sottopopolazione di macrofagi infettati con la funzione dell'APC modulata, costituisce una nicchia invisibile al sistema immunitario dove il batterio può sopravvivere e resistere.

Scopo della tesi

Schurr (Schurr 2007) si è chiesto se la tubercolosi fosse una malattia infettiva o ereditaria. L'autore sapeva molto bene che la tubercolosi può essere contratta solo in presenza del patogeno. Con la sua domanda (posta come titolo all'articolo), Schurr ha inteso mettere in evidenza il ruolo essenziale della componente ereditaria ai fini della resistenza alla tubercolosi. Indi per cui la ricerca si è incentrata sull'associazione (punto I) e interazione (punto II) tra i geni MyD88 e TIRAP e la tubercolosi. Entrambi i geni funzionano come trasmettitori del segnale dai recettori esterni alla cellula fino all'interno del nucleo dove avviene la trascrizione di geni capaci di innescare la risposta immune, innata e adattativa. Inoltre, la loro posizione su cromosomi distinti (indipendenza) e la loro comune funzione (trasmissione del segnale) rende plausibile l'ipotesi che i geni interagissero. Anche quest'anticipazione si è dimostrata valida, offrendo un esempio di epistasi (interazione tra geni non alleli). Inoltre, la letteratura scientifica inerente al coinvolgimento di MyD88 e TIRAP nella tubercolosi presenta notevoli discrepanze, soprattutto nel delicato campo degli studi di associazione.
Topi "knockout" per MyD88 mostrano una produzione di TNF-α, IL-12 e NO molto ridotta in risposta all'infezione dell'MTB e muoiono in 4 settimane (Fremond, Yeremeev et al. 2004). Topi "knockout" per TIRAP, invece, riescono a controllare efficacemente l'infezione da MTB (Fremond, Togbe et al. 2007). Il Single-nucleotide polymorphism (SNP) C558T di TIRAP è associato con la suscettibilità alla tubercolosi meningea (Hawn, Dunstan et al. 2006, Caws, Thwaites et al. 2008), e quello S180L conferisce protezione contro la malaria e la tubercolosi (Khor, Chapman et al. 2007). Studi successivi non hanno confermato il ruolo protettivo di S180L (Nejentsev, Thye

et al. 2008, Miao, Li et al. 2011), TIRAP (rs352165 and rs352167) e MyD88 (rs4988457 and rs6767684) contro la tubercolosi (Khor, Chapman et al. 2007, Nejentsev, Thye et al. 2008). Alla luce di queste discrepanze emerse, perfino all'interno dello studio dello stesso SNP (rs81777374) in differenti etnie, si è deciso di investigare il ruolo di questi SNPs nei malati di tubercolosi polmonare nella popolazione italiana. In aggiunta, il *M. tuberculosis* ha l'abilità di passare dalla forma attiva a quella dormiente. Pertanto i geni MyD88 e TIRAP potrebbero influenzare diversamente le due forme d'infezione. In questo caso, l'eterogeneità dei pazienti (con infezione attiva e con infezione latente) può ridurre notevolmente il potere discriminante del test statistico (caso-controllo). Per investigare meglio questo punto dello studio, si è pensato di spostare la ricerca su di un altro modello: i bovini. Da questi animali è stato possibile ottenere biopsie polmonari al momento della macellazione. L'indagine ha chiarito che – come ipotizzato – il genotipo può influenzare l'infezione, in termini di interazione tra i geni e la il tipo di risposta attuato.

Results

Study design

Per saggiare la riproducibilità dei risultati, è stato scelto uno studio a due fasi. Nella prima fase dello studio (hypotesis-generating) sono stati reclutati 100 casi e 100 controlli e genotipizzati per il sito polimorfico TIRAP rs8177374 e MyD88 rs6853. Entrambi i siti sono stati scelti perché sono stati gli unici tra i 5 saggiati per gene a mostrare una frequenza allelica >0.05. Inoltre entrambi i siti hanno mostrato associazione statisticamente significativa con la malattia (P-value <0.05). Sulla base dei risultati dello studio preliminare (MyD88: OR 0.40 e TIRAP: 0.48), è stato calcolato che - per assicurare allo studio una capacità discriminante del 96% con un livello di significatività dello 0.01- fosse necessario estendere lo studio ad un campione di 185 casi e 185 controlli (nel caso di MyD88) e 313 casi e 313 controlli (nel caso di TIRAP). Nella seconda parte dello studio sono stati coinvolti 400 casi e 400 controlli (indipendenti dai casi e dai controlli utilizzati nella fase preliminare dello studio). Per rendere la classe dei casi più omogenea possibile, sono stati reclutati solo pazienti con TB polmonare attiva confermata attraverso l'esame ai raggi X, il test batteriologico e la PCR. Tutti i casi sono stati trattati presso l'ospedale Monaldi (Napoli), il centro di referenza per la tubercolosi del Sud Italia. I controlli sono stati scelti tra mogli, mariti e conoscenti dei pazienti senza evidenze cliniche di tubercolosi (negativi al test con l'interferone gamma). In tal modo i controlli non sono correlati geneticamente ai casi e non risultano infettati, nonostante esposti al *Mycobacterium tuberculosis* (>2 anni). I criteri utilizzati per classificare casi e controlli sono stati gli stessi in entrambe le fasi dello studio. I casi ed i controlli sono stati collezionati durante più di 5 anni di collaborazione tra l'Ospedale ed il laboratorio della Prof.ssa Capparelli Rosanna. In questo tempo sei controlli sono diventati positivi al test con l'interferone e quindi sono stati esclusi dallo studio. I casi consistevano in 258 maschi e 142 femmine (media età 50±19 anni); i controlli in 222 maschi e 178 femmine (media età 49±17 anni). Lo studio è stato approvato dal comitato etico dell'Ospedale Monaldi. Il consenso informato è stato ottenuto da tutti i pazienti che hanno partecipato alla ricerca.

Eterozigosi è associata con la protezione contro la tubercolosi polmonare.

Le frequenze genotipiche dei markers rs6853 e rs8177374 sono in equilibrio di Hardy-Weinberg tra i controlli, ma non tra i casi. Ad entrambi i loci, l'eterozigosi (AG per MyD88 o CT per TIRAP) è associata con la resistenza alla tubercolosi polmonare. L'età rappresenta un fattore di rischio per la tubercolosi polmonare. Per conoscere come il rischio legato all'età varia attraverso le stratificazioni, i dati sono stati analizzati utilizzando il modello della regressione logistica. L'analisi è stata ristretta ai gruppi con un numero ≥ di 45 individui. A parte la categoria 31-40 vs 21-30 anni (apparentemente protetta, OR 0.31), il modello ha mostrato che il rischio di sviluppare la malattia aumenta con l'età. Chiaramente, l'età e le possibili variabili associate all'età - come il fumo, il diabete e la prolungata esposizione al patogeno - possono neutralizzare la resistenza genetica. Il modello di regressione logistica è stato anche usato per dissezionare il contributo di ogni singolo genotipo e la loro interazione. Particolarmente evidente è l'interazione tra i genotipi AG e CT (OR 0.09) e l'interazione in direzione opposta tra i genotipi GG e TT, AA e CC, AA e CT (OR 5.78, 5.78 e 7.46, rispettivamente).

L'eterozigosi controlla l'infiammazione

Per investigare sul meccanismo di come l'ospite può controllare l'infezione secondo l'assetto genotipico, i linfociti di donatori sani (controlli) (nove gruppi genotipici; 5 campioni/gruppo) sono stati stimolati con il ceppo *Mycobacterium tuberculosis* H37Rv inattivato al calore e poi sono stati misurati i livelli di TNF-α, IFN-ɣ e NO (nitric oxide) rilasciati nel mezzo attraverso un'ELISA test. Nel contesto del genotipo AA, i soggetti CT mostrano livelli intermedi di TNF-α, IFN-ɣ e NO rispetto ai soggetti CC e TT. Nel contesto genotipico AG, i dati mostrano la stessa tendenza, sebbene alcune differenze non siano significative. Inoltre, sono stati osservati bassi livelli di citochine in soggetti GG, come se l'allele "A" favorisse la produzione di citochine e l'allele "G" la attenuasse. Questi dati indicano che gli eterozigoti sono associati con un livello intermedio di citochine and NO. Questi risultati suggeriscono che i due loci cooperano fortemente a controllare la malattia. Questa evidenza è fortemente supportata dall'evidenza che TIRAP e MyD88 formano eterodimeri.

Analisi bioinformatica

Il sito rs6853 risiede nella regione 3' UTR del gene MyD88. Comparando le sequenze genomiche di diverse specie emerge che entrambi gli alleli A e G sono conservati in molte specie, suggerendo che essi sono mantenuti o almeno tollerati, dalla selezione naturale. Attraverso l'analisi con 4 ENCODE tracks è emerso che la regione 3' UTR del sito polimorfico potrebbe influenzare l'interazione tra l'mRNA ed fattori proteici. Il sito rs8177374, che risiede sull'esone 5 del gene TIRAP, ha permesso di valutare se il cambiamento di una serina con una leucina in posizione 180 potesse influenzare la struttura della proteina. Entrambi gli amminoacidi sono rappresentati in posizione 180 dei geni TIRAP di 22 specie di mammiferi. E' stato possibile predire che entrambi sono compatibili con l'attività della proteina. Questi risultati suggeriscono che le isoforme A e B sono originate dalla stessa molecola di mRNA che ha subito splicing alternativo conferendo una ulteriore plausibilità biologica al polimorfismo.

Discussione e conclusioni

Molto poco è conosciuto del "crosstalk" tra geni implicati nella resistenza ad un patogeno. In questo lavoro è stato investigato come i geni MyD88 e TIRAP si influenzano a vicenda. L'ipotesi che i due geni potrebbero interagire sembra plausibile in quanto entrambe le proteine MyD88 e TIRAP sono coinvolte nel "signaling" cellulare a valle dei TLRs. Sempre in questo studio è stato dimostrato che i due geni cooperano o si antagonizzano tra di loro sulla base della loro combinazione allelica. L'eterozigosi ad entrambi i loci fornisce una protezione più forte ($P= 1.3x10^{-12}$, age-corrected) rispetto all'eterozigosi ad un solo locus (MyD88 $P= 7.8x10^{-8}$; TIRAP $P= 2x10^{-6}$). Allo stesso tempo, negli individui AG/TT (MyD88/TIRAP) il genotipo TT neutralizza la protezione apportata dal genotipo AG. Questi dati, seppur in maniera limitata, mostrano come due o più geni indipendenti possono concorrere alla formazione e regolazione dello stesso fenotipo. Gli individui eterozigoti ai loci MyD88 o TIRAP mostrano livelli intermedi di TNF-α, IFN-ɣ e NO rispetto alle altre classi genotipiche (Figure 1). TNF-α, IFN-ɣ e NO giocano un ruolo fondamentale contro *Mycobacterium tuberculosis* (Casanova and Abel 2002, Scanga, Bafica et al. 2004, Velez, Hulme et al. 2009). NO esercita una forte attività anti-micobatterica ed insieme a TNF-α, favorisce la formazione dei granulomi (Miller and Ernst 2009). IFN-ɣ induce la produzione di NO, l'espressione delle molecole MHC II e la presentazione dell'antigene (Fortune, Solache et al. 2004, Scanga, Bafica et al. 2004). Tuttavia, vi è anche l'evidenza che la sovra-espressione di queste molecole favorisce la tubercolosi. Molti dei sintomi sono proprio causati dalla risposta immune dell'ospite, piuttosto che dal *Mycobacterium tuberculosis* (Glickman and Jacobs 2001). Infatti, la riattivazione della TB si è osservata dopo il trattamento terapeutico con TNF-α (Mankia, Peters et al. 2011) o in pazienti affetti da HIV dopo trattamento antiretrovirale (French and Price 2001). Anche l'ipo-espressione di TNF-α, IFN-ɣ e NO favorisce la TB, quindi il vantaggio biologico di averne un livello intermedio di espressione diventa chiaro. Questo vantaggio risulta molto più marcato nei doppi eterozigoti che mostrano come il crosstalk tra i geni si estenda dal livello epidemiologico a quello molecolare. Il vantaggio espresso dagli eterozigoti suggerisce che le frequenze alleliche ai siti polimorfici di rs6853 e rs8177374 sono mantenuti dal bilanciamento del polimorfismo, dove l'omozigosi è associato con la tubercolosi polmonare e l'eterozigosi alla resistenza. In accordo con questa ipotesi vi è il fatto che entrambi i siti polimorfici sono conservati attraverso la speciazione. Inoltre il fatto che la combinazione allelica influisce sui livelli di TNF-α, IFN-ɣ e NO suggerisce che l'associazione sia tra MyD88 e TIRAP piuttosto che tra geni strettamente correlati a loro. In conclusione, l'associazione di rs8177374 con la TB polmonare probabilmente sarà confermata anche in studi futuri, mentre rs6853 risulta associato in questo studio, ma non in due lavori precedenti (Miao, Li et al. 2011, Sanchez, Lefebvre et al. 2012), quindi al momento resta uno studio esplorativo hypothesis-testing study.

Commento

Purtroppo gli studi di associazione mancano di riproducibilità (Ioannidis, Ntzani et al. 2001). Durante la prima fase dello studio siamo incappati in ostacoli più o meno appianabili. Per ridurre al minimo tutte le fonti di errore è stato fatto in modo che tutto lo studio fosse costruito intorno a precisi punti: replicazione dei risultati in maniera indipendente (studio a 2 fasi); bassi P-value (10^{-6}-10^{-8}); selezione di casi omogenei

(lo studio ha arruolato pazienti con tubercolosi polmonare clinicamente diagnosticata e confermata con X-ray al torace, PCR e positività al test batteriologico); uso di appropriati controlli (individui senza segni evidenti della malattia, ma esposti al patogeno e geneticamente non correlati ai casi). Inoltre, l'evidenza che l'associazione è mantenuta in 3 differenti etnie rendono improbabile che l'associazione riscontrata tra i geni MyD88 e TIRAP e la TB sia un artefatto derivante dalla errata strutturazione demografica del campione esaminato. Ad ogni modo, la stessa associazione risulta dare risultati contrastanti quando si analizzano differenti popolazioni (Nejentsev, Thye et al. 2008, Miao, Li et al. 2011, Sanchez, Lefebvre et al. 2012). Quindi la riproducibilità di uno studio è necessariamente un artefatto? Noi pensiamo di no. Nel genoma umano nuovi alleli costantemente si presentano creando una vasta eterogeneità che si amplifica ulteriormente con l'interazione tra di essi e con l'ambiente. Tutta questa genetica eterogeneità è difficile da rilevare *a priori* e plausibilmente contribuisce all'irriproducibilità degli studi di associazione. Inoltre, durante tutta la sua lunga storia evolutiva, il *Mycobacterium tuberculosis* ha sviluppato un'efficace strategia per rendere difficile la sua eradicazione da parte del sistema immunitario dell'ospite: la latenza. Questo rappresenta un ulteriore grado di difficoltà nel costruire uno studio di associazione con la TB. Sulla base di queste considerazioni, il passo successivo è stato quello di disegnare uno studio caso-controllo scevro dai tutti i principali fattori di "bias" per quanto concerne il *Mycobacterium tuberculosis*. Il primo passo è stato cercare un modello che presentasse le seguenti caratteristiche:

- Azzeramento o minimizzazione dei fattori ambientali;
- Stessa etnia
- Possibilità di epurare i controlli da eventuali soggetti con TB latente;
- Un numero consistente di casi e controllo per un'adeguata potenza statistica).

Il modello scelto è stato quello dei bovini per la possibilità di biopsie polmonari al punto della morte grazie all'attiva collaborazione tra il laboratorio della Prof.ssa Capparelli e l'Istituto Zooprofilattico del Mezzogiorno. In aggiunta, grazie alla collaborazione con la Dott.ssa Berisio del Dipartimento di Chimica dell'Università di Napoli è stato possibile mettere a punto un "in-house assay" capace di discriminare tra batteri attivi e quelli dormienti.

Mycobacterium bovis e diagnosi di infezione polmonare

L'agente patogeno che causa la tubercolosi nei bovini è il *Mycobacterium bovis*. Nei paesi dove i programmi per l'eradicazione della tubercolosi bovina sono operativi (test periodici agli animali di allevamento, ispezioni delle carni e pastorizzazioni del latte) hanno ridotto a meno dell'1% i casi di tubercolosi umana attribuibile al *Mycobacterium bovis*, circoscrivendola soltanto alle persone affette da HIV o alle persone che vantano una prolungata esposizione ad animali infetti (persone del settore e veterinari). Il *Mycobacterium bovis* ha un ampio range di ospiti, il quale include numerose specie di allevamento e selvatiche. Esso è patogeno per l'uomo, mentre il *Mycobacterium tuberculosis* non è patogenico nei bovini (Ocepek, Pate et al. 2005). Questa caratteristica potrebbe essere attribuibile alla sola differente espressione genica tra di loro (Neill, Skuce et al. 2005) in quanto studi genetici hanno dimostrato l'elevata similarità tra le due specie batteriche (circa 99.5% a livello nucleotidico) (Garnier, Eiglmeier et al. 2003). Tutte queste osservazioni prese

insieme forniscono la plausibilità biologica del ruolo cruciale giocato dal gene MyD88 anche contro la tubercolosi bovina. Il gene TIRAP non è stato incluso nello studio perché la frequenza delle mutazioni risultava essere inferiore a 0.05.
L'infezione della tubercolosi polmonare può essere di tipo attivo (ATI) o latente (LTI); quest'ultima è caratterizzata dalla presenza di batteri dormienti (vitali, ma che non crescono sui normali mezzi di crescita) (Oliver 2010). I metodi comunemente usati per diagnosticare la tubercolosi sono il tuberculin skin test (TST) o l'IFN-ɣ assay. Tuttavia, questi metodi non distinguono tra ospiti ancora infetti e quelli che hanno controllato con successo l'infezione (Barry, Boshoff et al. 2009). Presi insieme questi gruppi, almeno nel presente studio, avrebbero potuto ridurne sensibilmente la capacità discriminante (Schurr 2007).

Results

Diagnosi di casi e controlli

Mycobacterium tuberculosis possiede 5 resuscitation-promoting genes (Rpf) che codificano per altrettante proteine (RpfA to RpfE), le quali in forma di proteine ricombinanti in *Escherichia coli*, inducono la risuscitazione del *Mycobacterium tuberculosis* (Biketov, Potapov et al. 2007) e M*ycobacterium marinum in vivo* ed *ex vivo (Parikka, Hammaren et al. 2012)*. Sulla base di questi risultati, è stato sviluppato un "in-house assay" capace di resuscitare i micobatteri dormienti attraverso l'impiego della proteina RpfB. È stato possibile recuperare micobatteri dormienti dal latte e polmoni provenienti da 7 animali trattati con RpfB, mentre nessuna colonia di batteri si è avuta dagli stessi campioni non trattati con RpfB. I risultati tra latte e polmoni sono stati pienamente concordanti. Altri 20 campioni (latte e polmone) sono stati utilizzati per validare l'assay. Il test è stato successivamente esteso a tutti gli animali utilizzando campione di polmone collezionati post-mortem. Un test di PCR discriminante tra *Mycobacterium tuberculosis, Mycobacterium bovis, o Mycobacterium avium*, ha determinato che tutti i campioni analizzati (con ATI o LTI) erano infettati da *Mycobacterium bovis.* In conclusione, i casi con ATI sono stati trovati positivi alla PCR ed al test batteriologico in assenza di RpfB; i casi con LTI sono stati trovati positivi alla PCR ed al test batteriologico in presenza di RpfB; i controlli sono soggetti esposti al *Mycobacterium bovis* (perché provengono dagli stessi allevamenti dove sono stati prelevati i casi), ma negativi alla PCR ed al test batteriologico sia in presenza che in assenza di RpfB.

Disegno sperimentale

Anche in questo caso lo studio è stato composto da due fasi. La fase preliminare ha coinvolto solo 50 animali di controllo, i quali sono stati separatamente confrontati con 50 casi con ATI o 50 casi con LTI. La fase preliminare ha mostrato una significativa associazione del sito polimorfico MyD88 A625C con ATI (P=0.01), ma non con LTI (P=0.84). Il sito A625C è situato sull'introne 1 del gene MyD88. Questa fase preliminare dello studio ci ha fornito due punti importanti: primo, che l'associazione sembra essere abbastanza robusta (poiché è stata rilevata utilizzando soltanto un numero piccolo di soggetti) e, secondo, che la stratificazione dei casi (tra la forma attiva o latente della TB) potrebbe fornire una maggiore potenza allo studio. Oltre ad A625C, non è stata rilevata la presenza di nessuno degli SNPs mostrati nella sequenza di riferimento. Per esplorare il ruolo di A625C, sono state allineate 11

diverse sequenze del gene di MyD88 appartenenti a diverse specie per studiarne la conservazione. Il basso livello di conservazione suggerisce che il sito polimorfico A625C non è sotto stringente selezione. Analizzando la sequenza dell'introne 1 con SCOPE sono stati evidenziati 4 motivi super-rappresentati nel genoma bovino, i quali includono i sito polimorfico. Questa evidenza potrebbe indicare un possibile ruolo regolatorio di A625C. Questi dati costituiscono un idoneo substrato per ulteriori future investigazioni.

L'eterozigosi ad MyD88 e la resistenza alla tubercolosi attiva bovina

Per la seconda fase dello studio è stato utilizzato un numero più ampio di campioni: 300 controlli, 150 casi con ATI e 150 casi con LTI, differenti da quelli della prima fase. I casi con ATI non sono in equilibrio Hardy-Weinberg (χ^2=4.4). Quando il test è stato ripetuto con i casi LTI, sia casi che controlli sono in equilibrio (χ^2controls =0.9; χ^2cases = 0.3). I dati suggeriscono un'associazione tra A625C e la ATI, ma non tra A625C e la LTI. Primo, il più stringente test esatto di Fisher mostra che l'eterozigosità (AC status) è fortemente associata con la resistenza alla ATI (OR 0.19, P=6.0x10^{-12}); secondo, l'associazione resta forte anche quando entrambe le classi omozigote (AA e CC) vengono unite (OR 0.22, P=1.8x10^{-10}); terzo, il sito A625C non ha influenzato la predisposizione alla LTI (OR 0.83, P=0.36 e 0.40). La regressione logistica binomiale ha supportato queste conclusioni.

L'eterozigosi ad MyD88 e l'infiammazione

TNF-α, IFN-ɣ e NOS influenzano profondamente la tubercolosi (Scanga, Bafica et al. 2004). È anche conosciuto che alti o bassi livelli di infiammazione hanno un impatto negativo sulla malattia (Glickman and Jacobs 2001, Doherty and Arditi 2004, Fremond, Yeremeev et al. 2004). Così, se l'eterozigote ad MyD88 mostrasse un livello intermedio di citochine rispetto a quello degli omozigoti, l'associazione tra A625C e la resistenza al *Mycobacterium bovis* acquisterebbe una fortissima plausibilità biologica. Per validare questa ipotesi sono stati misurati i livelli di mRNA TNF-α, IFN-ɣ e NOS in campioni di polmoni di soggetti con differente genotipo (AA, AC, CC) e status (controlli o animali con ATI o LTI) (6 classi; 5 animali/classe). I livelli di espressione dei soggetti con ATI o LTI sono stati comparati con quelli dei soggetti di controlli aventi lo stesso genotipo. Portatori dell'eterozigosi hanno espresso livelli di TNF-α, IFN-ɣ e NOS significativamente inferiori a quelli espressi dagli omozigoti AA. Al contrario, gli eterozigoti mostrano livelli solo leggermente superiori a quelli espressi dagli omozigoti CC, in questo caso, la differenza non ha raggiunto la significatività statistica. Presi insieme questi dati, supportano la conclusione che un'ottimale risposta infiammatoria è associata con il fenotipo di A625C.

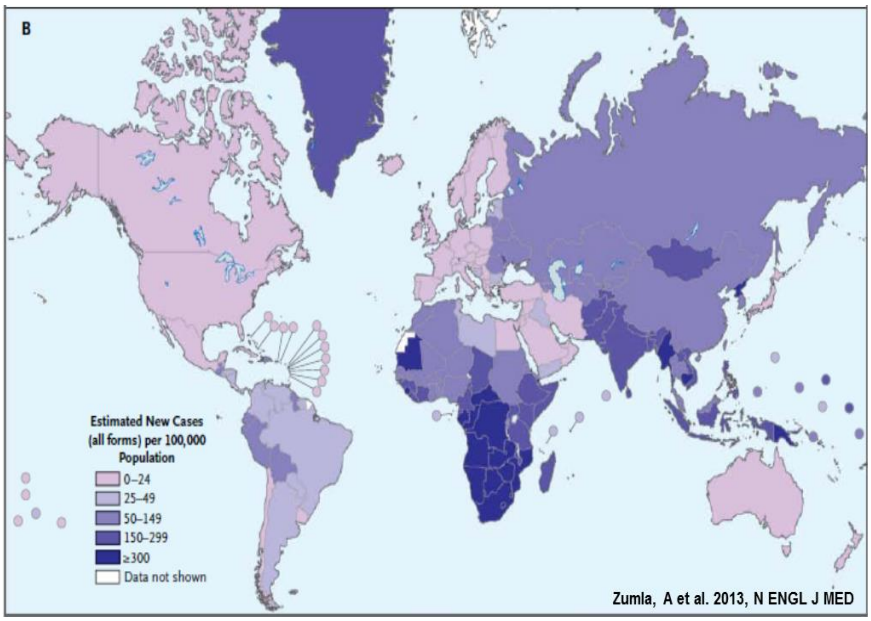
Discussione e conclusioni

Il presente studio ha dimostrato che nei bovini, animali eterozigoti al sito polimorfico MyD88 presentano un rischio ridotto di circa 5 volte di ATI (OR 0.19, P=6.0x10^{-12}). Tuttavia, la riduzione del rischio non si estende agli animali con LTI (OR 0.83, P=0.36 e 0.40). L'eterozigosi ad A625C è associato con livelli intermedi di TNF-α, IFN-ɣ e NOS. Lo studio ha anche mostrato differenze nell'espressione di mRNA delle citochine tra animali aventi lo stesso genotipo, ma con tubercolosi acuta o latente. La differenza è particolarmente evidente negli animali AA. Purtroppo non possiamo

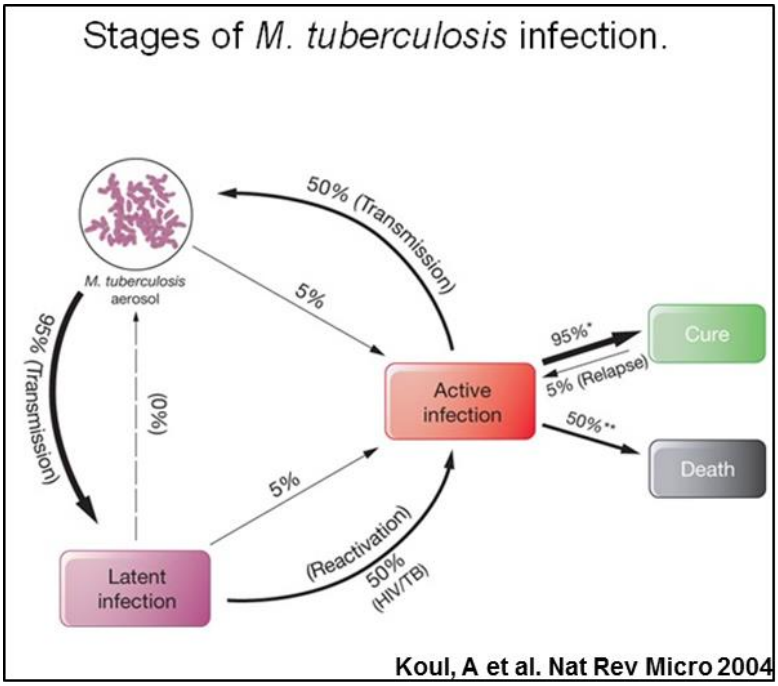
attribuire questi diversi livelli di espressione al patogeno o all'ospite, però i livelli di citochine rappresentano dei potenziali markers per la riattivazione della malattia. Il polimorfismo A625C è locato nell'introne 1 del gene MyD88, questo aggiunge evidenza che regioni non codificanti possono influenzare l'espressione genica. Non è sorprendente che questo accada nel caso dell'infiammazione, la quale ha bisogno di essere soggetta ad una fine e complessa regolazione. Nei bovini, l'esposizione ambientale ai micobatteri, che si verificano nella maggioranza dei soggetti, interferisce con la diagnosi della TB attraverso i TST o IFN-ɣ assay (Hope, Thom et al. 2005). La disponibilità dei reagenti, il tempo di incubazione, ed i livelli di cut-off possono influenzare la specificità e la sensibilità di questi assay (Pai, Riley et al. 2004). Il test batteriologico post-mortem resta, ad oggi, ancora il "gold standard" per le diagnosi di avvenuta infezione (Thacker, Harris et al. 2011). La tubercolosi è influenzata da molti geni che interagiscono tra di loro (Chang, Linderman et al. 2008) e con l'ambiente (Schurr 2007). La presenza del micobatterio è necessaria, ma non sufficiente ad acquisire la malattia, come mostrato dai soggetti di controllo (Diamond 1987). Fattori ambientali (clima, densità dell'allevamento, movimenti del bestiame etc.) sono conosciuti come fattori che promuovono la tubercolosi (Neill, Skuce et al. 2005). Perfino forti effetti genetici sul micobatterio possono essere mancati se non si prendono in considerazione gli effetti ambientali (Schurr 2007). Considerevoli OR e P value (OR = 0.19; P= $6.0x10^{-12}$) riportati in questo lavoro ci rendono cautamente ottimisti riguardo la possibilità di approcciare in modo corretto l'analisi genetica di questa complessa malattia. I casi sono stati resi omogenei (ATI ed LTI sono stati analizzati separatamente), i "confounders" ambientali sono stati o esclusi (sesso e razza) o "randomized" (età). Ancor più importante è che i controlli provengono dallo stesso allevamento dei casi e restano tuttavia "liberi" dall'infezione (negativi alla PCR e test batteriologico) nonostante abbiano avuto la stessa probabilità di infettarsi dei casi. Spesso la stratificazione della popolazione è presa in considerazione come responsabile dei falsi-positivi ottenuti dagli studi di associazione, ma raramente è stata dimostrata essere colpevole (Risch 2000, Colhoun, McKeigue et al. 2003). Studi umani hanno mostrato che la stratificazione potrebbe originarsi quando differenti etnie sono mescolate (Healy 2006). Nel presente studio è stata studiata una sola razza. Ulteriormente, gli stessi risultati provenienti da due campioni di popolazioni indipendenti offrono una prova considerevolmente convincente che non sia intervenuta alcuna stratificazione. Gli studi di associazione genetica sono caratterizzati da un alto tasso di risultati falsi-positivi (Risch 2000). Questa conclusione è spesso dovuta alla selezione di un gene candidato senza una relazione funzionale con la malattia (Lander and Schork 1994, Risch 2000). Nel presente studio, MyD88 è stato selezionato sulla base di un largo numero di evidenze sperimentali che mostrano, almeno nel topo, l'importanza di questo gene nel "signaling" a valle della rilevazione di componenti del micobatterio e dell'induzione degli effettori della risposta immunitaria innata da parte delle cellule dell'ospite (Doherty and Arditi 2004, Fremond, Yeremeev et al. 2004). In conclusione, l'elevata rilevanza biologica del gene da studiare, la scelta accurata dei criteri diagnostici, e la randomizzazione dei "confounders" ambientali, sono stati tutti attentamente tenuti in grandissima considerazione durante questo cammino nel complesso campo degli studi di associazione. Tuttavia, poiché l'associazione viene descritta per la prima volta, i risultati di questo studio sono da considerarsi come preliminari. Infine, il test qui utilizzato per distinguere tra malattia attiva e latente potrebbe potenzialmente essere esteso alla verifica periodica dei capi di bestiame per la tubercolosi. Il conteggio dei micobatteri dormienti risvegliato da RpfB in

campioni di latte sarebbe un modo semplice per conoscere l'incidenza della tubercolosi latente nella popolazione testata, un parametro fortemente influenzato dal controllo del patogeno da parte del sistema immunitario dell'ospite.

Introduction



Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB), also known as Koch's bacillus. It is the second cause of death from infectious diseases in the world, after the Human Immunodeficiency Virus (HIV). In 1993, TB was classified as a global emergency by the World Health Organisation (WHO), which estimated approximately 9 million of new cases in 2011 and 1.4 million deaths due to TB in 84. The same organisation considers Italy one of the Western countries where a significant increase in the number of TB cases between 1988 and 1990 was observed. Despite the availability of inexpensive and effective treatments, tuberculosis is prevalent among the poor people rather than the rich people, in both industrialized and developing countries. Almost 60% of total cases of TB are in Pakistan, South Africa, China and India. The highest incidence of tuberculosis is found in Sub-Saharan Africa, especially in association with HIV infection. The alarming increase of tuberculosis is also caused by the outbreak of anti-TB drug-resistant strains. Further disease spreading-factors are: population growth and difficulties to detect and treat cases among the poor masses, disease spreading in hospitals and congested prisons, and immigration of individuals from countries where TB is endemic (WHO 2012).



People with active TB represent the main source of infection. Only 10% of individuals show signs of active tuberculosis within 18 months from the infection. In the remaining cases, the host immune response can stop the growth of microorganism. However, the pathogen is eradicated in only 10% of cases, whereas in the remaining 90% of the cases, the

pathogen is able to evade the antibacterial mechanisms of the host and to survive in a non-replicative (dormant) state. This leads to a latent form of tuberculosis Infection (LTI), which affects about 2 billion people in the world (Koul, Herget et al. 2004) .

Symptomatology, Diagnosis and Management

Generally TB affects the lungs, although other organs may be involved. The development of the disease can occur slowly and symptoms may begin to appear months or even years after primary infection. Unlike asymptomatic latent infection, actively infected patients have the following symptoms:

- persistent cough, for more than three weeks (often bloody)
- high body temperature (above 38ºC)
- tiredness or fatigue
- breathlessness
- lack of appetite and weight loss
- night sweats

In some cases, TB may occur in extra-pulmonary sites. The incidence of extra-pulmonary TB varies between 10 and 42% of patients and also varies by ethnicity, age, strain of pathogen and the state of the host immune system (Zumla, Kim et al. 2013). TB infection can affect the lymph nodes, the bones, the joints, the digestive system, the bladder, the reproductive system and the nervous system.
Medical evaluation is needed for people suspected of having tuberculosis, such as:

- People with compromised immune system;
- People who come from countries where tuberculosis is endemic;
- People with HIV-infection;
- People who live or spent time with people with active disease.

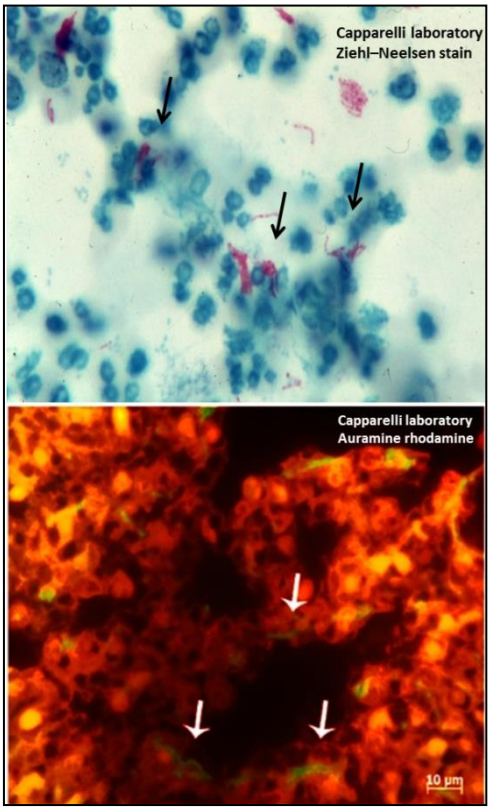
The first test used for TB detection is the Mantoux Tuberculin Skin Test (TST). In the lower part of the arm is injected a small amount of tuberculin (protein moiety of bacillus). Within 48/72 hours the body reaction to the test may appear. A swelling or hard area arises around the point of injection, whose intensity of reaction is correlated with the exposition to bacteria or the progression of disease. The second test is a TB blood test, also known as Interferon-ɣ Release Assay – IGRAs. This test measures how strong the person's immune system reacts to the protein moiety of bacillus. The next steps are the smear and the culture of sputum for the presence of bacilli and the pathogen DNA amplification. After that, the potentially patient needs chest radiograph to estimate the associated pulmonary outcomes. Once assessed the severity of infection, pharmacologic therapy is needed. This therapy involves a 4-drug regimen: Pyrazinamide, Isoniazid, Rifampicin and Ethambutol. When multiresistant tuberculosis is suspected, at least 3 of following antibiotics are administered, according to drug susceptibilities: Cycloserine, a fluoroquinolone (Ofloxacin, Ciprofloxacin and Levofloxacin), Terizidone, Bedaquiline. In extreme instances, surgical resection is suggested for the patients with multidrug resistant bacilli infection, whose medical outcome is poor.
Recently, the US Food and Drug Administration approved the use of Delamanid (an inhibitor of the synthesis of mycolic acids) in adults, against the infections caused by multidrug-resistant *Mycobacterium tuberculosis* (isionazid and rifampicin). It is the

first introduction on the market of an effective drug against TB after the advent of rifampicin in the ‘70s (Zumla, Kim et al. 2013). Patients affected by TB and resolved after a short period of prophylaxis betray a recurring within 1 or 2 years since the end of the drug treatment. This subgroup of individuals shows an increased risk of TB reinfection compared to the general population. Thanks to help of DNA fingerprinting, it has been shown that there is a new outbreak of TB not due to the failure of previous treatment, but to the reinfection of the host by a different bacterial strain (Verver, Warren et al. 2005). The main challenge to studying *Mycobacterium tuberculosis* is the lack of a test able to demonstrate the reinfection. The Tuberculin Skin Test and the Interferon- ɣ Release Assay cannot distinguish reinfection by the awakening of a latent infection (Andrews, Noubary et al. 2012).

Mycobacterium tuberculosis

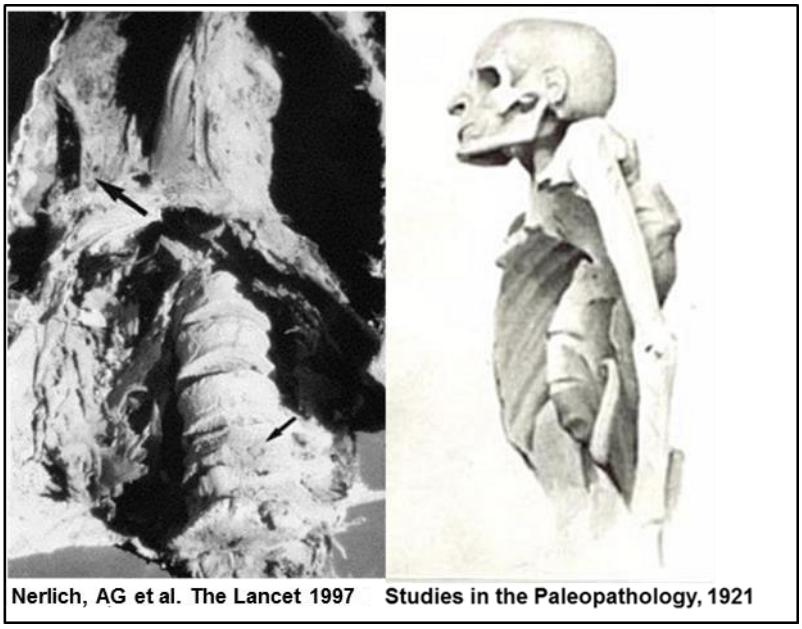
Mycobacterium tuberculosis is a member of a group named Mycobacterium Tuberculosis Complex (MTBC). At this group belong 6 other genetically related species (considered as a sub-species): *M. bovis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae* and *M. capretti*. Although all these strains can cause tuberculosis, they have different phenotypic traits and different host range (Cole, Brosch et al. 1998).

MTB is an aerobic bacterium characterized by slow growth and size of 0.2-0.6 µm. It is generally rod-shaped, without flagellum, although its shape may vary depending on the environment. In a study of 1940, the authors had demonstrated the different forms assumed by MTB under unfavourable environmental conditions (reduced supplement in the culture medium, low quantity of oxygen, etc.) (Vera and Rettger 1940). To date, by advanced microscopy techniques (SEM, TEM, etc.) it has been confirmed that Koch’s bacillus can assume different form from the classic rod-shaped. The wall of MTB is the main factor involved in the environmental changing. MTB complex structure provides resistance to many antibiotics and unfavourable environmental conditions. Starting from the inside, cell membrane is made up of several layers of peptidoglycan (PG), arabinogalactans (AG), lipomannans (LM), mannose- lipoarabinomannans (ManLAM) and mycolic acids (MA). All this layers are covalently linked one to each other forming a very complex structure. The PG consists of long chains of N-acetylglucosamine (NAG) linked to the chains of muramic acid (NAM) through the residues of L-alanyl-D-iso-glutaminyl-meso-diaminopimelic acid (DAP) (Velayati, Farnia et al. 2012). The long chain polysaccharides (LM, AG and LAM) form a bridge between the peptidoglycan and the mycolic acids, which are long chain fatty acids and the main component of the wall of MTB . The complex structure of the membrane gives to



the mycobacterium the singular colouring properties. These properties (acid-alcohol resistance) allow seeing the MTB through Zhiel-Nielsen (bright field) and Auromina-rhodamine (fluorescent field) stains.

Mycobacterium tuberculosis and its history

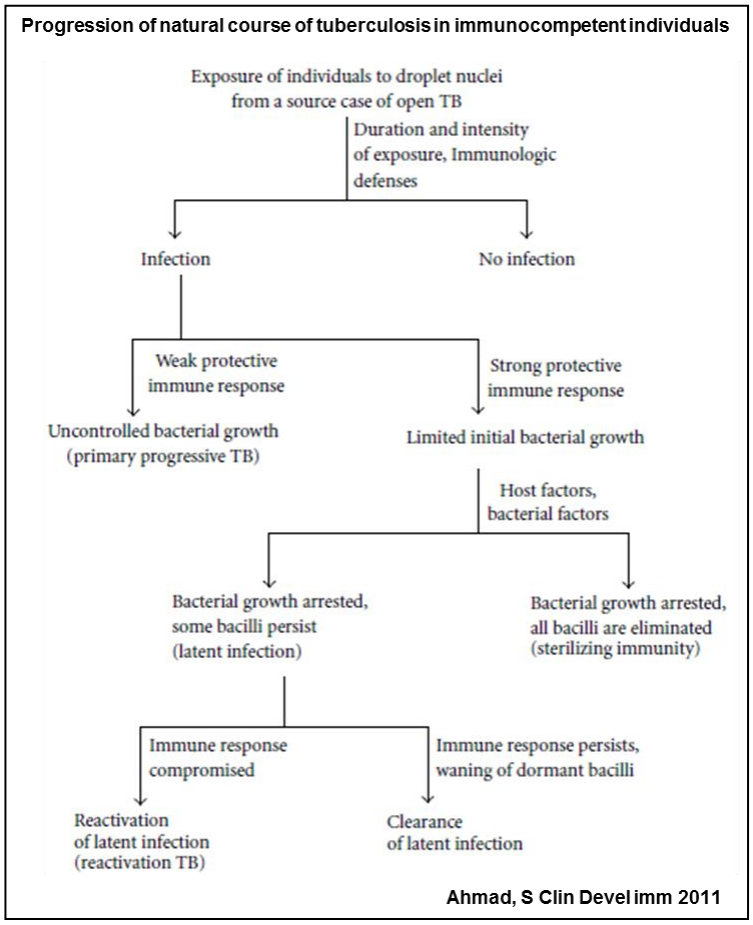

Nerlich, AG et al. The Lancet 1997 Studies in the Paleopathology, 1921

MTB has plagued the mankind since immemorial times. Probably, it is the bacterium that has killed more people than any other microbial pathogen. Its low rate of mutation allowed some scientists to suppose that the "*genus Mycobacterium*" might date 150 million years ago (Hayman 1984). All of the modern members of the Mycobacterium Complex (*M. bovis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae* and *M. capretti*) seem to be descended from a common ancestor from Africa dating back to about 35,000-15,000 years ago. First physical traces of MTB were found in Egypt and dated about 5,400 years old. In young Egyptian mummies was found the presence of typical skeletal deformities due to tuberculosis infection (Pott's disease). The advent of modern techniques for amplification of bacterial DNA from tissues did not left doubt about the cause of the skeletal deformities. Also, there are some clear references to tuberculosis in the biblical books like Deuteronomy and Leviticus. Well established in East Africa, tuberculosis began to spread in Europe and Asia thanks to migration of small groups of individuals between 35,000 and 89,000 years ago. It was found writings on tuberculosis dating back to 3,300 years ago in India and 2,300 years ago in China. As in Egypt, even in Peruvian mummies it was possible to observe the bone structure deformity due to mycobacteria infection. In Europe, the earliest writings date back to Hippocrates who defines tuberculosis with name "*Phtitis*" and emphasizes the preference of the disease for the young men. After the fall of the Roman Empire, the writings on tuberculosis seem to disappear. This does not mean that tuberculosis was not present. Archaeological findings dating to the fifth century after the fall of the Roman Empire indicate that tuberculosis was widespread. In 1266, the 44 years old St. Francesco of Assisi died of tuberculosis. Three centuries later, the French physician René Laennec (1781-1826), inventor of stethoscope, added significant evidence to the knowledge about tuberculosis. In his book "On Mediate Auscultation", the author clearly defines the pathogenesis of the disease and the concept of pulmonary and extra-pulmonary tuberculosis. He also describes many of the physical signs of lung disease still used today. After the "Laennec's era", TB inundated Europe as a real tsunami, with peaks of 800-1,000 deaths per 100,000 people in London, Stockholm and Hamburg. In the Northern part of Europe, TB has been classified as hereditary disease, while in the Southern part

as infectious disease. In 1865the French military surgeon Jean-Antoine Villemin provided evidence of the infectious nature of TB by inoculating a rabbit with a small amount of purulent fluid resulting from the lungs of a man who died of tuberculosis. Although the rabbit apparently looked like healthy, the lungs showed extensive signs of illness when sacrificed 3 months after inoculation. In 1882, the history of tuberculosis changed forever when Robert Koch presented his work "Die Aetiologie der Tuberculose" in Berlin. On that occasion, Robert Koch demonstrated that it is a bacillus which causes tuberculosis, and dictated the points of Koch's postulate that to date provide the gold standards for the classification of the infectious etiology of a disease. Also, Koch discovered in the body of living individuals a compound originated from the same bacilli, which rendered harmless the pathogen. The injection of this substance, called tuberculin, spread rapidly as a treatment against tuberculosis, but shortly after it was discredited as ineffective. Robert Koch injected himself with 0.25 cm^3 of tuberculin which caused him powerful fever. So he understood the diagnostic power of the tuberculin. In 1908, Charles Mantoux introduced the use of a needle and a syringe to inject tuberculin under the skin. In 1909, Von Pirquet, a Viennese pediatrician, after trying on himself the effects of tuberculin, published a work on the subcutaneous tuberculin injection used as a vaccine in children. He fixed the cut- point of 5 mm for the positive tuberculin reaction in children who did not exhibit signs of tuberculosis and he was the first person to coin the term latent tuberculosis. In the '20s, the Norwegian physicist Olaf Scheel noted the high incidence of TB among nursing students in their first year of clinical training. After 1924, Scheel, along with his colleague Heimbeck, performed the Tuberculin Skin Test (TST) on all of nursing center students, before the moment of entry to their training (Bjartveit 2003). After their three years training they noted a high incidence of infection and disease among the TST-negative students. Afterwards, this study was replicated in many hospitals in Europe and the United States of America. The high rate of infection and lack of treatments for latent tuberculosis (LTBI) allowed researchers to conduct an observational study, which could not be executed today (Andrews, Noubary et al. 2012). Shortly after the American biochemist Florence Seibert sinthesized purified the protein (PPD) that to date is still used as the standard test for TB. In 1952, Carroll Palmer and Leroy Bates used the PPD to conduct a study involving 3,000 patients with tuberculosis. Only in 1% of cases, the reaction to PPD by the immune system did not occur. Three years later, the WHO extends this skin test in children attending schools in various countries. This study brought to light that in countries with high prevalence of tuberculosis, such as Ethiopia and the Philippines, a large number of schoolchild without any symptoms of the disease showed a reaction to PPD similar to that of patients with active tuberculosis. These children were affected by latent tuberculosis, noted for the first time by Von Pirquet. A few years later, Edwards and Palmer performed the TST on 600,000 American soldiers. They concluded that latent tuberculosis was common in the United States of America and presumably also in other regions with low incidence of tuberculosis. While knowledge about tuberculosis was implemented through the efforts of Koch, Von Pirquet and other scientists, mortality rates began to decline in the mid-nineteenth century. The causes can be attributed to the improvement of social and living conditions and to the selection in breeding of species/populations genetically more resistant to the TB. The decline of tuberculosis continues to date, largely fought by the public health and the discovery of more effective drugs. The incidence of tuberculosis in Europe and America is

historically low. By contrast, in Sub-Saharan Africa, TB continues unopposed, strongly fueled by AIDS (Daniel 2006).

Transmission of *Mycobacterium tuberculosis* infection

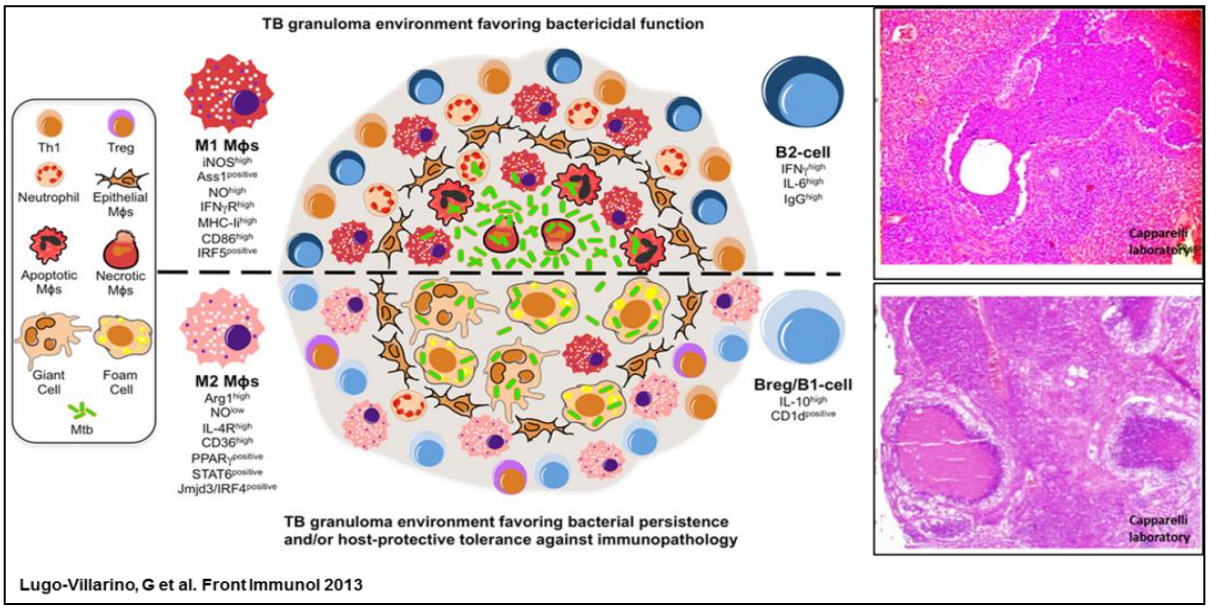


MTB infection starts with inhaling droplets containing the pathogen (size 1-6 µm), expectorated by patients with active tuberculosis after strong coughs. Many factors are involved in the risk of infection including the proximity to the patient, the inhaled bacterial load, the host immune system, and the genetic predisposition of the host. The droplets nuclei reach the alveoli of the lungs trough nasal/mouth passage. Their small size allows penetrating the upper respiratory tract where many organisms are removed by mucociliary mechanism. Here, the droplets containing pathogens are intercepted by phagocytic antigen-presenting cells (APC) such as dendritic cells and alveolar macrophages. Some studies report also the involvement of non-professional phagocytes cells (Bermudez and Goodman 1996, Garcia-Perez, Mondragon-Flores et al. 2003). The Pathogen-Associated Molecular Patterns (PAMPs) are recognized by specific MTB Pathogen Recognition Receptors (PRRs) located on the surface of the cells of the host immune system. These receptors, including Toll-like Receptors (TLRs), are essential to initiate and coordinate the innate immune responses of the host (Jo, Yang et al. 2007). The interaction between PAMPs of MTB and TLRs of the host triggers a signal transduction cascade that culminates in an inflammatory response by the host immune system (Harding and Boom 2010). However, mycobacteria have developed effective strategies to modulate or even inhibit this response. The most important ligands on the surface of the MTB that interact with TLRs are: 19 kDa lipoprotein, the lipomannans (LM) and the mannose-lipoarabinomannans (Jo, Yang et al. 2007). The interaction between these ligands with TLRs leads to the activation of the nuclear transcription factor B (NF-kB) and the consequent production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-ɣ) and nitric oxide. The NF-kB activation involves the myeloid differentiation primary response protein 88 (MyD88) -dependent and -independent pathways (Yamamoto, Sato et al. 2003, Jo 2008). In mice, prolonged exposition of alveolar macrophages' membrane to the MTB 19kDa lipoprotein attenuates the antigen processing, which in

turn reduces the expression of the major histocompatibility complex (MHC)-II, dampening T cell recruiting. In this way, a subpopulation of infected macrophages with a modulated APC function constitutes an invisible niche to the host immune system. Here, the bacteria survive, resist and sometimes proliferate. However, some other strategies are adopted by MTB to evade immune system's surveillance. The most studied is the effect of Man-LAM upon maturation of the phago-lysosome and the production of reactive nitrogen intermediate (RIN), lethal for MTB (Kang, Azad et al. 2005).

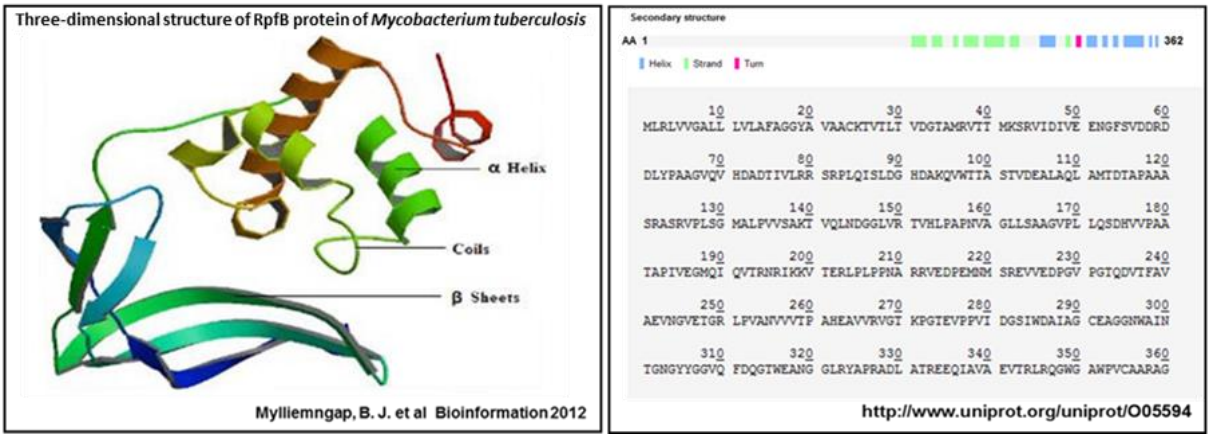
Latent Infection of *Mycobacterium tuberculosis*

The alveolar macrophages, after the entry of MTB, produce pro-inflammatory cytokines and chemokines as alarm signal for the host immune system. Thus, lymphocytes, monocytes and neutrophils can migrate to the site of infection. Despite well-orchestrated, sometimes the immune response could be insufficient. Some bacilli are able to evade the countermeasures of the immune system and multiply inside the cells causing necrosis (Chen, Gan et al. 2006). While T cells ($CD4^+$ and $CD8^+$) rush to the site of infection, engulfed dendritic cells migrate to the closest lymph node causing potential dissemination. Cytokines produced by T cells may contribute in a multitude of ways to the clearance of bacteria, including the activation of macrophages where the MTB resides. Specific structures called granuloma enclose the infection when it becomes chronic. These granulomas consisting of lymphocytes ($CD4^+$, $CD8^+$ and B cells) that surround macrophages, fibroblasts and other cells engulfed of bacilli. The function of the granuloma is to limit the spreading of the infection from the lung creating, in this way a local microenvironment of action for the cells of the immune system. Unfortunately, MTB has developed effective strategies to survive under these adverse conditions. The host immune system is not able to recognize the Bacilli in non-replicative state (dormancy), characterized by low metabolic status. Recent studies have shown physiologically different type of granulomas in different individuals. Some of these suppress the MTB (sterilizing immunity), while some others promote the persistence of viable bacilli in this environment (Young, Gideon et al. 2009). However, without formation of granuloma, the MTB could not be contained and could spread more rapidly in other organs. In HIV-infected patients (deficient of granuloma formation), TB appears to be more aggressive than people without HIV (Russell 2007). *Cynomolgus macaques* infected with a low dose of MTB lead to the formation of at least two types granulomas. Histopathological studies have shown the classic granuloma formed by epithelial macrophages, neutrophils and other immune system cells surrounded by fibroblasts, while the other type of granulomatous lesions, already seen in human latent TB, are composed almost exclusively by fibroblasts which contain few macrophages (Via, Lin et al. 2008, Lin, Rodgers et al. 2009). To date, no one knows if the MTB is located within macrophages or within the fibrotic tissue. If the host immune system is compromised, the dormant bacilli present in the granuloma can resuscitate during host's lifespan.



Resuscitating-promoting factor proteins

One-third of worldwide population suffers of tuberculosis infection. Only 10% of people show active signs of TB, while the remaining of infected people carry the pathogen in dormant form. TB pathogens have the ability to assume a non-replicative state after strong host immune response or post-drug treatment and, then, they are capable to resuscitate upon immune suppression or spontaneously (McCune, Feldmann et al. 1966, McCune, Feldmann et al. 1966). The reactivation of the mycobacterium is contributed by five secreted proteins named Resuscitating-promoting factors (RpfA-E). The hydrolyzing activity of these proteins on the peptidoglycan of bacterial cell wall allows the transition of mycobacteria from the dormant state to the viable one. Aged mycobacterial cultures have shown greater sensitivity than viable MTB to the exogenously administration of Rpf proteins *in vitro* (Shleeva, Mukamolova et al. 2004). A physiologically role of these proteins, especially under bacterial stress condition, was confirmed in various *in vivo* and *ex vivo* animal models (Biketov, Potapov et al. 2007, Parikka, Hammaren et al. 2012). The Resuscitation-promoting factor B (RpfB) is the most complex and studied protein dedicated to bacterial reactivation (Ruggiero, Tizzano et al. 2007). The understanding of the molecular mechanism behind the transition from non-replicative to viable state could help the global strategy to prevent the spread of TB.

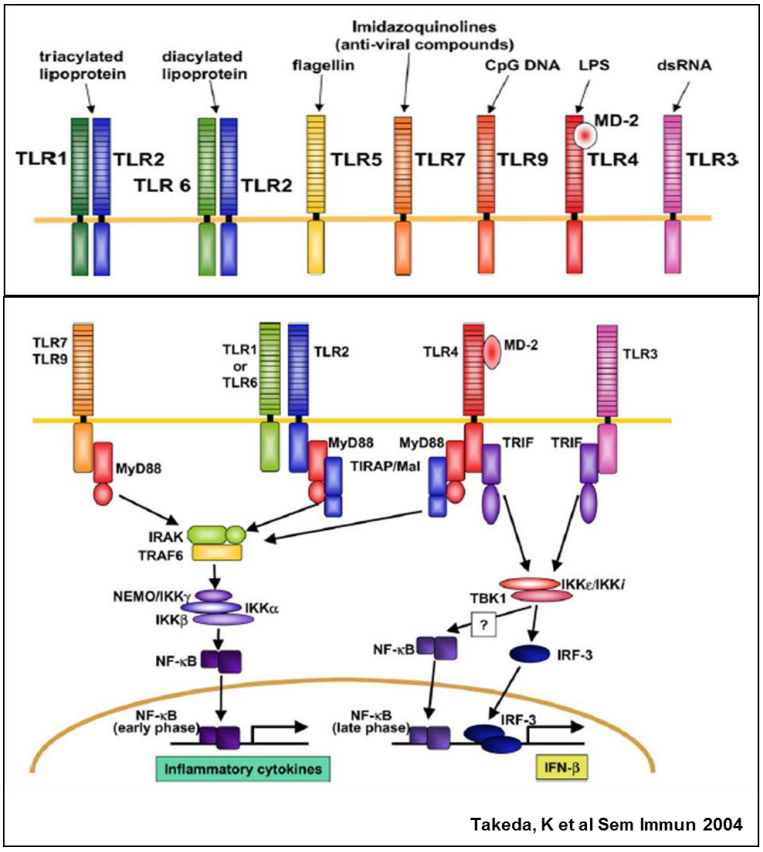


Vaccine strategies and Bacilli Calmette-Guérin

MTB has an extraordinary ability to hide itself within the host immune system cells. This ability makes inadequate most of vaccine strategies, for example those ones based on the neutralisation of virulence proteins or genes. Another strategy for vaccine development introduces in the host an avirulent bacterium that triggers immune response, but not the disease. To date, numerous laboratories are focusing on the development of attenuated strains of MTB through genetic manipulations. *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) is unable to cause disease in the human and, until today, it is the current vaccine administered in infants at birth in countries where the TB is endemic. BCG vaccine shares ample homology with MTB (<95%), and has protective effects against disseminate TB in children. Unfortunately, BCG vaccination seems to be inadequate because it does not prevent primary infection and reactivation of latent pulmonary infection (WHO 2012). In 1908, at the Institute Pasteur de Lille in France, a physician, Albert Calmette, and the veterinary Camille Guérin noted that bacilli cultured in glycerinated/beef/bile/potato medium had lost their own virulence. After 13 years and over 200 subcultures later, the bacilli were attenuated enough to be considered for use as a vaccine. In 1921, after the World War I, both scientists were ready to try the vaccine. Over the next 7 years, more than 100,000 children were immunized and BCG was widely accepted in the most part of Europe. More than 30 vaccines, all as primary immunogens to replace BCG and as boosters for BCG, are in development or have entered in clinical trials (Raviglione, Marais et al. 2012). Understanding the molecular mechanisms behind the persistence of MTB inside the cells, the recruitment and the activation of T and B cells in the site of infection, and the host genetic resistance are important keys for the development of an effective vaccine against TB.

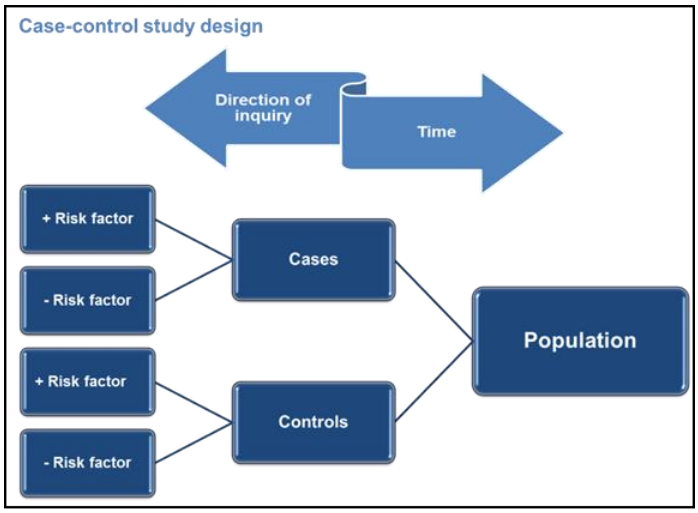
Activation of inflammation through MyD88 and TIRAP

The inflammation is a complex biological mechanism involved in response to different harmful stimuli. It is the effort of the mammalian body to restore damaged cells, tissues or organs. The inflammation is triggered by activation of many receptors located on the surface of the cells. The most studied receptors are the Toll-like receptors (TLRs). They were discovered for the first time in *Drosophila melanogaster*, where they are involved in the formation of dorso-ventral patterning of embryos (Hashimoto, Hudson et al. 1988). Later, it was discovered that flies mutant for this receptor were very susceptible to fungal infections (Lemaitre, Nicolas et al. 1996).



TLRs homologues have been found also in mammals and are able to recognize specific microbial patterns conserved among the pathogens. The activation of signaling pathways via TLRs triggers several genes involved in immune response. TLR family comprises 11 members (TLR 1-11) in both human and mouse. Each TLR shows specific responses to the microbial component involving specific molecules from the cell's surface to the nucleus. One of these molecules is a common adaptor called MyD88, which was the first essential component characterized for the induction of inflammatory cytokines (TNF-α and IL-12). TLRs signaling pathways initiate when the cytoplasmic portion of TLRs, named Toll/IL-1 receptor domain (TIR), interacts with the TIR domain of MyD88. Activated MyD88 recruits IL-1 Receptor-Associated Kinase (IRAK) and then the TNF Receptor-Associated Factor 6 (TRAF6). TRAF6 triggers two distinct pathways: c-Jun N-terminal Kinases (JNK) and Nuclear Factor-kB (NF-kB) - MyD88-dependent pathways, that lead to the transcription of several pro-inflammatory genes. Mice knockout for MyD88 did not show production of pro-inflammatory cytokines and activation of NF-kB and JNK upon activation of TLR2, TLR7, and TLR9. However, mice stimulated with LPS display activation of NF-kB and JNK, although without production of cytokines (Kawai, Adachi et al. 1999). Following studies show without doubts that there is a MyD88-independent pathway downstream the activation of TLRs. In this pathway, LPS stimulation activates Interferon Regulatory Factor 3, a transcription factor of several IFN-inducible genes. Later, the analysis of the MyD88-independent pathway has allowed to identifying another TIR domain-containing Adaptor Protein (TIRAP) or MyD88-adaptor-like (Mal). Similarly to MyD88 knockout mice, TIRAP knockout mice displayed compromised pro-inflammatory cytokines production and delayed activation of NF-kb and JNK. Further studies clearly established that TIRAP plays a crucial role in the MyD88-dependent signaling pathway via TLR2 and TLR4 but not in the MyD88-independent signaling activated by TLR3, TLR5, TLR7, and TLR9 ligands (Takeda and Akira 2004).

Case-control study



		Disease		
		Cases	Control	Total
Risk factor	+	A	B	A+B
	-	C	D	C+D
	Total	A+C	B+D	A+B+C+D

Odds Ratio = $\frac{A/B}{C/D}$

OR >1 = Risk factor confers susceptibility to the disease

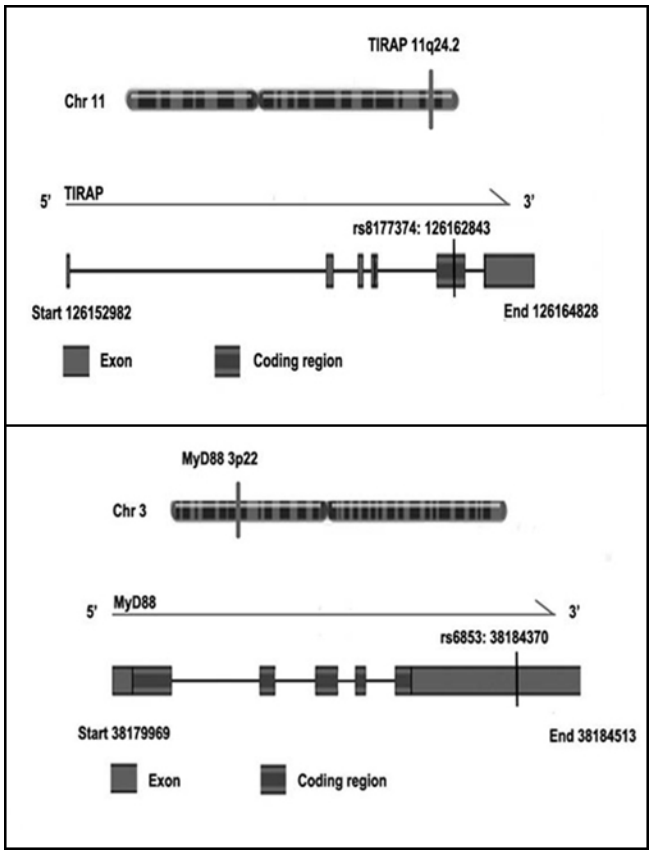
OR =1 = Risk factor does not affect the disease

OR <1 = Risk factor confers protection to the disease

A case-control study is a statistical method designed to identify potential risk factors. In this type of study two groups of people are identified by referring their health status: the cases are people with the disease or the condition of interest and controls are people with no history of disease or without the condition of interest. Usually, a case-control study is cheap, quick and performed as initial assessment before undertaking larger and more expensive studies. Case-control studies are not susceptible to loss during the follow-up. Cases and controls are compared to establish whether a particular risk factor is more frequent in one group than in the other. The most commonly measure of this frequency measured between the exposure (+/- Risk factor) and outcome (+/- disease) is called odds ratio (**OR**). In other words, OR determines whether a risk factor is associated or not with a particular disease and compares the magnitude of that association. The OR must be accompanied by 95% Confidence Interval (**95% CI**) that estimates the accuracy of the OR. To note, the 95% CI, in contrast to p value, is not a measure of statistical significance. Often, many risk factors are not measured and can influence the OR in case-control studies. These risk factors are called **confounding** and, when unequally distributed between the groups, can distort the association between the exposure and the condition of interest. To minimise the effect of confounding factors the OR can be adjusted using a statistical method known as **logistic (or binomial) regression**. Unlike in linear and multiple regression analyses, where the outcome variable is continuous, in logistic regression it is binary (yes or not). Generally, the predictor variables (age, gender, etc.) are any combination of binary, continuous, or categorical variables, independent of each other. The logistic regression assesses the association between a dependent variable (disease or outcome) and one or more predictor variables concurrently. The results of logistic regression are presented as unadjusted and adjusted odds ratios for potential confounding effect of predictor variables. That means the measure of association between the disease and a dependent variable when all other dependent variables are constant. The extent of confounding is determined by comparing of the unadjusted and adjusted OR. The explanatory variable is defined independently associated with the medical condition when the association is significant after adjustment for confounding. Another value is also estimated with logistic regression: the coefficient of regression (β). The exponential function of β (e^{β}) corresponds to the OR associated with one unit increase in the value of exposure. The potential

confounding effect can be minimized more efficiently by reducing the systematic differences between the groups (matching cases and controls) at the design stage of the study, rather than in the subsequent statistical analysis.

Aim



Only 10% of total infected people will develop active TB. At the base of the susceptibility of disease there are the interactions of host's genetics with environmental factors and the pathogen. Understanding these interactions represents a huge challenge facing human genetics. Schurr wondered if the susceptibility to tuberculosis was acquired or inherited (Schurr 2007). The author knew very well that tuberculosis could develop only upon exposure to the pathogen. With his question (posted as the title to the article), Schurr intended to highlight the essential role of the hereditary component for resistance to tuberculosis.

The host's membrane receptors TLRs recognize many PAMPs of MTB and, through adapter proteins, lead to the activation of nuclear factor kB and the successive immune response, innate and adaptive. The detection of mycobacterial DNA requires TLR9 and MyD88; 19LP, LM, LAM and STF requires TLR2 and MyD88, whereas HSP65 requires TLR4, MyD88 and Toll-Interleukin-Receptor Adapter Protein (TIRAP). Mice knockout for MyD88 show a low production of TNF-α, IL-12 and NO in response to the infection of MTB and die by 4 weeks (Fremond, Yeremeev et al. 2004). Instead, Mice knockout for TIRAP control effectively MTB infection (Fremond, Togbe et al. 2007). MyD88 and TIRAP have a clear biological plausibility. In addition, both work as transmitters of the signal from the receptors of the TLR family to the transcription factor NF-kB. Therefore, their location on separate chromosomes (independence) and their common function (signal transmission) make it plausible the hypothesis that these genes interact between them. If the above proposition is correct, the study could provide an example of epistasis (interaction between independent genes). The Single Nucleotide Polymorphism (SNP) C558T of TIRAP is associated with the susceptibility to meningeal tuberculosis (Hawn, Dunstan et al. 2006, Caws, Thwaites et al. 2008) and, again, the same SNP studied in this work (rs81777374) confers protection against malaria and tuberculosis (Khor, Chapman et al. 2007). Following studies, however, do not confirm the protective role of S180L against tuberculosis (Nejentsev, Thye et al. 2008, Miao, Li et al. 2011). One more study shows that neither TIRAP (rs352165 and rs352167) nor MyD88 (rs4988457 and rs6767684) influence tuberculosis (Sanchez, Lefebvre et al. 2012). In the light of these discrepancies

emerged from the study of these same genes in different populations, even studying the same SNP (rs81777374), it was decided to investigate the role of these genes in patients with pulmonary tuberculosis in the Italian population.

Premise

The present study analyses the same disease (tuberculosis) in two different species, humans and cattle. The study however should not be intended as consisting of two independent investigations or - worse – as the duplication in cattle of the study originally carried out in humans. The studies in humans and cattle should be seen as tightly linked, rather than independent. The study in humans necessarily preceded that in cattle. Tuberculosis – in humans as well as in cattle – can assume the active or latent forms. The genes chosen for study (MyD88 and TIRAP) could differently influence the two forms of the disease. However, patients enrolled in case-control studies need to be highly homogeneous (all with the same disease form). Mixing cases with latent and active tuberculosis infection would have sensibly reduced the power of the study or, worse, open the way to type I errors and false results. Currently there are no methods that can discriminate between the two forms of tuberculosis with the level of accuracy required by the kind of study we planned to carry out. We therefore were forced to set up an assay able to distinguish between active and latent infection. To do this we needed lungs biopsies. These specimens could be obtained only from cattle, at the time of slaughter. The assay demonstrated that, at least in cattle, the genes influence the active –but not the latent – form of infection.

Results

Study design

To reduce the type I error and to limit multiple comparisons, it was chosen a study in two phases. In the first phase of the study (hypothesis - generating) 100 cases and 100 controls were recruited and genotyped for the polymorphic sites on MyD88 and TIRAP genes. The site rs8177374 on TIRAP was included in the study because in the literature there are conflicting results, the rs6853 was chosen because it was the only one among the 5 tested to show an allele frequency > 0.05. Both sites showed statistically significant association with the disease (P-value < 0.05) (Table 1). Based on these preliminary results (MyD88: OR 0.40 and a proportion of controls with susceptible genotypes 0.61; TIRAP: OR 0.48 and a proportion of controls with susceptible genotypes 0.64), it was calculated that a sample of 185 cases and 185 controls for MyD88 and another one of 313 cases and 313 controls for TIRAP would be necessary to assuring to the study a statistical power of 96%, with a significance level of 0.01. In the second part of the study 400 cases and 400 controls were involved (independent from the cases and controls used in the preliminary phase of the study). In order to get a cohort as homogeneous as possible, the cases were recruited only among patients with evident pulmonary TB, confirmed by chest X -ray, bacteriological tests and PCR. The bacteriological tests were performed on sputum samples. All cases were treated by Monaldi Hospital (Naples), the reference center for tuberculosis of the southern part of Italy. Controls were selected among wives, husbands and friends of patients without any clinical evidence of tuberculosis (negative test with interferon-ɣ). In that way, controls are not infected and genetically

unrelated to the cases, despite the exposure to *Mycobacterium tuberculosis* (> 2 years). The criteria used to classify cases and controls were the same in both phases of the study. Cases and controls were collected in more than five years of collaboration between the Hospital and the laboratory of Prof. Rosanna Capparelli. At that time six controls became positive to test with interferon and were excluded from the study. The cases consisted of 258 males and 142 females (mean age 50 ± 19 years), the controls of 222 males and 178 females (mean age 49 ± 17 years). The study was approved by the Ethics Committee of Monaldi.
The informed consent to the study was obtained from all patients who participated to the study.

Heterozygosis is associated with protection against pulmonary tuberculosis

The genotype frequencies of the markers rs6853 and rs8177374 were in Hardy-Weinberg equilibrium among the controls, but not among the cases (Table 2). The association of polymorphisms was confirmed by Fisher's exact test (Table 2). Heterozygous status (AG for MyD88 and TIRAP CT) has been associated with resistance to pulmonary tuberculosis for both loci. The protection persists when the two homozygous classes are pooled (AG vs. AA + GG and CT vs. TT + TT) (Table 2). Age is known as a risk factor for pulmonary tuberculosis. Therefore, ORs associated with different genotypes (age confounding effect) were corrected by the Mantel-Haenszel test (Table 3). After correction, the double heterozygous (MyD88/TIRAP AG/CT) still offer high level of protection (OR 0.16) compared with single heterozygous (AG: OR 0.4, CT: OR 0.42) (Table 3). To assess how to age-related risk varies across the “strata”, the data were re-analyzed using the logistic regression model. The analyses were restricted to only the groups with a number of individuals of ≥45. Apart from the category 31-40 vs. 21-30 years (apparently protected, OR 0.31), the model showed that the risk of developing the disease increases with age, as shown by the increase in ORs (1.32 to 13:59) (Table 4). Clearly, the age and the possible variables associated with age, such as smoking, diabetes and prolonged exposure to the pathogen, can overcome the resistance conferred by genes. We did not have access to all patient records and therefore we could not investigate the role of these potential "confounders". The logistic regression model was also used to dissect the contribution of each genotype and their interaction with the ORs. Particularly evident was the interaction between the AG and CT genotypes (OR estimated 0.09), and the interaction, although in the opposite direction, between the GG and TT genotypes, AA and CC, and AA and CT (OR 5.78, 5.78 and 7:46 respectively) (Table 5).

Table 1. Association of the MyD88 rs6853 and TIRAP rs8177374 polymorphic sites with pulmonary tuberculosis. Exploratory study in an Italian population sample

Genes	*Status*	*Number of individuals in each genotype*			*Total*	*HWE (P)*	*Allelic frequency*		*OR (95% CI)*[a]	*P-value*[a]
							Co	*Ra*		
		AA	AG	GG					AG vs (AA + GG)	
MyD88	Cases	73	21	6	100	0.017	0.84	0.16	0.40 (0.22–0.77)	0.008
	Control	48	39	13	100	0.267	0.68	0.32		
		CC	CT	TT					CT vs (CC + TT)	
TIRAP	Cases	76	22	5	103	0.058	0.84	0.16	0.48 (0.25–0.90)	0.029
	Control	60	36	4	100	0.624	0.78	0.22		

Abbreviations: Co, common allele (MyD88: A; TIRAP: C); Ra, rare allele (MyD88: G; TIRAP: T). [a]CI (confidence intervals) and *P*-values were calculated with the Fisher's exact test.

Table 2. Association of the MyD88 rs6853 and TIRAP rs8177374 polymorphic sites with pulmonary tuberculosis

	Genes	Status	Number of individuals in each genotype			Total	HWE (P)	Allelic frequency		OR (95% CI)[a]		OR (95% CI)[a]	P-value[a]
								Co	Ra				
Italian			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	297	89	14	400	0.029	0.85	0.15	0.40 (0.28–0.55)	5.8×10^{-9}	0.42 (0.31–0.59)	7.8×10^{-8}
		Control	213	160	27	400	0.68	0.73	0.27				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	318	71	11	400	0.006	0.88	0.12	0.44 (0.31–0.62)	1.2×10^{-6}	0.44 (0.31–0.63)	2×10^{-6}
		Control	258	130	12	400	0.36	0.81	0.19				
Romanian			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	104	34	12	150	8×10^{-4}	0.81	0.19	0.44 (0.26–0.74)	0.003	0.47 (0.28–0.79)	0.005
		Control	78	57	15	150	0.34	0.71	0.29				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	100	40	10	150	0.04	0.8	0.2	0.55 (0.33–0.90)	0.01	0.53 (0.32–0.86)	0.01
		Control	84	61	5	150	0.12	0.76	0.24				
Ukrainian			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	117	27	6	150	0.01	0.87	0.13	0.45 (0.26–0.78)	0.004	0.48 (0.28–0.82)	0.01
		Control	92	47	11	150	0.16	0.77	0.11				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	119	26	5	150	0.03	0.88	0.12	0.41(0.24–0.71)	0.002	0.42 (0.24–0.71)	0.002
		Control	95	50	5	150	0.61	0.8	0.2				
All ethnicities			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	518	150	32	700	5×10^{-6}	0.85	0.15	0.42 (0.32–0.53)	8×10^{-13}	0.45 (0.35–0.57)	3×10^{-11}
		Control	383	264	53	700	0.42	0.74	0.26				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	537	137	26	700	1.8×10^{-5}	0.87	0.13	0.46 (0.35–0.59)	5.3×10^{-10}	0.46 (0.36–0.69)	4.6×10^{-10}
		Control	437	241	22	700	0.10	0.80	0.20				

Abbreviations: Co, common allele (MyD88: A; TIRAP: C); Ra, rare allele (MyD88: G; TIRAP: T). [a]CI (confidence intervals) and *P*-values were calculated with the Fisher's exact test.

Table 3. Age confounding effect assessed by weighted Mantel–Haenszel test

	MyD88			TIRAP			MyD88/TIRAP								
	AA	AG	GG	CC	CT	TT	AA/CC	AA/CT	AA/TT	AG/CC	AG/CT	AG/TT	GG/CC	GG/CT	GG/TT
OR	2.65	0.41		2.27	0.42		1.99	2.12			0.16				
CI	(1.9–3.6)	(0.3–0.6)	n.s.	(1.6–3.2)	(0.3–0.6)	n.s.	(1.5–2.7)	(1.7–3.6)	n.s.	n.s.	(0.1–0.3)	n.s.	n.s.	n.s.	n.s.
P	2.8×10^{-10}	1.8×10^{-8}		7.6×10^{-7}	6.5×10^{-7}		3.2×10^{-6}	5.5×10^{-3}			1.3×10^{-12}				

Abbreviations: CI, 95% confidence interval; OR, odds ratio; P, significance level; n.s., non significant.

Table 4. Influence of age on pulmonary tuberculosis assessed by the logistic regression model procedure, which includes 2000 bootstrap re-sampling

Regression term	OR	P-value
Age class		
31–40 vs 21–30	0.31	5.0×10^{-4}
41–50 vs 31–40	1.32	0.29
51–60 vs 41–50	8.71	5.0×10^{-4}
61–70 vs 51–60	13.59[a]	5.0×10^{-4}
71–80 vs 61–70	1.38	0.27
Constant	0.63	2.0×10^{-3}

Abbreviation: OR, odds ratio estimated by the logistic regression model. [a]Example of OR calculation. OR of people aged 65 years compared with people belonging to the 51–60 years category is 13.59 (61–70 vs 51–60).

Table 5. Influence of the MyD88 and TIRAP genotypes on pulmonary tuberculosis assessed by the logistic regression model procedure, which includes 2000 bootstrap re-samplings

Regression term	OR[a]	P-value
MyD88		
MyD88 (AG vs AA)	0.50	0.01
MyD88 (AG vs GG)	0.64	0.09
MyD88 (GG vs AA)	0.78	0.41
TIRAP		
TIRAP (CT vs CC)	0.58	0.03
TIRAP (CT vs TT)	0.80	0.45
TIRAP (TT vs CC)	0.73	0.29
Effects between loci		
MyD88(AG) by TIRAP(CT)[b]	0.09	5.0×10^{-4}
MyD88(AG) by TIRAP(TT)[b]	3.05	0.13
MyD88(GG) by TIRAP(CT)[b]	0.77	0.71
MyD88(GG) by TIRAP(TT)[c]	5.78	0.02
MyD88(AA) by TIRAP(CC)[c]	5.78	0.03
MyD88(AA) by TIRAP(CT)[c]	7.46	1.5×10^{-3}
MyD88(AG) by TIRAP(CC)[c]	1.90	0.34
Constant	0.63	2.0×10^{-3}

Abbreviations: OR, odds ratio estimated by the logistic regression model; vs, within locus comparisons; by, between loci interactions; /, reference genotype (note C) is double heterozygous. [a]Double heterozygotes (AG/CT), compared with double homozygotes (AA/CC) have the OR: 0.5 (AG vs AA) x 0.58 (CT vs CC) x 0.09 (AG by CT) = 0.026. [b]The reference genotype is MyD88(AA)/TIRAP(CC). [c]The reference genotype is MyD88(GG)/TIRAP(TT).

The association resists to the demographic stratification

The study was extended to one sample of population from Romania and one from Ukraine. The study, although underpowered, confirmed the protective role of the state of heterozygous to the site MyD88 rs6853 (P= 0.003-0.004) and the site TIRAP rs8177374 (P= 0.01 to 0.02) (Table 2). The protective role provided by heterozygous status persisted when the samples from Romania, Ukraine and Italy were mixed (P = $8x10^{-13}$, rs8177374 P= $5.3x10^{-10}$) (Table 2). The association of rs6853 and rs8177374 with pulmonary tuberculosis extended to three nationalities tested in this study resisted to the artificial stratification determined by the pooling different ethnicities.

The heterozygous controls inflammation

To investigate the mechanism of how the host controls the infection depending on genotype basis, Peripheral Blood Mononuclear Cells (PBMCs) from healthy blood donors (controls) (nine genotype groups; 5 samples/group) were extracted. PBMC were stimulated with heat-killed Mycobacterium tuberculosis strain (H37Rv). The levels of TNF-α, IFN-ɣ and NO (nitric oxide) released into the medium were measured by ELISA test. In the context of the AA genotype, the CT subjects showed intermediate levels of TNF-α, IFN-ɣ and NO compared with the CC and TT subjects. In the context of genotype AG, the data show the same trend, although some differences are not significant. Furthermore, the lowest levels of cytokines were observed in subjects GG, as if possessing the allele "A" facilitates the production of cytokines and the allele "G" to reduce it. These data indicate that heterozygotes are associated with an intermediate level of cytokines and NO (Figure 1). The experimental data were then analyzed with a generalized linear model. This methodology allows us to study the relationship between a variable (in this study, TNF-α, IFN-ɣ and NO) and a set of predictors (MyD88 and TIRAP) to extract a model. In addition, this methodology can be applied to data that presumably do not have a normal distribution (like in this case, where only a small number of donors were recruited). The model calculate β1, β2 and β3, which measure as MyD88, TIRAP and MyD88-TIRAP interaction influence the levels of TNF-α, IFN-ɣ and NO released. Surprisingly, the model predicted the cytokine levels on the basis of their genotypic structures with exceptional accuracy (Table 6). These results suggest that the two loci cooperate strongly to control the disease. This evidence is strongly supported by the evidence that TIRAP and MyD88 form heterodimers.

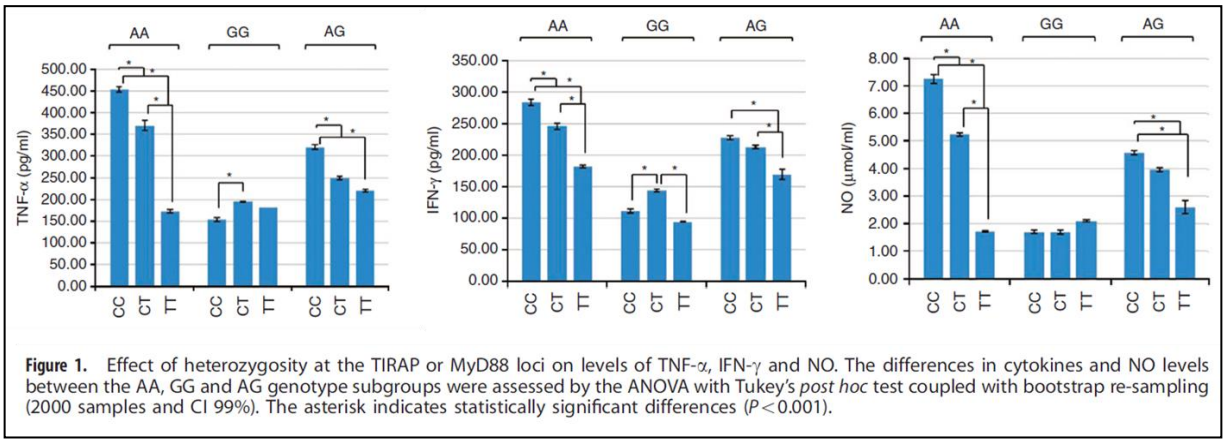


Figure 1. Effect of heterozygosity at the TIRAP or MyD88 loci on levels of TNF-α, IFN-γ and NO. The differences in cytokines and NO levels between the AA, GG and AG genotype subgroups were assessed by the ANOVA with Tukey's *post hoc* test coupled with bootstrap re-sampling (2000 samples and CI 99%). The asterisk indicates statistically significant differences ($P < 0.001$).

Table 6. Effects of the MyD88 and TIRAP genotypes on IFN-γ, TNF-α and NO levels estimated by the Generalized Linear Model procedure

Genotype predictors	$[IFN\text{-}\gamma]_{(pg\,ml)}$				$[TNF\text{-}\alpha]_{(pg\,ml)}$				$[NO]_{(pg\,ml)}$			
	Parameter (β)	P-value	Predicted mean	Observed mean[a]	Parameter (β)	P-value	Predicted mean	Observed mean[a]	Parameter (β)	P-value	Predicted mean	Observed mean[a]
Constant (k)	6.55	1.00×10^{-89}	na[b]		7.51	1.00×10^{-99}	na		1.07	1.00×10^{-82}	na	
MyD88 (β_1)[c]												
AA	0.95	1.00×10^{-78}	na	248.22 (a)	−0.07	9.43×10^{-2}	na	363.22 (a)	−0.29	4.16×10^{-7}	na	5.34 (a)
AG	0.85	1.00×10^{-99}	na	206.01 (b)	0.28	3.92×10^{-11}	na	258.92 (b)	0.30	3.13×10^{-7}	na	3.78 (b)
GG	0.00		na	112.29 (c)	0.00		na	172.33 (c)	0.00		na	1.83 (c)
TIRAP (β_2)[c]												
CC	0.23	3.69×10^{-87}	na	203.06 (a)	−0.25	3.21×10^{-12}	na	306.80 (a)	−0.31	1.00×10^{-94}	na	4.49 (a)
CT	0.61	1.00×10^{-69}	na	208.45 (a)	0.10	1.70×10^{-2}	na	274.80 (b)	−0.32	4.57×10^{-8}	na	3.88 (b)
TT	0.00		na	140.52 (b)	0.00		na	190.61 (c)	0.00		na	2.13 (c)
Effect between loci (β_3)[c]												
AA by CC	0.40	2.30×10^{-15}	280.14	283.33 (a)	1.63	1.00×10^{-77}	451.94	453.08 (a)	2.39	1.00×10^{-93}	7.26	7.24 (a)
AA by CT	−0.18	1.73×10^{-3}	243.88	245.55 (b)	0.99	1.00×10^{-85}	369.65	370.00 (b)	1.93	1.00×10^{-99}	5.24	5.23 (b)
AA by TT	0.00		181.02	182.00 (c)	0.00		173.65	173.33 (c)	0.00		1.72	1.71 (c, d, e)
AG by CC	0.20	4.12×10^{-4}	227.54	227.50 (b)	0.78	1.00×10^{-83}	319.57	320.50 (d)	1.13	1.00×10^{-81}	4.56	4.57 (f)
AG by CT	−0.28	2.93×10^{-7}	212.31	212.23 (d)	0.07	1.92×10^{-1}	249.00	249.53 (e)	0.93	1.00×10^{-87}	3.94	3.94 (g)
AG by TT	0.00		168.90	169.00 (c)	0.00		221.32	220.83 (e, f)	0.00		2.58	2.59 (h)
GG by CC	0.00		109.90	110.58 (e)	0.00		153.28	153.66 (c)	0.00		1.69	1.70 (c, i, j)
GG by CT	0.00		143.01	143.33 (f)	0.00		195.36	195.16 (c)	0.00		1.68	1.69 (d, i, k)
GG by TT	0.00		93.70	93.89 (e)	0.00		182.28	182.00 (c, f)	0.00		2.10	2.10 (e, h, j, k)

Within each dependent variable (IFN-γ, TNF-α and NO levels), the β_1, β_2 and β_3 parameters weigh the effect of predictors (MyD88 and TIRAP genotypes) on the dependent variable fitting the model $Y = 2^{k+\beta1(MyD88)+\beta2(TIRAP)+\beta3(MyD88*TIRAP)}$. Abbreviation: na, not applied. [a]Different letters indicate statistically significant differences between genotypes at Tukey's HSD *post hoc* test ($P<0.001$). [b]The model predicts cytokines and NO levels based on the MyD88 and TIRAP genotypes and their interactions. The model was not accurate when applied to one single genotype. [c]Redundant parameters are set equal to 0.

In *silico* analysis of both polymorphic sites

The site rs6853 is located on three prime Untranslated Region (3' UTR) of the MyD88 gene. Comparison of different genome sequences revealed that both alleles A and G are conserved in many species, suggesting that they are maintained, or at least tolerated by natural selection (Figure 2). The analysis with 4 ENCODE tracks showed that the 3' UTR of the polymorphic site could have effect on interaction between the MyD88 mRNA and other protein factors. The residing of rs8177374 site on exon 5 of the gene TIRAP has allowed us to evaluate whether the change of a serine with a leucine at position 180 may have an impact on the structure of the protein. Both amino acids are represented in position 180 of the TIRAP genes of 22 species of mammals and through SIFT algorithm has been predicted that both are compatible with the activity of the protein (Figure 3). These results suggest that the isoforms A and B originate from the same mRNA molecule that has undergone alternative splicing (Figure 4), giving a further biological plausibility to the polymorphism.

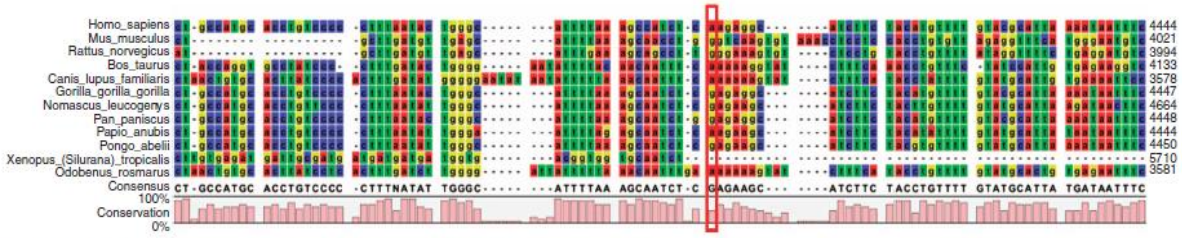


Figure 2. Multi-alignment and conservation analysis of 13 MyD88 gene sequences within a region surrounding the polymorphic site rs6853. The A and G nucleotides are both conserved across species.

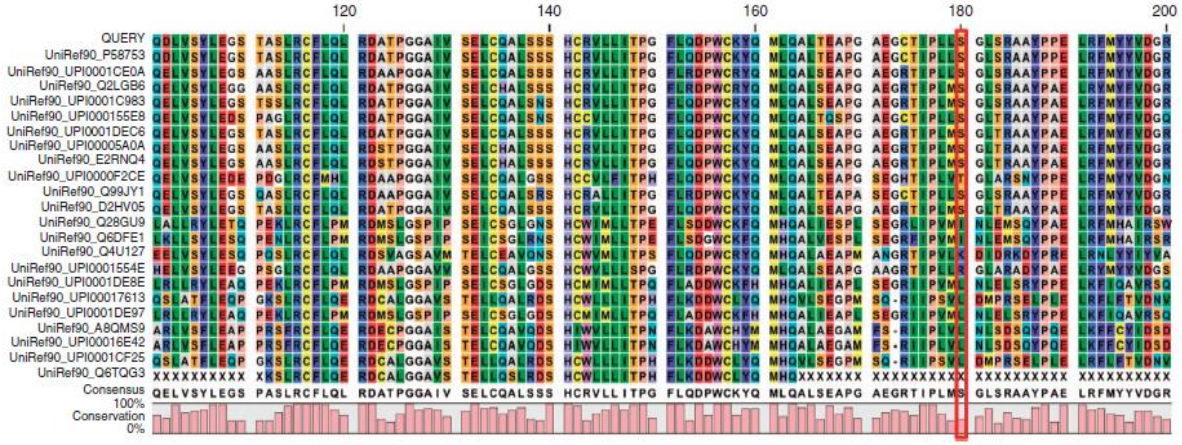


Figure 3. Multi-alignment and conservation analysis of 22 TIRAP protein sequences within a region surrounding the polymorphic site under study (marked with a red line). The serine and leucine amino acid at position 180 are both conserved across species. The automatic search for similar sequences and alignment against the human TIRAP protein was obtained through the SIFT tool (http://sift.jcvi.org/).

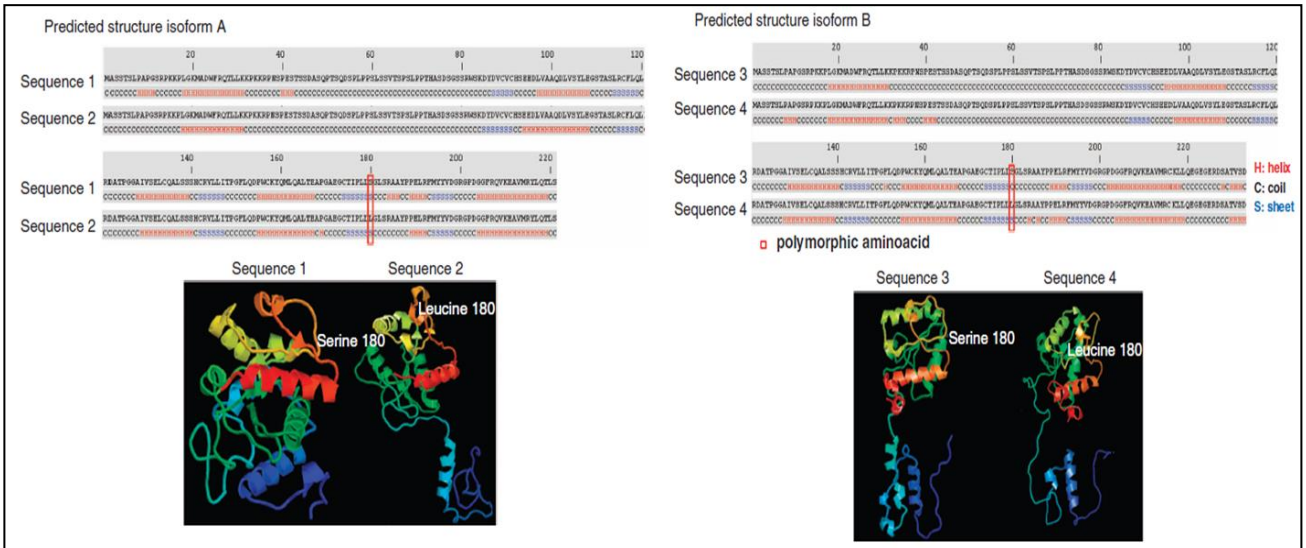


Figure 4. Predictive comparative analysis of TIRAP proteins translated from the common and rare alleles polymorphic at the site rs8177374. Sequences from the common allele (Sequence 1 and 3) and the rare allele (Sequence 2 and 4) are compared based on bi- and tridimensional models generated by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), which predicts secondary and tertiary structures.

Discussion and Conclusion

Very little is known of the "crosstalk" between genes involved in the resistance to the pathogen. In this study, it was investigated how MyD88 and TIRAP genes influence each other in the pulmonary tuberculosis. The hypothesis that the two genes may interact seems plausible because both proteins MyD88 and TIRAP are involved in the "signalling" downstream of TLRs. Also, it was demonstrated that the two genes cooperate or antagonize each other on the basis of their allelic combination. The heterozygosis at both loci provides a stronger protection ($P= 1.3x10^{-12}$, age-corrected, Table 3) compared to a single locus heterozygous ($P= 7.8x10^{-8}$ MyD88, TIRAP $P= 2x10^{-6}$, Table 2). At the same time, in individuals AG/TT (MyD88/TIRAP) the TT genotype neutralizes protection afforded by the AG genotype (Table 5). These data, although in a limited way, show how two or more independent genes may contribute to the formation and regulation of the same phenotype. Individuals heterozygous at MyD88 or TIRAP loci show intermediate levels of TNF-α, IFN-ɣ and NO compared to other genotypic classes (Figure 1). These molecules play a crucial role against the *Mycobacterium tuberculosis* infection (Casanova and Abel 2002, Velez, Hulme et al. 2009). NO exerts a strong anti-mycobacterial activity and, together with TNF-α, promotes the formation of granulomas (Miller and Ernst 2009). IFN-ɣ induces the production of NO, the expression of MHC II molecules and antigen presentation (Fortune, Solache et al. 2004, Scanga, Bafica et al. 2004). Furthermore, there are a lot of evidence that the over-expression of these molecules favours the tuberculosis. Many of the symptoms are caused by host's immune response, rather than by *Mycobacterium tuberculosis* (Glickman and Jacobs 2001). Indeed, reactivation of TB was observed after therapeutic treatment with TNF-α (Mankia, Peters et al. 2011) or in patients with HIV infection after antiretroviral treatment (French and Price 2001). Even the hypo-expression of TNF-α, IFN-ɣ and NO promotes TB. The biological advantage to have an intermediate level of expression of these molecules becomes clear. This advantage is much more pronounced in the double heterozygotes (Table 5), showing that the crosstalk between genes extends from the epidemiology to the molecular level. The advantage expressed by heterozygotes suggests that the allele frequencies at polymorphic sites of rs6853 and rs8177374 are maintained by balancing selection, where homozygosis is associated with pulmonary tuberculosis and heterozygosis with resistance. In agreement with this hypothesis is the fact that both polymorphic sites are conserved through speciation (Figures 2 and 3). In conclusion, the association of rs8177374 and rs6853 with pulmonary TB seems promising on Italian samples, but should be confirmed in future studies.

Aim II

This kind of study revealed itself much trickier than expectations and, unfortunately, association studies lack of reproducibility (Ioannidis, Ntzani et al. 2001). During the first phase of the study we ran into different obstacles. In order to minimize all sources of error the study was built on specific points:

- Independent findings replication (Two-steps study);
- Low P-value (10^{-6}-10^{-8});
- Selection of homogenous cases (the study enrolled patients with active pulmonary tuberculosis confirmed by X -ray chest, PCR and bacteriological positivity);
- Use of appropriate controls (individuals negative to the IFN-ɣ assay, but exposed to the pathogen and genetically unrelated to the cases).

Furthermore, the evidence that the association is maintained in 3 different ethnic groups (Table 2) makes unlikely that the observed association between MyD88 and TIRAP genes and TB is an artifact resulting from incorrect demographic structure of the sample. However, the same genes appear to give conflicting results when analyzing different populations (Nejentsev, Thye et al. 2008, Miao, Li et al. 2011, Sanchez, Lefebvre et al. 2012). Is the irreproducibility of a study necessarily an artifact? We think no. New alleles in the human genome are constantly generating a wide heterogeneity that is amplified by the interaction between them and with the environment. All this genetic heterogeneity is difficult to detect in advance, and plausibly contributes to the unreliability of association studies. In addition, throughout its long evolutionary history, MTB has developed an effective strategy to make difficult own eradication by the host's immune system: the latency. This represents an extra level of difficulty in genetic association study design. On the basis of these considerations, the next step was to design a case-control study lacking by the major "bias or confounding" factors. The first step was to find an animal model that presented the following characteristics:

- Null or minimization environmental factors;
- Same ethnicity;
- Discrimination between population with active and latent TB;
- A consistent number of cases and controls for acceptable statistical power.

The pulmonary tuberculosis infection can be active (ATI) or latent (LTI), the latter is characterized by the presence of dormant bacteria (viable, but not-cultivable on normal growth media) (Oliver 2010). The methods commonly used to diagnose latent tuberculosis are the tuberculin skin test (TST) or IFN-ɣ assay. However, these methods do not distinguish between hosts still infected and those who have successfully controlled the infection (Barry, Boshoff et al. 2009). Therefore, MyD88 and TIRAP genes could influence differently the two forms of infection To define the focal point of this research, it was decided to shift on animals from which it should be possible to obtain lung biopsies when they were slaughtered. Finally, the investigation of bovines was limited to only MyD88 gene for case of force majeure. In the population studied, the TIRAP rarer allele has a frequency <0.01 and thus could be maintained in the population by recurrent mutations, rather than by natural selection.

Mycobacterium bovis infection and diagnosis of pulmonary infection

Mycobacterium bovis is the pathogen that causes TB in cattles. In countries where programs for the eradication of bovine tuberculosis are efficient (periodic testing for farm animals, meat inspection and pasteurization of milk), human tuberculosis due to *Mycobacterium bovis* was reduced to less than 1% of cases, limiting it only people with HIV or persons who have prolonged exposure to the infected animals (manufacturing people and veterinarians). *Mycobacterium bovis* has a wide host range, which includes numerous wild and breeding species. It is pathogenic for humans, while *Mycobacterium tuberculosis* is not pathogenic in cattle (Ocepek, Pate et al. 2005). This feature could be attributed solely to the different gene expression between them (Neill, Skuce et al. 2005). Genetic studies have demonstrated the high genetic similarity between the two bacterial species (approximately 99.5 % at the nucleotide level) (Garnier, Eiglmeier et al. 2003). All of these observations taken together provide the biological plausibility of the crucial role played by MyD88 gene also against bovine tuberculosis.

Results

Cases and controls diagnosis

The first point was developed an "in-house assay" able to resuscitate dormant mycobacteria. Through the use of protein RpfB, which are produced in recombinant form in *Escherichia coli*. It was possible to recover dormant mycobacteria from milk and lungs from 7 animals treated with RpfB, while no colony of bacteria there was in the same samples not treated with RpfB. The results of the milk and lungs were fully concordant. Twenty more samples (milk and lungs) were used to validate the assay. The test was subsequently extended to all animals using lung sample collected post-mortem (Figure 5-6). A PCR assay was used to discriminate between *Mycobacterium tuberculosis*, *Mycobacterium bovis* or *Mycobacterium avium* (Figure 7). That test has determined that all samples analyzed (with ATI or LTI) were infected with *Mycobacterium bovis*. In conclusion, cases with ATI were found positive by PCR and bacteriological examination in the absence of RpfB; cases with LTI were found positive by PCR and bacteriological examination in the presence of RpfB, but not in presence of RpfB, the controls are subjects exposed to *Mycobacterium bovis* (because they come from the same farms where they were taken cases), but PCR and the bacteriological test both negative in the presence and absence of RpfB.

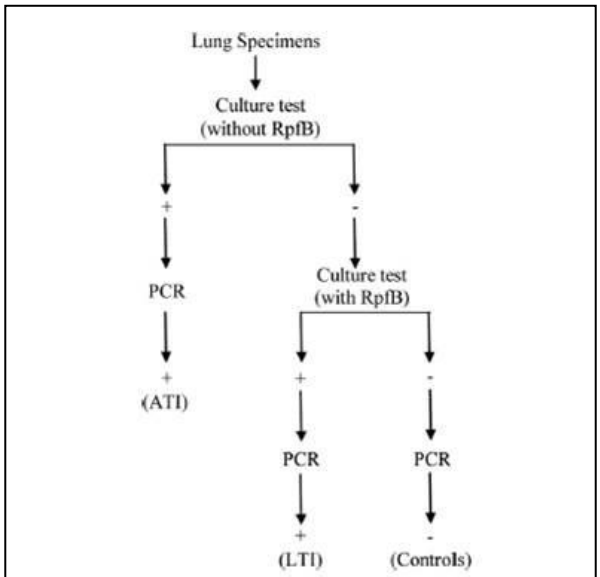
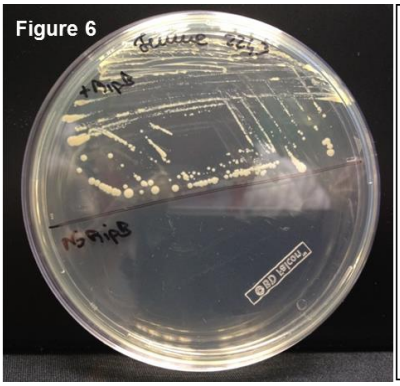
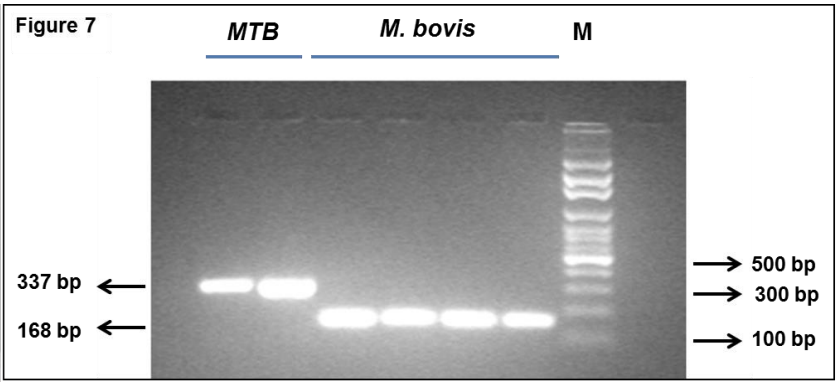


FIG 5 Diagnostic criteria used to classify subjects into controls or subjects with ATI or LTI. Controls, subjects with and without RpfB negative by culture and PCR tests; ATI, subjects without RpfB positive by culture and PCR tests; LTI, subjects with RpfB positive by culture and PCR tests.





Experimental design

Also in this case the study was composed of two phases. The first phase involved only 50 control animals, which were compared with 50 cases with 50 cases with ATI or LTI. Preliminary results showed a significant association of the polymorphic site of MyD88 A625C with ATI (P = 0.01, Table 7), but not with LTI (P = 0.84, Table 7). The site A625C is located on the intron 1 of the MyD88 gene (Figure 8). This preliminary phase of the study has provided two important assumptions: first, that the association seems to be quite robust (detected by using only a small number of samples) and, second, that the stratification of cases (between the active or latent TB) could provide more power to the study. Besides A625C, it was not detected the presence of any of the SNPs shown in the reference sequence. To explore the role of A625C, have been aligned with 11 different gene sequences of MyD88 belonging to different species, in order to study themselves conservation (Figure 9). The low level of conservation suggests that the polymorphic site A625C is not under stringent selection. Surprisingly, analyzing the sequence of cattle by SCOPE, it was seen 4 over-represented sequences in the whole bovine genome, which includes the polymorphic site A625C (Table 8). That could indicate a possible regulatory role of the polymorphism. These data constitute a suitable substrate for further future investigations.

TABLE 7 Heterozygosity at the A625C SNP influences active pulmonary tuberculosis infection

Study stage	TB type[a]	Status	No. of cows with the following genotype: AA	AC	CC	Total	χ^{2b}	AC vs AA: OR (CI[c])	AC vs AA: P	AC vs (AA + CC): OR (CI)	AC vs (AA + CC): P
1	Active	Cases	36	10	4	50	5.2	0.30 (0.12–0.74)	0.001	0.29 (0.12–0.71)	0.01
		Controls	25	23	2	50	1.4				
	Latent	Cases	28	21	1	50	1.7	0.81 (0.36–1.81)	0.68	0.85 (0.38–1.87)	0.84
		Controls	25	23	2	50	1.4				
2	Active	Cases	123	23	4	150	4.4	0.19 (0.11–0.32)	6×10^{-12}	0.22 (0.12–0.37)	1.8×10^{-10}
		Controls	140	135	25	300	0.9				
	Latent	Cases	75	60	15	150	0.3	0.83 (0.53–1.28)	0.40	0.81 (0.53–1.23)	0.36
		Controls	140	135	25	300	0.9				

[a] TB type, type of *M. bovis* pulmonary tuberculosis infection.
[b] $\chi^2_{0.05}$ (1 degree of freedom) = 3.8.
[c] CI, 95% confidence interval.

The heterozygosity at MyD88 and resistance to the active bovine tuberculosis

In the second phase of the study, it was used a larger number of samples: 300 controls, 150 cases with ATI and 150 cases with, different from those of the first phase. Cases with ATI are not in Hardy-Weinberg equilibrium ($\chi 2$ = 4.4, Table 7). When the test was repeated with the LTI cases, both cases and controls are in equilibrium ($\chi 2$ controls= 0.9; $\chi 2$ cases= 0.3, Table 7). The data suggest an association between A625C and ATI, but not between A625C and LTI. First, the most stringent Fisher's exact test shows that the heterozygosity (AC status) is strongly associated with resistance to ATI (OR 0.19; P= 6.0×10^{-12}, Table 7), and second, the association remains strong even when both homozygous classes are pooled (OR 0:22; P= 1.8×10^{-10}, Table 7), and thirdly, the site A625C did not affect the susceptibility to the LTI (OR 0.83; P= 0:36 and 0:40; Table 7). The binomial logistic regression has supported these conclusions (Table 9).

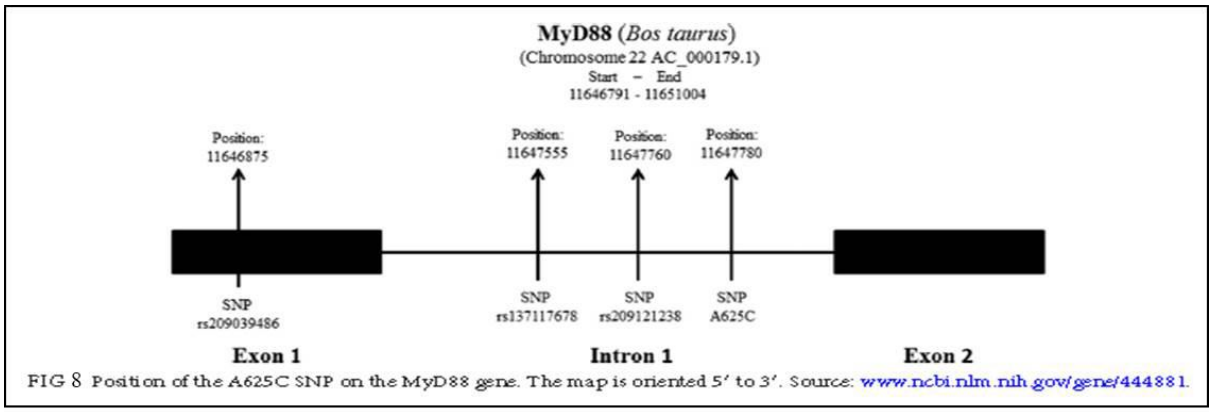


FIG 8 Position of the A625C SNP on the MyD88 gene. The map is oriented 5' to 3'. Source: www.ncbi.nlm.nih.gov/gene/444881.

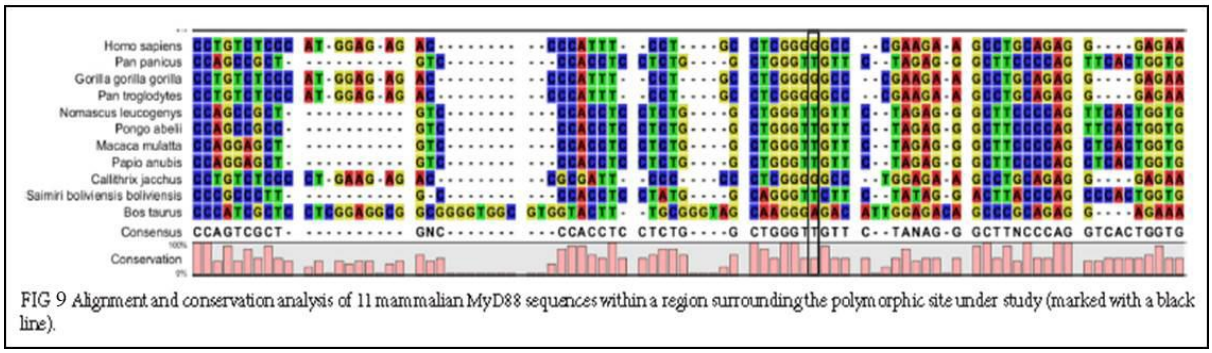


FIG 9 Alignment and conservation analysis of 11 mammalian MyD88 sequences within a region surrounding the polymorphic site under study (marked with a black line).

TABLE 8 Nucleotide motifs overrepresented in the bovine intron 1 sequence[a]

Sequence[b]	Searched consensus sequence	Counts within the *Bos taurus* genome
GGGTAGCAAGGG<u>A</u>GACA	GGGNVNVDDDSSHSACA	5
GGG<u>A</u>GACATTGGAGACA	GGGNVNVDDDSSHSACA	5
AGGG<u>A</u>	AGGGA	7
AGGG<u>A</u>GACAT	AGGGRBVCAT	3

[a] Motifs were identified using the SCOPE motif finder (http://genie.dartmouth.edu/scope/). All strands were plus strands, and 100% coverage was achieved for all sequences.
[b] The polymorphic nucleotide is underlined.

TABLE 9 Heterozygosity at the A625C SNP and resistance to active pulmonary tuberculosis shown by binary logistic regression

Reference genotype	TB[a]	Binary logistic regression analysis result: Wald	*P*	$\hat{e}^{\beta}$	H-L *P*[c]
AA	Active	40	1.8×10^{-10}	0.19	1
	Latent	0.78	0.37	0.83	1
CC	Active	0.01	0.91	1	1
	Latent	0.68	0.40	0.74	1

[a] TB, *M. bovis* pulmonary tuberculosis infection.
[b] Odds ratios estimated by the binary analyses.
[c] The nonsignificance of the Hosmer-Lemeshow (H-L) *P* value indicates that the model predicted by the logistic regression fits the observed data.

The heterozygosis at the MyD88 locus and inflammation

TNF-α, IFN-ɣ and NOS profoundly affect tuberculosis (Scanga, Bafica et al. 2004). It is also known that high or low levels of inflammation have a negative impact on tuberculosis (Glickman and Jacobs 2001, Doherty and Arditi 2004, Fremond, Yeremeev et al. 2004). Thus, if the heterozygous to MyD88 shows an intermediate level of cytokines than the homozygous, the association between A625C and resistance to the *Mycobacterium bovis* acquires a strong biological plausibility. To validate this hypothesis, we measured the mRNA levels of TNF-α, IFN-ɣ and NOS in subjects with different genotypes (AA, AC, CC) and status (controls or animals with ATI or LTI) (6 classes; 5 animals/class). The expression levels of the subjects with ATI or LTI were compared with those of control subjects with the same genotype. The heterozygous carrier expressed levels of TNF-α, IFN-ɣ and NOS significantly lower than those expressed by the AA homozygotes. In contrast, the heterozygous show levels only slightly higher than those expressed by the homozygous CC, in this case, the difference between them did not reach statistical significance (Figure 10). A possible explanation for this is that the technique used for the quantification of mRNA levels does not discriminate below a determined threshold. Taken together, these data support the conclusion that an optimal inflammatory response is associated with the phenotype of A625C.

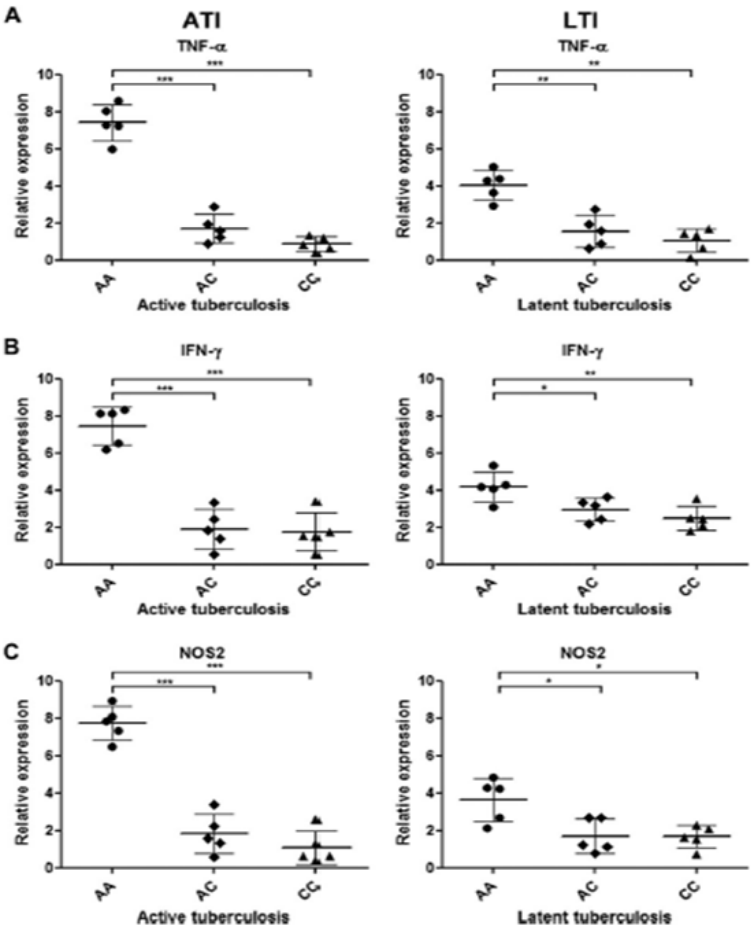


FIG 10 Proinflammatory cytokine mRNA levels measured by the TaqMan gene expression assay. Specimens were from controls or animals with active (ATI) or latent (LTI) *M. bovis* infection. Animals were grouped according to genotype (AA, AC, or CC) and class (controls, subjects with ATI or LTI). Each group consisted of 5 subjects. (A to C) Levels of TNF-, IFN-, and NOS mRNA expression, respectively. Relative sample quantification was carried out by the comparative $2^{-\Delta\Delta CT}$ method. The endogenous control was the -actin gene.

Discussion and Conclusions

The present study has shown that in heterozygous animals at the polymorphic site MyD88 have a reduced risk of about 5 times than the ATI (OR 0.19; P= $6.0x10^{-12}$, Table 7). However, the reduction in risk does not extend to the animals with LTI (OR 0.83, P= 0:36 and 0:40; Table 7). The heterozygosity at A625C is associated with intermediate levels of TNF-α, IFN-ɣ and NOS (Figure 10). Also, the study showed differences in the expression of cytokines mRNA between animals of the same genotype, but with acute or latent tuberculosis. The difference is particularly evident in animals AA (Figure 4). Unfortunately, we cannot attribute these different levels of expression at the pathogen or at the host; however, the levels of cytokines are potential markers for the reactivation of the disease. The A625C polymorphism, which located on the intron 1 of the gene MyD88, adds evidence that non-coding regions may affect genes expression. It is not surprising that this happens in the case of inflammation, which needs to be subject to a fine and complex regulation. In cattle, environmental exposure to the mycobacteria, which occur in the majority of subjects, interferes with the diagnosis of TB by TST or IFN- ɣ assay (Hope, Thom et al. 2005). The availability of reagents, incubation timing, and the levels of cut-off can affect the specificity and sensitivity of these assays (Pai, Riley et al. 2004). The bacteriological test post-mortem still remains, to date, the "gold standard" for the diagnosis of this infection (Thacker, Harris et al. 2011). Environmental factors (climate, density farming, herds' movements, etc.) are known as factors that promote tuberculosis (Neill, Skuce et al. 2005). Even strong genetic effects on mycobacteria may be missed if it does not take into account the environmental effects (Schurr 2007). Considerable OR and P value (OR = 0:19; P= $6.0x10^{-12}$) reported in this study make us cautiously optimistic about the possibility of correctly approaching the genetic analysis of this complex disease. The cases were made homogeneous (cases ATI and LTI were individually analyzed), the "environmental confounders" were excluded (gender and race) or "randomized" (age). More important is that the controls come from the same breeding of cases, however, remain infection-free (negative to the PCR and bacteriological test) despite having the same probability of becoming infected as in the cases. Further, in the present study, we investigated only one race. Additionally, the same results from two independent samples of populations offer considerably convincing evidence that there has not been stratification. In conclusion, the high biological relevance of the gene to study, the careful choice of diagnostic criteria, and randomization of environment confounders had been carefully taken into great consideration during this journey in the complex field of association studies. However, because the association is described for the first time, the results of this study are to be considered as preliminary. Finally, the test used here to distinguish between latent and active disease could potentially be extended to periodic testing of cattle for tuberculosis. The counting of dormant mycobacteria awakened by RpfB in milk samples would be an easy way to assess the incidence of latent tuberculosis in the population tested, a parameter strongly influenced by the control of the pathogen from the host's immune system.

Comment

Tuberculosis is influenced by many genes that interact with each other (Chang, Linderman et al. 2008) and the environment (Schurr 2007) . The presence of mycobacteria is necessary, but not sufficient to acquire the disease (Diamond 1987). Often, the stratification of the population is considered as responsible for the false-positive results obtained from association studies, but rarely has been proven to be guilty (Risch 2000, Colhoun, McKeigue et al. 2003). Human studies have shown that the stratification could arise when different ethnic groups are mixed (Healy 2006). The genetic association studies are characterized by a high rate of false-positive results (Risch 2000). This conclusion is often due to the selection of candidate genes without a functional relationship with the disease (Lander and Schork 1994, Risch 2000). In the present studies, MyD88 and TIRAP in the human and MyD88 in bovine, have been selected on the basis of a large number of experimental evidences. These studies show, at least in the mouse, the importance of gene conserved in the "signaling" downstream of the detection of the mycobacterial components and induction of immune response (Doherty and Arditi 2004, Fremond, Yeremeev et al. 2004). In conclusion, these studies have clarified that - as hypothesized – the host's genotype influences strongly the type of infection.

Materials and method

Genotyping

DNA was extracted from human blood samples by the phenol-chloroform method (Sambrook J, 1989). The quality of DNA was assessed by run on agarose gel and by Nanodrop (ratio 260/280 and 260/230 nm). The PCR was performed using the One-Step Real-Time PCR system and the TaqMan universal PCR master mix from Applied Biosystems (Life Technologies, Monza, Italy). The PCR program included one step at 50 ° C for 2 min, one at 95 ° C for 10 min and 40 cycles at 92 ° C for 15 s and 60 ° C for 1 min. The probes used for the gene MyD88 are: rs2585635, rs41285117, rs2200055, rs6853, rs7744; for gene TIRAP: rs646005, rs8177352, rs625413, rs614700, rs8177374.

Measurement of levels of TNF-α and IFN-ɣ by ELISA

Cytokine levels were determined before the blood donors were genotyped. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood on Ficoll-Hypaque gradient (Sigma, Milan, Italy), centrifuged (400g, 30 min), washed with RPMI 1640, distributed (10^5 PBMCs per well) on a 96-well plate (Falcon, Milan, Italy) and incubated with heat-killed *Mycobacterium tuberculosis* strain H37/Rv (10^6 CFU per well) for 48 hours (the optimal number of *Mycobacterium tuberculosis* to be used in the assay was set by a preliminary dose-response experiments). The supernatant was centrifuged (2000g, for 5 min) and added (50 µl per well) in a 96-well plate previously coated with mouse anti-human TNF-α- mouse or anti-human -IFN-ɣ (BDPharmingen, Milan, Italy; 50 µl diluted to $2x10^3$ per well, incubated for 4h) and quenched with 3% BSA (100 µl per well, 2h). Following incubation of the supernatant for 4h, the plate was washed with PBS and incubated with: mouse anti-human TNF-α or mouse anti-human-IFN-ɣ 50 µl diluted $2x10^3$ per well; 4h); subsequently it was used rat-anti mouse bounding the peroxidase diluted 10^3 (50 µl per well, 2h) and TMB peroxidase substrate (100 µl per well; Biorad, Milan, Italy). The optical density was measured at 405 nm. The samples were performed in triplicate.

Nitric oxide quantification

The PBMCs were incubated with heat-killed *Mycobacterium tuberculosis* strain H37/Rv (10^6 CFU per well) for 48 hours, centrifuged and the supernatant (100 µL) was mixed with 100 µl of Griess reagent (10 min at Room Temperature). The optical density was measured at 570 nm. The concentration of nitrite was measured using as a standard of 1, 10, 25 and 50 µM of a solution of sodium nitrite.

Bioinformatics and statistical analysis

OR and 95% confidence intervals were calculated with the Fisher's exact test using the statistical package GraphPad Prism version 5 (GraphPad). Sample Size was calculated with the module OpenEpi 3.03.17 and the Hardy- Weinberg equilibrium was calculated using the Hardy-Weinberg equilibrium calculator (http://www.oege.org/software/HWE-mr-calc.shtml). The Mantel-Haenszel test, logistic regression analysis coupled with resampling (2000 samples and 99%

confidence intervals), the generalized linear model (Garrett, Madden et al. 2004) and ANOVA with HSD test of Tuckey's post hoc analysis were performed using the statistical package SPSS version 19 MyD88 and TIRAP orthologs were retrieved by BLAST analysis (blast.ncbi.nlm.nih.gov/). Alignments were performed with the software MAFFT v7.045b (http://mafft.cbrc.jp/allineamento/software) and maps CLC Main Workbench 6.8.2 software. Mutations in the TIRAP and protein function was predicted by SIFT (http://sift.jcvi.org/) (Kumar, Henikoff et al. 2009) and I-TASSER tools (http://zhanglab.ccmb.med .umich.edu/I- TASSER) (Roy, Kucukural et al. 2010). The human sequence 3'UTR of MyD88 gene was scanned for the identification of regulatory elements using the Encyclopedia of DNA Elements (ENCODE; http://encodeproject.org/ENCODE/).

Diagnosis of pulmonary infection of bovine

The samples were collected post-mortem in accordance with the guidelines set out by the European Food Safety Authority (Serratosa, Arbelot et al. 2004). A portion of the infected tissue and afferent lymph node were removed from animals which showed macroscopic lung lesions. In the case of animals without visible lesions were collected mediastinal, bronchial and retropharyngeal lymph node. Samples from each animal consisted of 1 gr or more of tissue. To distinguish between subjects with active pulmonary tuberculosis (ATI) and latent pulmonary tuberculosis (LTI), were made serial dilutions (10^{-1} to 10^{-8}) of individual lung homogenates in sterile PBS and plated (10 µL/ spot; 5 spots/dilution) on agar-Middlebrook (MB) and incubated at 37 ° C for 4 to 5 weeks. At the end of the incubation period were counted colonies forming units (CFU). Negative samples were further incubated for 10 days in liquid medium (MB) supplemented with mycobacterial resuscitation-promoting factor B (RpfB) (5 gr/ mL) (Ruggiero, Tizzano et al. 2007) and then counted the CFU. The optimal concentration of RpfB used in the test was obtained through preliminary experiments. The growth of colonies in the absence of RpfB was indicative of ATI, and colony growth only in the presence of RpfB was indicative of LTI. The controls were negative for both tests (Figure 6).

The identification of mycobacterial species by PCR analysis.

A colony of *Mycobacterium bovis* was resuspended in 200 µl of distilled H_2O containing lysozyme (20 mg/ml, Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C for 2h. After the incubation time, the DNA was isolated using a DNeasy-kit of Qiagen (Hilden, Germany). The PCR was performed as previously described (Bakshi, Shah et al. 2005) (Figure 7).

Cases and Controls

The animals included in the study, both cases and controls, come from three herds declared to be infected. sex and age as potential confounders, the animals were all lactating cows between 40 and 90 months of age. This age interval was selected to represent subjects matched for age (as much as it was realistic) and, at the same time, a population sample sufficiently numerous to provide adequate power to the study. The average ages of the cases and controls were 65.4±5.2 and 69.6±3.9 months, respectively. To curb stratification, both cases and controls were from the same herds and the same breed (Friesian); to keep cases and controls genetically

unrelated to each other, when mother and daughter were present, one of the two was excluded.

MyD88 genotyping

The intron/exon boundaries of the bovine MyD88 gene were established by matching the published mRNA sequence of the bovine MyD88 gene (GenBank accession number NM_001014382.2) and the DNA sequence of the human MyD88 gene (GenBank accession number NC_000003.11). Alignment was carried out using DNAsis software (Hitachi Solutions America, San Francisco, CA). DNA was extracted from lung specimens with a QIAamp DNA kit (Qiagen, Hilden, Germany). The primers were 5'-TGAAGGAGTACCCCGCGC-3' (forward) and 5'-GATGCCTGCCATGTCATT-3' (reverse). Conditions of the PCR were 7 min at 97°C and then 45 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C (35 cycles), with a final extension for 5 min at 72°C. The 1,210-bp fragments from 20 cases and 20 controls were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and aligned by use of Chromas software (Technelysium, Queensland, Australia). The sequences were used to design primers and TaqMan probes targeting specifically the single nucleotide polymorphism (SNP) located 625 bp downstream of exon 1 (A625C). The forward and reverse unlabeled primers were 5'-GGTGGCGTGGTACTTTGC-3' and 5'-TTTCTCCTCTACGGGCTGTCT-3=, respectively. The TaqMan VIC- and 6-carboxyfluorescein-labeled probes were 5'-TAGCAAGGGAGACATT-3' and 5'-TAGCAAGGGCGACATT-3', respectively, where the underlining and boldface indicate the polymorphic nucleotide. PCR conditions were 30 s at 60°C, 10 min at 95°C, and then 40 cycles each lasting 15 s at 95°C and 1 min at 60°C. Genotyping was carried out with the investigator blinded to the case or control status of the animals being tested.

TaqMan gene expression assay

TNF-α, IFN-ɣ, and NOS2 mRNA levels in the lung specimens were measured using the TaqMan gene expression assay and a StepOne instrument (Applied Biosystems, Foster City, CA). Total RNA (2µg) from mediastinal, bronchial or retropharyngeal lymph node was reverse transcribed using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The real-time quantitative PCRs were carried out following the manufacturer's protocol. The identification numbers of the probes are Bt03259155_g1 (TNF-α bovine), Bt03212722_g1 (IFN-ɣ), Bt03249602_g1 (NOS2), and Bt03279175_g1 (α-actin). Five animals for each genotype (AA, AC, CC) and class (control, active and latent tuberculosis) were tested in triplicate. Relative sample quantification was carried out by the comparative $2^{-\Delta\Delta CT}$ method (where CT represents the threshold cycle). The endogenous control gene was α-actin. The amplification efficiency of target (TNF-α, IFN-ɣ, and NOS) and reference (α-actin) genes was approximately the same (slope <0.1).

Sample size calculation

The data for 50 cases with active tuberculosis and 50 controls (odds ratio [OR], 0.3; proportion of controls with susceptible genotype, 0.46) showed that a sample of 127 cases and 127 controls would provide 96% power and a two-sided significance level

of 0.01. The study enrolled 150 animals with acute tuberculosis, 150 animals with latent tuberculosis, and 300 controls.

Other methods

Logistic regression

The binomial logistic regression analysis is used in epidemiology to predict the outcome (variable dependent) on the basis of a set of descriptive variables (independents), both qualitative and quantitative. The dependent variable is measured with value of “yes” or “no” (dichotomy variable) and describes the outcome occurring of random event. Logistic model estimates the logit transformation of probability of an event occurring (disease) on the basis of other variables (e.g. age, gender, SNP etc.). The output of logistic regression is confined to values between 0 and 1and hence it is interpreted as probability:

$$\textbf{Logit (P)} = \ln\left(\frac{P}{1-P}\right) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_n X_n$$

Where **P**= Probability of condition of interest (disease). **α** is the intercept; Coefficient **β** (and its standard errors and significance level) estimates how quickly the probability changes with changing X a single unit; **X** represents independent variables along with other covariates, ***e*** is the base of the natural logarithm (about 2.718). The logit transformation works as a link function between the probability and the linear regression expression. Fisher’s exact test and analysis of variance with the Tukey post hoc test were performed with GraphPad Prism software, version 5. Binary logistic regression was performed with the SPSS statistical package, version 18. Hardy-Weinberg equilibrium and relative risk reduction were calculated as described previously (Modiano, Luoni et al. 2001). Conservation analysis was carried out on 11 MyD88 genomic sequences retrieved from the Nucleotide BLAST database (blast-.ncbi.nlm.nih.gov/). The alignment was performed using the T-COFFEE multiple-sequence alignment server (tcoffee.crg.cat/) and map visualized with CLC Main Workbench software, version 6.8.2. The bovine intron 1 sequence was scanned for identification of overrepresented motifs by using the SCOPE (Suite for Computational identification of Promoter Elements) motif finder (http://genie.dartmouth.edu/scope/). Regulatory elements were searched for using the Encyclopedia of DNA Elements (ENCODE; http://encodeproject.org/ENCODE/).

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Publication

- Rosanna Capparelli, **Francesco De Chiara**, Antonio Di Matteo, Chiara Medaglia, Domenico Iannelli. “The MyD88 rs6853 and TIRAP rs8177374 Polymorphic Sites are Associated with Resistance to Human Pulmonary Tuberculosis”. **Genes Immun. 2013** Dec; 14(8):504-11.

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Laboratories

Dates (from – to)	**01 January 2013 – to date**
Occupation or position held	Visiting PhD Student
Main activities and responsibilities	“Establishing Stable inflammatory Cell Lines”
Name and address of employer	Royal Free Hospital - University of College of London
Dates (from – to)	**20 September 2012 – 31 December 2012**
Occupation or position held	PhD Student
Main activities and responsibilities	“MyD88 and TIRAP allelic interaction against Pulmonary TB
Name and address of employer	Immunology laboratory - University of Naples “Federico II”
Dates (from – to)	**19 June 2012 – 19 September 2012**
Occupation or position held	Visiting PhD Student
Main activities and responsibilities	Role of α-adrenergic receptors in human Infection Disease
Name and address of employer	Medicine division University of College of London
Dates (from – to)	**13 April 2011 – 18 June 2012**
Occupation or position held	PhD Student
Main activities and responsibilities	“MyD88 and TIRAP allelic interaction against Pulmonary TB
Name and address of employer	Immunology laboratory - University of Naples “Federico II”

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ORIGINAL ARTICLE

The MyD88 rs6853 and TIRAP rs8177374 polymorphic sites are associated with resistance to human pulmonary tuberculosis

R Capparelli, F De Chiara, A Di Matteo, C Medaglia and D Iannelli

Toll-like receptors recognize several components of *Mycobacterium tuberculosis*, the main causative agent of tuberculosis. The signaling pathways leading to activation of the immune response require the MyD88 and TIRAP genes. The hypothesis that polymorphic variants of these genes influenced resistance to pulmonary tuberculosis was tested by a case–control study (400 cases and 400 controls). Heterozygosity at the polymorphic sites MyD88 rs6853 (alleles: A, G) or TIRAP rs8177374 (S180L) (alleles: C, T) is associated with resistance to pulmonary tuberculosis (P: 7.8×10^{-8} and 2×10^{-6}, respectively). Double heterozygosity confers higher protection levels (P: 10^{-14} to 2×10^{-16}). The logistic regression model displayed that the double homozygous genotype GG/TT predisposes to the disease (odds ratio (OR): 5.78) and the AG/TT genotype combination neutralizes the protective activity exerted by AG (OR: 3.05). The same model showed that the risk of developing the disease increases with age from 31–40 years to 71–80 years (OR: 1.32–13.59).

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Keywords: MyD88; TIRAP; *Mycobacterium tuberculosis*; gene interaction

INTRODUCTION

Toll-like receptors (TLRs) are pattern recognition receptors (PRR), which sense invading pathogens by recognizing pathogen-associated molecular patterns (PAMPS). TLRs recognize several PAMPS of the *Mycobacterium tuberculosis*, such as the 19 kD lipoprotein (19LP), soluble tuberculosis factor (STF), lipomannan (LM), lipoarabinomannan (LAM) and the heat shock protein 65 (HSP65). In the presence of adapter proteins, PAMPs recognition initiates signaling pathways that lead to activation of the factor NF-kB and induction of a rapid immune response, innate and adaptive. Sensing of the mycobacterial DNA requires TLR-9 and the adapter protein myeloid differentiation factor 88 (MyD88); 19LP, LM, LAM and STF require TLR-2 and MyD88, whereas the HSP65 requires TLR-4, MyD88 and the TIR domain-containing adapter protein (TIRAP; also known as Mal).[1] The MyD88 and TIRAP genes map to chromosomes 3p21.3-p2[2] and 11q24.2,[3] respectively. MyD88-deficient mice ($MyD88^{-/-}$) display defective production of TNF-α, IL-12 and NO, and upon infection with *M. tuberculosis* die within 4 weeks.[4] $TIRAP^{-/-}$ mice instead can efficiently control *M. tuberculosis* infection.[5] The TIRAP single-nucleotide polymorphism C558T is associated with susceptibility to meningeal tuberculosis[6,7] and the single-nucleotide polymorphism S180L (rs8177374) of the same gene with protection against malaria, tuberculosis, bacteremia and invasive pneumococcal disease.[3] Subsequent studies however did not confirm the protective role of S180L against tuberculosis.[8,9] One more study[10] reported that neither TIRAP (rs352165 and rs352167) nor MyD88 (rs4988457 and rs6767684) genes influence tuberculosis. The discrepancies that emerged when these genes—or even the same single-nucleotide polymorphism (rs81777374)[3,9]—were studied in different ethnicities intrigued the present authors, who decided to investigate the association of MyD88 and TIRAP with pulmonary tuberculosis in a sample of Italian population. Here they show that in this population heterozygosity at the polymorphic sites MyD88 rs6853 or TIRAP rs81777374 is associated with resistance to pulmonary tuberculosis. The two genes cooperate or antagonize each other, depending upon the allelic combination.

RESULTS

Study design

To reduce the type I error and the drawback of multiple comparison corrections, a two-stage study was followed. The first stage was limited to 100 cases and 100 controls and it included the polymorphic sites TIRAP rs8177374 and MyD88 rs6853. The rs8177374 site was included because in previous studies it yielded contrasting results;[3,9] the rs6853 site was included because it was the only one, among the five tested, to display a frequency of the rarer allele >0.05. Both polymorphic sites displayed a statistically significant association with the disease (P-value <0.05) (Table 1). Based on the data from this preliminary study (MyD88: odds ratio (OR): 0.40 and proportion of controls with susceptible genotypes: 0.61; TIRAP: OR: 0.48 and proportion of controls with susceptible genotypes: 0.64), it was calculated that a sample of 185 cases and 185 controls (in the case of MyD88) and 313 cases and 313 controls (in the case of TIRAP) would provide 96% power and a two-sided significance level of 0.01. The second stage of the study was conducted on 400 cases and 400 controls (independent from cases and controls included in the preliminary test). To limit the study to a homogeneous disease spectrum, cases were all patients with a clinical diagnosis of pulmonary tuberculosis, confirmed by chest X-ray, bacteriological and PCR-positive tests. The bacteriological test was carried out on sputum samples. Cases were all treated at the Monaldi hospital (Naples), the southern Italy reference center for tuberculosis. Controls were household contacts (spouses or husbands of patients) without clinical signs of tuberculosis

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(negative to the interferon test). Thus, controls were genetically unrelated subjects, who remained healthy though exposed (>2 years) to the pathogen. Criteria for definition of cases and controls were the same for both stages of the study. Cases and controls were collected over a 5-year time period. In this time interval six controls became positive to the interferon test and were excluded. Cases consisted of 258 males and 142 females (mean age: 50 ± 19 years); controls consisted of 222 males and 178 females (mean age: 49 ± 17 years). The study was approved by the ethic committee of the Monaldi hospital. Informed consent was obtained from all subjects who participated to the study.

Heterozygosity is associated with protection against pulmonary tuberculosis

Genotype frequencies at the rs6853 and rs8177374 markers were in Hardy–Weinberg equilibrium among controls but not among cases (Table 2), as expected if the single-nucleotide polymorphisms and pulmonary tuberculosis were associated. The association was confirmed by Fisher's exact test (Table 2). At both loci, heterozygosity (the AG or CT status) was associated with protection from pulmonary tuberculosis; protection persisted when the two classes of homozygotes were pooled (AG vs AA + GG or TC vs TT + CC) (Table 2).

Age is a known risk factor for pulmonary tuberculosis.[11] Therefore, ORs associated with different genotypes were corrected from the age confounding effect by the Mantel-Haenszel test (Table 3). After correction, double heterozygosity (AG/CT) still afforded a higher level of protection (OR: 0.16) compared with single heterozygosity (AG, OR: 0.41; CT, OR: 0.42) (Table 3). To know how the age risk varied across strata, the data were then re-analyzed according to the logistic regression model. The analysis was restricted to the numerically more representative

Table 1. Association of the MyD88 rs6853 and TIRAP rs8177374 polymorphic sites with pulmonary tuberculosis. Exploratory study in an Italian population sample

Genes	*Status*	*Number of individuals in each genotype*			*Total*	*HWE (P)*	*Allelic frequency*		*OR (95% CI)*[a]	P-*value*[a]
							Co	*Ra*		
		AA	AG	GG					AG vs (AA + GG)	
MyD88	Cases	73	21	6	100	0.017	0.84	0.16	0.40 (0.22–0.77)	0.008
	Control	48	39	13	100	0.267	0.68	0.32		
		CC	CT	TT					CT vs (CC + TT)	
TIRAP	Cases	76	22	5	103	0.058	0.84	0.16	0.48 (0.25–0.90)	0.029
	Control	60	36	4	100	0.624	0.78	0.22		

Abbreviations: Co, common allele (MyD88: A; TIRAP: C); Ra, rare allele (MyD88: G; TIRAP: T). [a]CI (confidence intervals) and *P*-values were calculated with the Fisher's exact test.

Table 2. Association of the MyD88 rs6853 and TIRAP rs8177374 polymorphic sites with pulmonary tuberculosis

	Genes	*Status*	*Number of individuals in each genotype*			*Total*	*HWE (P)*	*Allelic frequency*		*OR (95% CI)*[a]		*OR (95% CI)*[a]	*P-value*[a]
								Co	*Ra*				
Italian			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	297	89	14	400	0.029	0.85	0.15	0.40 (0.28–0.55)	5.8×10^{-9}	0.42 (0.31–0.59)	7.8×10^{-8}
		Control	213	160	27	400	0.68	0.73	0.27				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	318	71	11	400	0.006	0.88	0.12	0.44 (0.31–0.62)	1.2×10^{-6}	0.44 (0.31–0.63)	2×10^{-6}
		Control	258	130	12	400	0.36	0.81	0.19				
Romanian			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	104	34	12	150	8×10^{-4}	0.81	0.19	0.44 (0.26–0.74)	0.003	0.47 (0.28–0.79)	0.005
		Control	78	57	15	150	0.34	0.71	0.29				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	100	40	10	150	0.04	0.8	0.2	0.55 (0.33–0.90)	0.01	0.53 (0.32–0.86)	0.01
		Control	84	61	5	150	0.12	0.76	0.24				
Ukrainian			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	117	27	6	150	0.01	0.87	0.13	0.45 (0.26–0.78)	0.004	0.48 (0.28–0.82)	0.01
		Control	92	47	11	150	0.16	0.77	0.11				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	119	26	5	150	0.03	0.88	0.12	0.41(0.24–0.71)	0.002	0.42 (0.24–0.71)	0.002
		Control	95	50	5	150	0.61	0.8	0.2				
All ethnicities			AA	AG	GG					AG vs AA		AG vs AA + GG	
	MyD88	Cases	518	150	32	700	5×10^{-6}	0.85	0.15	0.42 (0.32–0.53)	8×10^{-13}	0.45 (0.35–0.57)	3×10^{-11}
		Control	383	264	53	700	0.42	0.74	0.26				
			CC	CT	TT					CT vs CC		CT vs CC + TT	
	TIRAP	Cases	537	137	26	700	1.8×10^{-5}	0.87	0.13	0.46 (0.35–0.59)	5.3×10^{-10}	0.46 (0.36–0.69)	4.6×10^{-10}
		Control	437	241	22	700	0.10	0.80	0.20				

Abbreviations: Co, common allele (MyD88: A; TIRAP: C); Ra, rare allele (MyD88: G; TIRAP: T). [a]CI (confidence intervals) and *P*-values were calculated with the Fisher's exact test.

Table 3. Age confounding effect assessed by weighted Mantel–Haenszel test

	MyD88			*TIRAP*			*MyD88/TIRAP*								
	AA	*AG*	*GG*	*CC*	*CT*	*TT*	*AA/CC*	*AA/CT*	*AA/TT*	*AG/CC*	*AG/CT*	*AG/TT*	*GG/CC*	*GG/CT*	*GG/TT*
OR	2.65	0.41		2.27	0.42		1.99	2.12			0.16				
CI	(1.9–3.6)	(0.3–0.6)	n.s.	(1.6–3.2)	(0.3–0.6)	n.s.	(1.5–2.7)	(1.7–3.6)	n.s.	n.s.	(0.1–0.3)	n.s.	n.s.	n.s.	n.s.
P	2.8×10^{-10}	1.8×10^{-8}		7.6×10^{-7}	6.5×10^{-7}		3.2×10^{-6}	5.5×10^{-3}			1.3×10^{-12}				

Abbreviations: CI, 95% confidence interval; OR, odds ratio; P, significance level; n.s., non significant.

Table 4. Influence of age on pulmonary tuberculosis assessed by the logistic regression model procedure, which includes 2000 bootstrap re-sampling

Regression term	*OR*	P-*value*
Age class		
31–40 vs 21–30	0.31	5.0×10^{-4}
41–50 vs 31–40	1.32	0.29
51–60 vs 41–50	8.71	5.0×10^{-4}
61–70 vs 51–60	13.59[a]	5.0×10^{-4}
71–80 vs 61–70	1.38	0.27
Constant	0.63	2.0×10^{-3}

Abbreviation: OR, odds ratio estimated by the logistic regression model. [a]Example of OR calculation. OR of people aged 65 years compared with people belonging to the 51–60 years category is 13.59 (61–70 vs 51–60).

Table 5. Influence of the MyD88 and TIRAP genotypes on pulmonary tuberculosis assessed by the logistic regression model procedure, which includes 2000 bootstrap re-samplings

Regression term	*OR*[a]	P-*value*
MyD88		
MyD88 (AG vs AA)	0.50	0.01
MyD88 (AG vs GG)	0.64	0.09
MyD88 (GG vs AA)	0.78	0.41
TIRAP		
TIRAP (CT vs CC)	0.58	0.03
TIRAP (CT vs TT)	0.80	0.45
TIRAP (TT vs CC)	0.73	0.29
Effects between loci		
MyD88(AG) by TIRAP(CT)[b]	0.09	5.0×10^{-4}
MyD88(AG) by TIRAP(TT)[b]	3.05	0.13
MyD88(GG) by TIRAP(CT)[b]	0.77	0.71
MyD88(GG) by TIRAP(TT)[c]	5.78	0.02
MyD88(AA) by TIRAP(CC)[c]	5.78	0.03
MyD88(AA) by TIRAP(CT)[c]	7.46	1.5×10^{-3}
MyD88(AG) by TIRAP(CC)[c]	1.90	0.34
Constant	0.63	2.0×10^{-3}

Abbreviations: OR, odds ratio estimated by the logistic regression model; vs, within locus comparisons; by, between loci interactions; /, reference genotype (note C) is double heterozygous. [a]Double heterozygotes (AG/CT), compared with double homozygotes (AA/CC) have the OR: 0.5 (AG vs AA) x 0.58 (CT vs CC) x 0.09 (AG by CT) = 0.026. [b]The reference genotype is MyD88(AA)/TIRAP(CC). [c]The reference genotype is MyD88(GG)/TIRAP(TT).

age categories, consisting of $\geqslant 45$ subjects. Apart from the category 31–40 years vs 21–30 years (apparently protected, $P = 0.31$), the model showed that the risk of developing the disease increases with age from the 31–40 years to the 71–80 years categories, as shown by the steadily increasing ORs (from 1.32 to 13.59) (Table 4). Clearly, aging—and possibly aging-associated covariates (smoking, diabetes and prolonged exposure to the pathogen)—can overcome genetic resistance. The authors did not have complete access to the clinical records of the patients and therefore could not investigate the role of these potential confounders.

The logistic regression model was also used to dissect the contribution of single genotypes and their interactions with the ORs. Particularly evident is the interaction between the AG and CT genotypes (estimated OR: 0.09) and in the opposite direction the interactions between the GG and TT, AA and CC, and AA and CT genotypes (OR: 5.78, 5.78, 7.46) (Table 5).

The association resists stratification

The study was extended to one sample of people from Romany and one from Ukraine. The study, though underpowered, confirmed the protective role of the heterozygous status at the rs6853 ($P = 0.003$–0.004) and rs8177374 ($P = 0.01$–0.02) sites (Table 2). The protective role persisted when the samples from Romany, Ukraine and Italy were pooled (rs6853: $P = 8 \times 10^{-13}$; rs8177374: $P = 5.3 \times 10^{-10}$) (Table 2). Thus, the association of rs6853 and rs8177374 with pulmonary tuberculosis extended to the several ethnicities tested in this study and resisted the artificial stratification determined by pooling different ethnicities.

Heterozygosity curbs inflammation

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers (nine genotypic groups; five samples/group) were stimulated with heat-killed *M. tuberculosis* strain H37Rv and the levels of TNF-α, IFN-γ and NO were then measured. In the AA genotype context, CT subjects displayed intermediate levels of TNF-α, IFN-γ and NO compared with CC or TT subjects. In the AG genotype context, the data showed the same tendency, though the differences between subgroups were not always significant. Further, the lowest cytokines levels were observed in the subjects carrying the GG genotype, as if the A allele favors cytokines production, whereas the G allele curbs it. On the whole, the data indicate that heterozygosity is associated with an intermediate level of cytokines and NO (Figure 1).

The experimental data were then analyzed by the generalized linear model. This methodology permits to study relationships between response variables (in the present study IFN-γ, TNF-α or NO) and a set of predictor variables (in the present study MyD88 and TIRAP) to extract patterns. In addition, the methodology can be applied to data that presumably are not normally distributed (as in the present case, where only a small number of blood samples was examined). The model calculated the parameters $\beta 1$, $\beta 2$ and $\beta 3$, which measure how MyD88, TIRAP and the MyD88–TIRAP interactions influence the levels of IFN-γ, TNF-α and NO. Phrased another way, the model quantified the relationship between predictor variables and their interactions with one response variable (the effect of the MyD88 or TIRAP genotypes on each

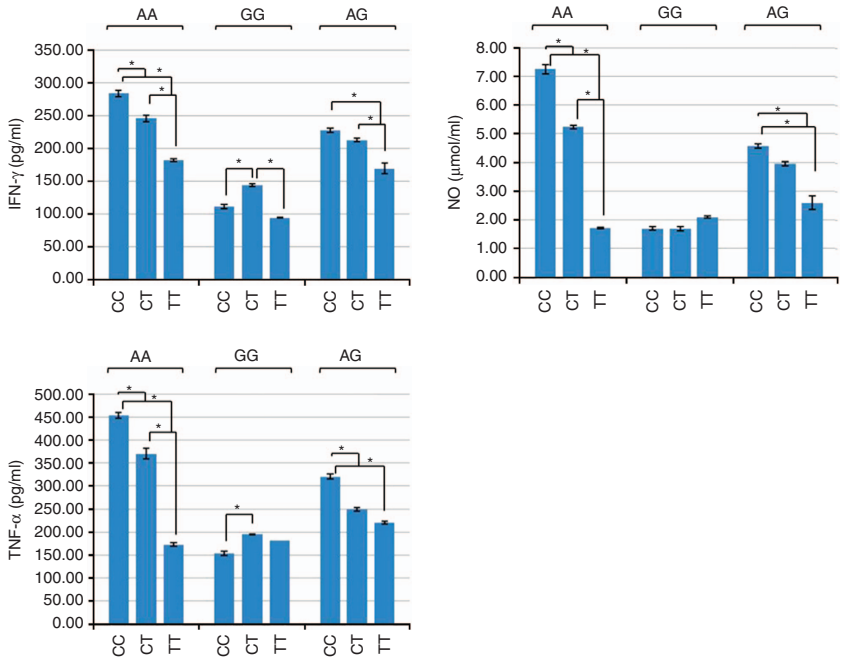

Figure 1. Effect of heterozygosity at the TIRAP or MyD88 loci on levels of TNF-α, IFN-γ and NO. The differences in cytokines and NO levels between the AA, GG and AG genotype subgroups were assessed by the ANOVA with Tukey's *post hoc* test coupled with bootstrap re-sampling (2000 samples and CI 99%). The asterisk indicates statistically significant differences ($P<0.001$).

cytokine). The model predicted the levels of the cytokines and NO of volunteers on the basis of their genotypes at the MyD88 and TIRAP loci, with surprising accuracy (Table 6). The predictive power of the model collapsed when the two loci were analyzed separately. This finding suggests that the two loci strongly cooperate to control the disease. This conclusion is strongly supported by the evidence that TIRAP and MyD88 form heterodimers.[12]

In silico analysis of polymorphic sites

The site rs6853 resides in the 3′UTR region of the MyD88 gene. Comparison of genomic sequences from different species showed that both the A and G nucleotides are conserved across species, suggesting that they are maintained—or at least tolerated—by natural selection (Figure 2). Further, the rs6853 was found to overlap with 4 ENCODE tracks (H3K4Me1, H3K4Me3, H3K27Ac and Broad Chromatin HMM marks) detected by Chromatin Immuno Precipitation Sequencing (ChIP-Seq) technique. This finding points out the potential of the rs6853 polymorphic site—though it is located in the 3′UTR—to influence the interaction of the MyD88 mRNA with protein factors.

The site rs8177374, residing on the exon 5 of the TIRAP gene, provided the opportunity to test whether the change of a serine to leucine at position 180 alters the protein structure. The amino acids were both frequently represented at position 180 among the TIRAP proteins from 22 mammalian species and the SIFT algorithm predicted that they both are compatible with protein activity (Figure 3). At the same time, the I-TASSER server predicted that the amino-acid change (S to L) at position 180 affects the secondary and tertiary structures of the TIRAP protein. This finding extends to the A and B isoforms originating from the same mRNA molecule by alternative splicing (Figure 4) and provides further ground to the biological plausibility of the polymorphism.

DISCUSSION

Very little is known about the crosstalk occurring among genes influencing pathogen resistance. In this article we explored how the genes MyD88 and TIRAP influence each other. The hypothesis that the two genes might interact seemed plausible, as the MyD88 and TIRAP proteins both act as signaling molecules of the TLRs.[1] We found that the two genes cooperate or antagonize each other, depending upon the allele combination. Heterozygosity at both loci provides stronger protection ($P=1.3\times10^{-12}$, age-corrected; Table 3) than heterozygosity at one single locus (P(MyD88) $=7.8\times10^{-8}$; P(TIRAP) $=2\times10^{-6}$, Table 2). At the same time, in AG/TT subjects the genotype TT neutralizes the protection afforded by the genotype AG (Table 5). These data, though limited, clearly show how the concurrent analysis of two or more genes working along the same pathway helps to better define the phenotype (show how independent genes may contribute to shape the same phenotype). The approach therefore can help to design case–control studies that are clinically more relevant (studies more likely to predict disease outcome). The evidence that the gene under study can be influenced by background genes can also contribute to explain why the same gene might be associated with protection to a pathogen in one population but not in another one.

In general, subjects heterozygous at the MyD88 or the TIRAP loci display intermediate levels of IFN-γ, TNF-α or NO (Figure 1). IFN-γ, TNF-α and NO have a major role against *M. tuberculosis* infection.[13–16] NO exerts strong anti-mycobacterial activity and, along with TNF-α, favors granuloma formation.[17] IFN-γ induces the production of NO,[16] expression of MHC II molecules and antigen presentation.[15] However, there is also evidence that overexpression of these molecules favors tuberculosis. Many of the symptoms of tuberculosis are caused by the immune response of the host, rather than by the mycobacterium.[18] Disease reactivation has been observed after therapeutic treatment with TNF-α[19] or in HIV-infected patients after antiretroviral therapy.[20] As deficiency as well as overexpression of TNF-α, IFN-γ or NO favor tuberculosis, the biological advantage of an intermediate level of pro-inflammatory cytokines and NO production becomes clear. This biological advantage is more manifest in the double heterozygotes (Table 5), showing that the crosstalk between MyD88 and TIRAP extends from the epidemiological level to the

Table 6. Effects of the MyD88 and TIRAP genotypes on IFN-γ, TNF-α and NO levels estimated by the Generalized Linear Model procedure

Genotype predictors	*[IFN-γ](pg ml)*				*[TNF-α](pg ml)*				*[NO](pg ml)*			
	Parameter (β)	P-value	*Predicted mean*	*Observed mean*[a]	*Parameter (β)*	P-value	*Predicted mean*	*Observed mean*[a]	*Parameter (β)*	P-value	*Predicted mean*	*Observed mean*[a]
Constant (k)	6.55	1.00×10^{-89}	na[b]		7.51	1.00×10^{-99}	na		1.07	1.00×10^{-82}	na	
MyD88 (β_1)[c]												
AA	0.95	1.00×10^{-78}	na	248.22 (a)	−0.07	9.43×10^{-2}	na	363.22 (a)	−0.29	4.16×10^{-7}	na	5.34 (a)
AG	0.85	1.00×10^{-99}	na	206.01 (b)	0.28	3.92×10^{-11}	na	258.92 (b)	0.30	3.13×10^{-7}	na	3.78 (b)
GG	0.00		na	112.29 (c)	0.00		na	172.33 (c)	0.00		na	1.83 (c)
TIRAP (β_2)[c]												
CC	0.23	3.69×10^{-87}	na	203.06 (a)	−0.25	3.21×10^{-12}	na	306.80 (a)	−0.31	1.00×10^{-94}	na	4.49 (a)
CT	0.61	1.00×10^{-69}	na	208.45 (a)	0.10	1.70×10^{-2}	na	274.80 (b)	−0.32	4.57×10^{-8}	na	3.88 (b)
TT	0.00		na	140.52 (b)	0.00		na	190.61 (c)	0.00		na	2.13 (c)
Effect between loci (β_3)[c]												
AA by CC	0.40	2.30×10^{-15}	280.14	283.33 (a)	1.63	1.00×10^{-77}	451.94	453.08 (a)	2.39	1.00×10^{-93}	7.26	7.24 (a)
AA by CT	−0.18	1.73×10^{-3}	243.88	245.55 (b)	0.99	1.00×10^{-85}	369.65	370.00 (b)	1.93	1.00×10^{-99}	5.24	5.23 (b)
AA by TT	0.00		181.02	182.00 (c)	0.00		173.65	173.33 (c)	0.00		1.72	1.71 (c, d, e)
AG by CC	0.20	4.12×10^{-4}	227.54	227.50 (b)	0.78	1.00×10^{-83}	319.57	320.50 (d)	1.13	1.00×10^{-81}	4.56	4.57 (f)
AG by CT	−0.28	2.93×10^{-7}	212.31	212.23 (d)	0.07	1.92×10^{-1}	249.00	249.53 (e)	0.93	1.00×10^{-87}	3.94	3.94 (g)
AG by TT	0.00		168.90	169.00 (c)	0.00		221.32	220.83 (e, f)	0.00		2.58	2.59 (h)
GG by CC	0.00		109.90	110.58 (e)	0.00		153.28	153.66 (c)	0.00		1.69	1.70 (c, i, j)
GG by CT	0.00		143.01	143.33 (f)	0.00		195.36	195.16 (c)	0.00		1.68	1.69 (d, i, k)
GG by TT	0.00		93.70	93.89 (e)	0.00		182.28	182.00 (c, f)	0.00		2.10	2.10 (e, h, j, k)

Within each dependent variable (IFN-γ, TNF-α and NO levels), the β_1, β_2 and β_3 parameters weigh the effect of predictors (MyD88 and TIRAP genotypes) on the dependent variable fitting the model $Y = 2^k + \beta1(\text{MyD88}) + \beta2(\text{TIRAP}) + \beta3(\text{MyD88*TIRAP})$. Abbreviation: na, not applied. [a]Different letters indicate statistically significant differences between genotypes at Tukey's HSD *post hoc* test ($P < 0.001$). [b]The model predicts cytokines and NO levels based on the MyD88 and TIRAP genotypes and their interactions. The model was not accurate when applied to one single genotype. [c]Redundant parameters are set equal to 0.

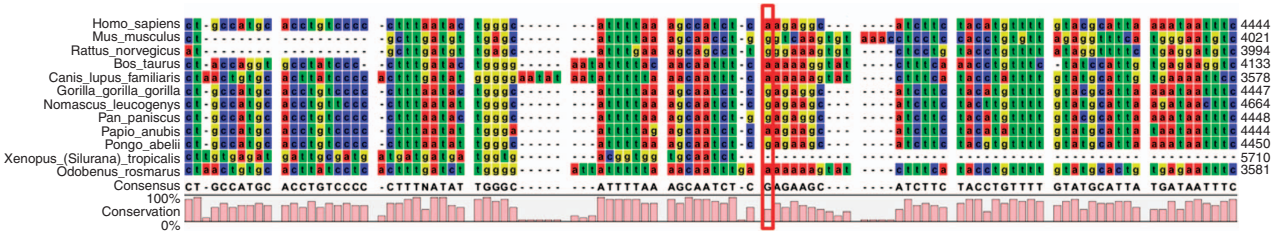

Figure 2. Multi-alignment and conservation analysis of 13 MyD88 gene sequences within a region surrounding the polymorphic site rs6853. The A and G nucleotides are both conserved across species.

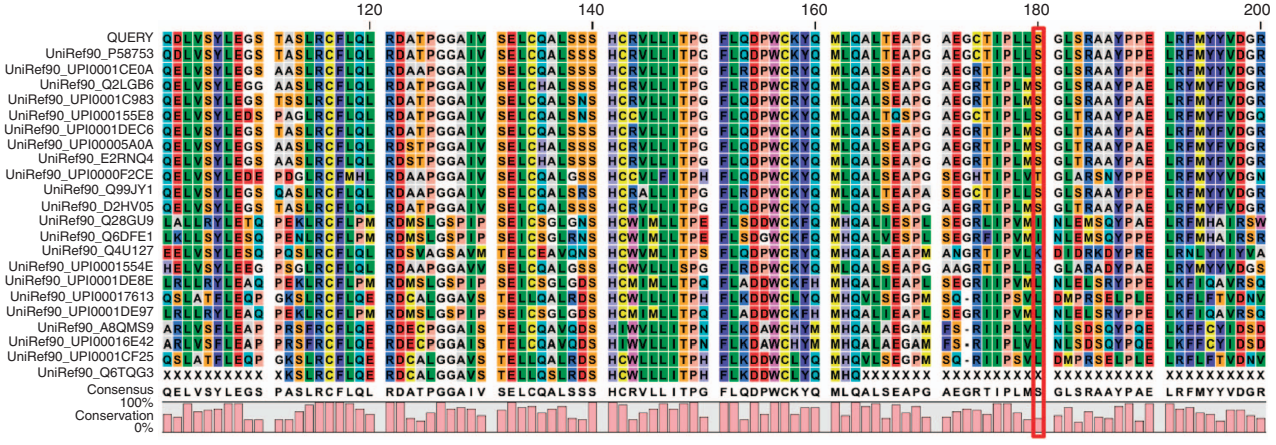

Figure 3. Multi-alignment and conservation analysis of 22 TIRAP protein sequences within a region surrounding the polymorphic site under study (marked with a red line). The serine and leucine amino acid at position 180 are both conserved across species. The automatic search for similar sequences and alignment against the human TIRAP protein was obtained through the SIFT tool (http://sift.jcvi.org/).

molecular level (Figure 1). The advantage expressed by heterozygotes suggests that allelic frequencies at the rs6853 and rs8177374 polymorphic sites are maintained by balanced polymorphism, where homozygosity is associated with pulmonary tuberculosis and heterozygosity with resistance. The hypothesis of the balanced polymorphism agrees with the evidence that nucleotides (AG and CT) at the rs6853 and rs8177374 sites respectively are highly conserved throughout speciation (Figures 2 and 3). As the TIRAP gene confers resistance to several diseases,[3] allelic frequencies at this locus might reflect multiple selective

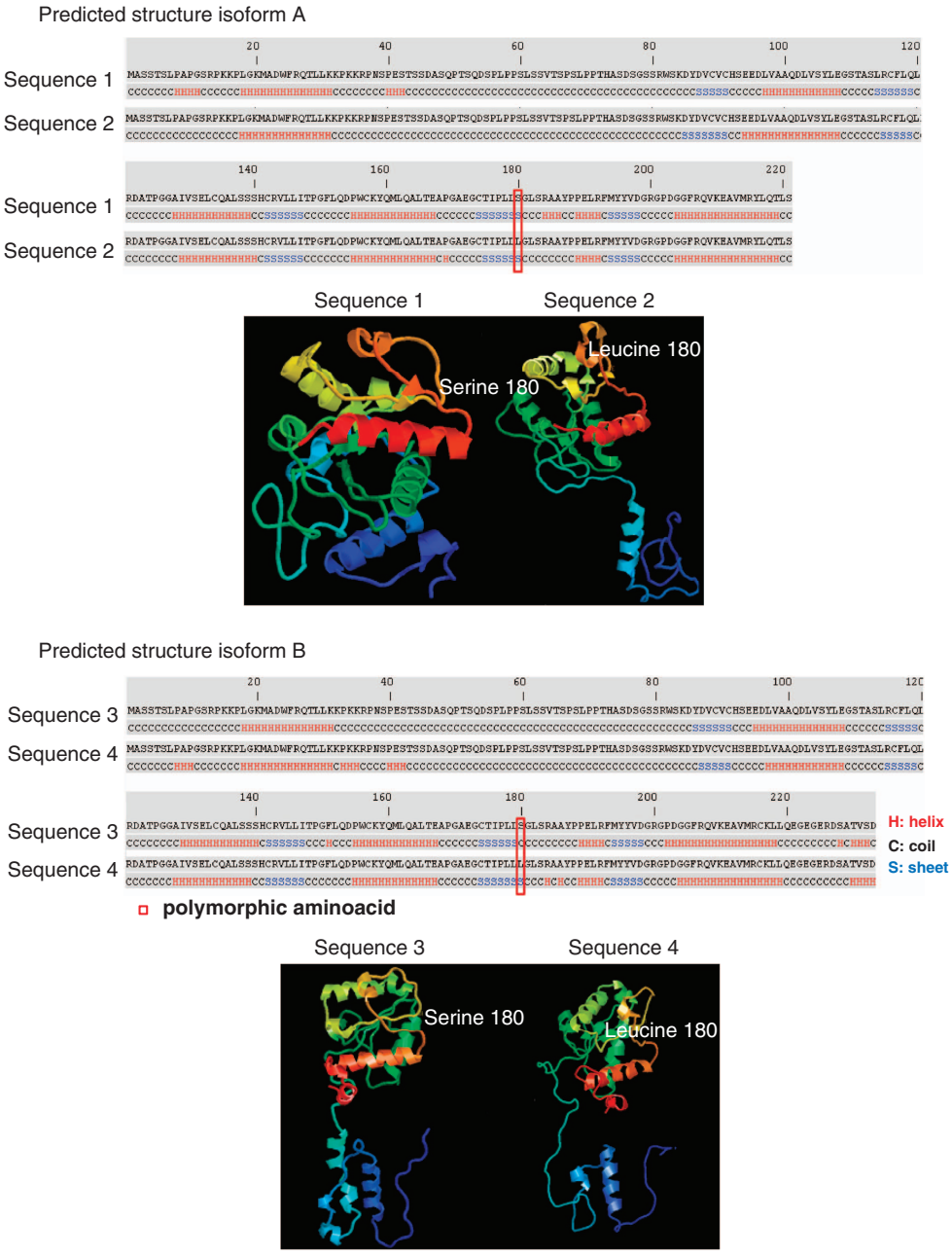

Figure 4. Predictive comparative analysis of TIRAP proteins translated from the common and rare alleles polymorphic at the site rs8177374. Sequences from the common allele (Sequence 1 and 3) and the rare allele (Sequence 2 and 4) are compared based on bi- and tridimensional models generated by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), which predicts secondary and tertiary structures.

pressures. Finally, the crucial role exerted by TNF-α, IFN-γ and NO on tuberculosis[14–19] and the influence of the MyD88 and TIRAP genes on the control of these factors (Figure 1) suggest that the association is between MyD88 and TIRAP, rather than genes closely linked to them.

Disturbingly, association studies lack reproducibility.[21,22] The following characteristics of the present study invite to a cautious optimism about its reproducibility: independent replication (adoption of a two-stage study design, which directly tested reproducibility of the association); low *P*-value (10^{-6}–10^{-8}); selection of homogeneous cases (the study enrolled only patients with pulmonary tuberculosis, clinically diagnosed and confirmed by chest X-ray and positive bacteriological and PCR tests); use of appropriate controls (subjects without the disease, but exposed to *M. tuberculosis* and genetically unrelated to cases); biological plausibility of the genes selected for study[1,16] (Figure 1). Further, the evidence that the association is found in three independent ethnicities (Table 2) makes it unlikely that it is an artifact arising from the demographic structure of the sample population examined.

The association of the MyD88 and TIRAP genes with pulmonary tuberculosis provided contrasting results when examined in different populations.[3,8–10] Is non-reproducibility necessarily an artifact? We think not. New alleles constantly arise in the human genome,[23,24] creating a vast genetic heterogeneity, which interactions between genes and the environment further amplify. All this genetic heterogeneity is difficult to detect *a priori* and plausibly contributes to the irreproducibility of

association studies. This concept is not new[25] and recently has been proposed again.[26] Replication studies are one way for distinguishing between artifacts and true associations.[27,28] One single study may not be trusted, but two or more concurrent and methodologically meaningful studies reinforce each other. Metanalysis of 25 association studies indicates that two independent studies both with $P<0.001$ have high replication probability.[29]

In conclusion, the association of rs8177374 with pulmonary tuberculosis (characterized by *P*-values $<9.6\times10^{-8}$ in the study by Khor *et al.*[3] and 2×10^{-6} in this article; Table 2) very likely will be confirmed to affect the risk of the disease also in future studies, possibly as a population-limited risk factor. The rs6853 site instead—associated with tuberculosis in this study ($P=7.8\times10^{-8}$; Table 2) but not in two previous ones[8,10]—at present must stand as an exploratory, hypothesis-testing study.

MATERIALS AND METHODS

Genotyping

DNA was extracted from blood samples with the phenol–chloroform method.[30] PCR was carried out using probes, the step-one real-time PCR system and the TaqMan universal PCR master mix from Applied Biosystems (Life Technologies, Monza, Italy). The PCR program included one step at 50 °C for 2 min, one at 95 °C for 10 min and 40 cycles at 92 °C for 15 s and 60 °C for 1 min.

ELISA measurement of IFN-γ and TNF-α

Cytokine levels were determined before the blood of donors was genotyped. PBMCs were separated from whole blood on Ficoll-Hypaque (Sigma, Milan, Italy), centrifuged (400 *g*, 30 min), washed with RPMI 1640, distributed (10^5 PBMC per well) on a 96-well plate (Falcon, Milan, Italy) and incubated with heat-killed *M. tuberculosis* strain H37/Rv (10^6 CFU per well) for 48 h (the optimal number of *M. tuberculosis* to use was set in advance by a dose-response experiment). The supernatant was centrifuged (2000 *g* for 5 min) and added (50 μl per well) to a 96-well plate previously sensitized with mouse anti human TNF-α or mouse anti human IFN-γ (BD-Pharmingen, Milan, Italy; 50 μl diluted 2×10^{-3} per well; 4 h) and quenched with 3% BSA (100 μl per well; 2 h). Following incubation of the supernatant for 4 h, the plate was washed with PBS and incubated (in the order) with mouse anti-human TNF-α or mouse anti-human IFN-γ diluted 2×10^{-3} (50 μl per well; 4 h), rat anti mouse labeled with horse radish peroxidase diluted 10^{-3} (50 μl per well; 2 h) and TMB peroxidase substrate (100 μl per well; Biorad, Milan, Italy). Optical density was measured at 405 nm. Samples were run in triplicate.

Measurement of NO

Following incubation with *M. tuberculosis*, the PBMCs were centrifuged and the supernatant (100 μl) was mixed with 100 μl of Griess reagent (10 min). Optical density was measured at 570 nm. Nitrite concentration was measured using as standards 1, 10, 25 and 50 μM sodium nitrite solutions.

Statistical analysis and bioinformatics

ORs and 95% confidence intervals were calculated by Fisher's exact test using the statistical package GraphPad Prism version 5 (GraphPad, La Jolla, CA, USA). Sample size was calculated with the OpenEpi module 3.03.17 and the Hardy–Weinberg equilibrium by the Hardy–Weinberg calculator (http://www.oege.org/software/hwe-mr-calc.shtml). The Mantel–Haenszel test, the binary logistic regression analysis coupled with bootstrap re-sampling (2000 samples and 99% confidence intervals), the Generalized Linear Model procedure[31] and the univariate ANOVA with Tucky's HSD *post hoc* test were carried out using the statistical package SPSS version 19 (IBM Corporation, Armonk, NY, USA). MyD88 and TIRAP orthologs were retrieved by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments were carried out with MAFFT software v7.045b (http://mafft.cbrc.jp/alignment/software) and maps visualized with the CLC Main Workbench 6.8.2 software. The relevance of mutations on the TIRAP protein function was predicted by the SIFT (http://sift.jcvi.org/)[32] and I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) tools.[33] The human 3′UTR sequence of the MyD88 gene was scanned for the identification of regulatory elements using the Encyclopedia of DNA Elements (ENCODE; http://encodeproject.org/ENCODE/).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Infection and Immunity

Heterozygosity at the A625C Polymorphic Site of the MyD88 Gene Is Associated with Mycobacterium bovis Infection in Cattle

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Heterozygosity at the A625C Polymorphic Site of the MyD88 Gene Is Associated with *Mycobacterium bovis* Infection in Cattle

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The study demonstrates that in cattle, animals heterozygous at the MyD88 A625C polymorphic marker have a 5-fold reduced risk for active pulmonary tuberculosis (odds ratio [OR] = 0.19; $P = 6 \times 10^{-12}$). The reduced risk, however, does not extend to animals with latent pulmonary tuberculosis (OR = 0.83; $P = 0.40$). Heterozygosity at the A625C single nucleotide polymorphism is associated with intermediate levels of tumor necrosis factor alpha, gamma interferon, and nitric oxide synthase (NOS). Accordingly, deficiency as well as overexpression of proinflammatory cytokines or NOS favor tuberculosis, while heterozygosity provides the animals with the optimal level of inflammation.

The causative agent of bovine tuberculosis, *Mycobacterium bovis*, has a broad host range, which includes numerous wild and farm animal species. *M. bovis* is also pathogenic in humans. *M. tuberculosis*, the main agent of human tuberculosis, instead is nonpathogenic in cattle (1). This characteristic is attributed to differences in gene expression between the two bacterial species (2). In countries where programs for the eradication of bovine tuberculosis are operative, the periodic testing of cattle herds for tuberculosis infection, meat inspection, and milk pasteurization have reduced to <1% the cases of human tuberculosis attributable to *M. bovis* and confined them primarily to people infected with HIV or exposed to prolonged contact with animals (veterinarians or abattoir workers) (3). Nevertheless, bovine tuberculosis remains relevant as a zoonosis and because of the major economic losses that it causes to the cattle industry from the slaughter of infected—and often valuable—animals, quarantine of infected herds, and restrictions on animal export.

Innate and adaptive immune responses to mycobacteria rely on Toll-like receptors (TLRs), which sense several mycobacterial components. Sensing of the mycobacterial DNA requires TLR9, while heat shock protein 65 (HSP65) requires TLR4 and the lipomannan (LM), lipoarabinomannan (LAM), 19-kDa lipoprotein (19LP), and soluble tuberculosis factor (STF) require TLR2 (4). All TLRs (with the exception of TLR3) critically depend upon myeloid differentiation factor 88 (MyD88) to link bacterial recognition by TLRs with NF-κB activation and cytokine production (5). Evidence of the crucial role played by MyD88 as a signal transducer is provided by MyD88-knockout ($MyD88^{-/-}$) mice, which die within 4 weeks from the time of infection with *M. tuberculosis* (4, 5). $MyD88^{-/-}$ mice infected with *M. tuberculosis* display reduced expression of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and nitric oxide synthase (NOS). This observation has suggested that MyD88 controls the infection by regulating the production of these mediators (6).The above-described studies and the high genetic similarity (99.95% identity at the nucleotide level) of the *M. tuberculosis* and *M. bovis* genomes (7) collectively provided biological plausibility to the hypothesis of a functional role of the MyD88 gene against bovine tuberculosis infection. The present study shows that heterozygosity at the MyD88 A625C polymorphic site is associated with resistance against active—but not latent—*M. bovis* infection in cattle.

MATERIALS AND METHODS

Diagnosis of pulmonary infection. Postmortem samples were collected according to European Food Safety Authority (EFSA) recommendations (8). In the case of animals displaying macroscopic pulmonary lesions, a portion of the diseased tissue and afferent lymph node was collected. In the case of animals without visible lesions, the mediastinal, retropharyngeal, and bronchial lymph nodes were collected. Individual lung homogenates consisted of 1 g or more of pooled specimens collected from each animal. To distinguish between subjects with active tuberculosis pulmonary infection (ATI) or latent tuberculosis pulmonary infection (LTI), 10-fold dilutions (10^{-1} to 10^{-8}) of individual lung homogenates in sterile phosphate-buffered saline were spotted (10 μl/spot; 5 spots/dilution) on agar-Middlebrook (MB) medium and incubated at 37°C for 4 to 5 weeks. At the end of the incubation time, the numbers of CFU were counted. Negative samples were incubated for 10 days in liquid MB medium supplemented (5 μg/ml) with the mycobacterial resuscitation-promoting factor B (RpfB) (9), spotted on agar-MB medium, and incubated for 4 to 5 weeks, and the numbers of CFU were then counted. The optimal concentration of RpfB to use in the assay was found during preliminary experiments. The growth of colonies in the absence of RpfB was indicative of ATI, and the growth of colonies only in the presence of RpfB was indicative of LTI. Controls were negative by both tests.

Identification of mycobacterial species by PCR. One colony of *M. bovis* was dispersed in 200 μl of distilled H_2O containing lysozyme (20 mg/ml; Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 2 h. After incubation, DNA was isolated using a DNeasy blood and tissue kit from Qiagen (Hilden, Germany). PCR was carried out as described previously (10).

Cases and controls. The animals included in the study—both cases and controls—were from three herds declared to be infected. To exclude

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sex and age as potential confounders, the animals were all lactating cows between 40 and 90 months of age. This age interval was selected to represent subjects matched for age (as much as it was realistic) and, at the same time, a population sample sufficiently numerous to provide adequate power to the study. The average ages of the cases and controls were 65.4 ± 5.2 and 69.6 ± 3.9 months, respectively. To curb stratification, both cases and controls were from the same herds and the same breed (Friesian); to keep cases and controls genetically unrelated to each other, when mother and daughter were present, one of the two was excluded.

MyD88 genotyping. The intron/exon boundaries of the bovine MyD88 gene were established by matching the published mRNA sequence of the bovine MyD88 gene (GenBank accession number NM_001014382.2) and the DNA sequence of the human MyD88 gene (GenBank accession number NC_000003.11). Alignment was carried out using DNAsis software (Hitachi Solutions America, San Francisco, CA). DNA was extracted from lung specimens with a QIAamp DNA kit (Qiagen, Hilden, Germany). The primers were 5′-TGAAGGAGTACCCCGC GC-3′ (forward) and 5′-GATGCCTGCCATGTCATT-3′ (reverse). Conditions of the PCR were 7 min at 97°C and then 45 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C (35 cycles), with a final extension for 5 min at 72°C. The 1,210-bp fragments from 20 cases and 20 controls were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and aligned by use of Chromas software (Technelysium, Queensland, Australia). The sequences were used to design primers and TaqMan probes targeting specifically the single nucleotide polymorphism (SNP) located 625 bp downstream of exon 1 (A625C). The forward and reverse unlabeled primers were 5′-GGTGGCGTGGTACTTTGC-3′ and 5′-TTT CTCCTCTACGGGCTGTCT-3′, respectively. The TaqMan VIC- and 6-carboxyfluorescein-labeled probes were 5′-TAGCAAGGG**A**GACAT T-3′ and 5′-TAGCAAGGG**C**GACATT-3′, respectively, where the underlining and boldface indicate the polymorphic nucleotide. PCR conditions were 30 s at 60°C, 10 min at 95°C, and then 40 cycles each lasting 15 s at 95°C and 1 min at 60°C. Genotyping was carried out with the investigator blinded to the case or control status of the animals being tested.

TaqMan gene expression assay. TNF-α, IFN-γ, and NOS2 mRNA levels in the lung specimens were measured using the TaqMan gene expression assay and a StepOne instrument (Applied Biosystems, Foster City, CA). Total RNA (2 μg) was reverse transcribed using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The real-time quantitative PCRs were carried out following the manufacturer's protocol. The identification numbers of the probes are Bt03259155_g1 (TNF-α bovine), Bt03212722_g1 (IFN-γ), Bt03249602_g1 (NOS2), and Bt03279175_g1 (β-actin). Five animals for each genotype (AA, AC, CC) and class (control, active and latent tuberculosis) were tested in triplicate. Relative sample quantification was carried out by the comparative $2^{-\Delta\Delta CT}$ method (where C_T represents the threshold cycle). The endogenous control gene was β-actin. The amplification efficiency of target (TNF-α, IFN-γ, and NOS) and reference (β-actin) genes was approximately the same (slope < 0.1).

Sample size calculation. The data for 50 cases with active tuberculosis and 50 controls (odds ratio [OR], 0.3; proportion of controls with susceptible genotype, 0.46) showed that a sample of 127 cases and 127 controls would provide 96% power and a two-sided significance level of 0.01. The study enrolled 150 animals with acute tuberculosis, 150 animals with latent tuberculosis, and 300 controls.

Other methods. Fisher's exact test and analysis of variance with the Tukey *post hoc* test were performed with GraphPad Prism software, version 5. Binary logistic regression was performed with the SPSS statistical package, version 18. Hardy-Weinberg equilibrium and relative risk reduction were calculated as described previously (see references 11 and 12, respectively). Conservation analysis was carried out on 11 MyD88 genomic sequences retrieved from the Nucleotide BLAST database (blast.ncbi.nlm.nih.gov/). The alignment was performed using the T-COFFEE multiple-sequence alignment server (tcoffee.crg.cat/) and map visualized with CLC Main Workbench software, version 6.8.2. The bovine intron 1 sequence was scanned for identification of overrepresented motifs by using the SCOPE (Suite for Computational identification of Promoter Elements) motif finder (http://genie.dartmouth.edu/scope/). Regulatory elements were searched for using the Encyclopedia of DNA Elements (ENCODE; http://encodeproject.org/ENCODE/).

RESULTS

Diagnosis of cases and controls. Pulmonary tuberculosis infection can be active (ATI) or latent (LTI); the latter is characterized by the presence of dormant bacteria (viable but not culturable on usual growth media) (13). The methods commonly used to diagnose latent tuberculosis are the tuberculin skin test (TST) or the IFN-γ release assay. However, these methods do not distinguish between hosts still infected and those which successfully controlled infection (14). In the present study, grouping together different phenotypes would sensibly reduce the power of the study (15). *M. tuberculosis* has 5 resuscitation-promoting factor (*rpf*) genes coding for as many redundant proteins (RpfA to RpfE) which, in the form of recombinant proteins expressed in *Escherichia coli*, induce resuscitation of *M. tuberculosis* (16) and *M. marinum* (17) *in vitro* and *ex vivo*. On the basis of these findings, an in-house assay aimed at resuscitating dormant mycobacteria with the RpfB protein was developed. It was possible to recover dormant *M. bovis* from seven milk and seven lung specimens from animals treated with the RpfB protein but not from any of the specimens from animals untreated with RpfB when 20 of the animals included in the study were tested. The results for milk and lung specimens from the 20 animals were fully concordant. This material was used to validate the method. The test was therefore extended to all the animals, using lung specimens collected postmortem. A PCR assay discriminating between *M. tuberculosis*, *M. bovis*, or *M. avium* established that all cases (with ATI or LTI) were infected with *M. bovis*. In conclusion, the cases with ATI were subjects positive by the PCR assay and the bacteriological test in the absence of RpfB; the cases with LTI were positive by the PCR assay and the bacteriological test in the presence of RpfB; controls were subjects exposed to *M. bovis* infection (since they were from the same herds that also supplied the cases) but free from infection (negative by the PCR assay and the bacteriological tests in the presence or absence of RpfB) (Fig. 1).

Study design. The criticism more often leveled at association studies is that they lack reproducibility (18, 19). To curb this drawback, a two-stage study was designed. The preliminary (hypothesis-generating) stage involved 50 control animals, which were separately confronted with 50 cases with ATI or 50 cases with LTI. This preliminary study displayed a significant association of the MyD88 polymorphic site A625C with ATI ($P = 0.01$; Table 1) but not with LTI ($P = 0.84$; Table 1). The A625C polymorphic site is located in intron 1 of the MyD88 gene (Fig. 2). The study also yielded the following valuable data: first, that the association is potentially robust (since it was detected using a small number of subjects) and, second, that case stratification according to the form (active or latent) of the infection would definitively provide more power to the study. Other than A625C, the SNPs shown in the reference sequence (Fig. 2) were not present in the sample population studied.

To explore the functional role of A625C further, 11 Myd88 genomic sequences were analyzed for conservation across species (Fig. 3). The low level of conservation suggested that the A625C position is not under stringent selection. Scanning of the bovine intron 1 sequence with SCOPE highlighted 4 overrepresented nu-

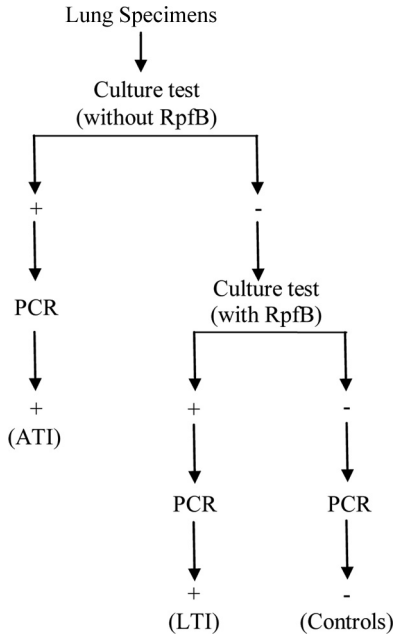

FIG 1 Diagnostic criteria used to classify subjects into controls or subjects with ATI or LTI. Controls, subjects with and without RpfB negative by culture and PCR tests; ATI, subjects without RpfB positive by culture and PCR tests; LTI, subjects with RpfB positive by culture and PCR tests.

cleotide motifs, which included the polymorphic site (Table 2), evoking a possible regulatory role of the site. However, ENCODE did not find regulatory elements within intron 1 in the bovine MyD88, except for a CpG track. The analysis, repeated on intron 1 of the human MyD88, tracked the transcription factor binding site V$BACH1_01 and the chromatin immunoprecipitation (ChIP) fragment for RNA polymerase II. Interestingly, both these tracks overlap the bovine A625C site. Collectively, the data invited further investigation of the potential influence of A625C on *M. bovis* infection.

MyD88 heterozygosity and resistance to ATI. The study was repeated on a larger and independent sample consisting of 300 controls, 150 cases with ATI, and 150 cases with LTI. A separate experiment (with animals not included in the case-control study) showed that crosses between subjects homozygous for the A or C factor (AA × CC) yielded only heterozygous (AC) offspring. The experiment proved that A and C are transmitted as codominant alleles (data not shown). Cases with ATI were not in Hardy-Weinberg equilibrium ($\chi^2 = 4.4$; Table 1). When the test was repeated on the cases with LTI, both cases and controls were in equilibrium (χ^2 for controls = 0.9; χ^2 for cases = 0.3; Table 1). The results suggested an association of the MyD88 marker with ATI but not with LTI. First, the more cogent Fisher's exact test showed that heterozygosity (the AC status) is strongly associated with resistance to ATI (OR = 0.19, $P = 6.0 \times 10^{-12}$; Table 1); second, the association remained strong when the homozygous classes were pooled (OR = 0.22; $P = 1.8 \times 10^{-10}$; Table 1); third, the MyD88 marker did not influence the predisposition to LTI (OR = 0.81 and 0.83; $P = 0.36$ and 0.40; Table 1). The binary logistic regression test supported these conclusions (Table 3). Given the frequency of the AC heterozygotes among controls (135/300 = 0.45; Table 1) and the level of protection afforded (OR = 0.19; Table 1), this genotype prevented 36% [0.45 × (1 − 0.19) = 0.36] of the potential cases of ATI in the population examined (12).

MyD88 heterozygosity and inflammation. TNF-α, IFN-γ, and NOS are known to profoundly influence tuberculosis (6). It is also known that high as well as low levels of inflammation negatively impact this disease (4, 5, 20). Thus, if the MyD88 heterozygotes displayed intermediate cytokine levels compared to those of homozygotes, the association between A625C heterozygosity and resistance to *M. bovis* infection would gain strong biological plausibility. To test this hypothesis, the levels of TNF-α, IFN-γ, and NOS of subjects with different genotypes (AA, AC, CC) and status (controls or animals with ATI or LTI) (6 classes; 5 animals/class) were measured. The expression levels of the subjects with ATI or LTI were then compared with those of control subjects having the same genotype. Heterozygous carriers expressed levels of TNF-α, IFN-γ, and NOS significantly lower than those expressed by the AA homozygotes. Instead, carriers expressed levels only slightly higher than those expressed by the CC homozygotes; in this case, the difference did not reach statistical significance (Fig. 4). One possible explanation for this heterogeneity is that the technique used does not discriminate below a threshold level. Taken together, the data support the conclusion that an optimal inflammatory response is associated with the intermediate A625C phenotype.

TABLE 1 Heterozygosity at the A625C SNP influences active pulmonary tuberculosis infection

Study stage	TB type[a]	Status	No. of cows with the following genotype:				χ^{2b}	AC vs AA		AC vs (AA + CC)	
			AA	AC	CC	Total		OR (CI[c])	*P*	OR (CI)	*P*
1	Active	Cases	36	10	4	50	5.2	0.30 (0.12–0.74)	0.001	0.29 (0.12–0.71)	0.01
		Controls	25	23	2	50	1.4				
	Latent	Cases	28	21	1	50	1.7	0.81 (0.36–1.81)	0.68	0.85 (0.38–1.87)	0.84
		Controls	25	23	2	50	1.4				
2	Active	Cases	123	23	4	150	4.4	0.19 (0.11–0.32)	6×10^{-12}	0.22 (0.12–0.37)	1.8×10^{-10}
		Controls	140	135	25	300	0.9				
	Latent	Cases	75	60	15	150	0.3	0.83 (0.53–1.28)	0.40	0.81 (0.53–1.23)	0.36
		Controls	140	135	25	300	0.9				

[a] TB type, type of *M. bovis* pulmonary tuberculosis infection.
[b] $\chi^2_{0.05}$ (1 degree of freedom) = 3.8.
[c] CI, 95% confidence interval.

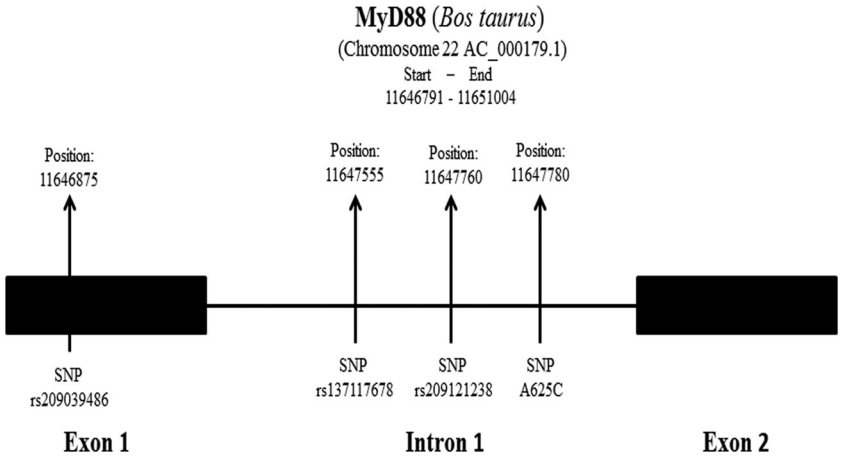

FIG 2 Position of the A625C SNP on the MyD88 gene. The map is oriented 5′ to 3′. Source: www.ncbi.nlm.nih.gov/gene/444881.

DISCUSSION

The present study demonstrates that in cattle, animals heterozygous at the MyD88 A625C polymorphic marker benefit from a 5-fold reduced risk for ATI (OR = 0.19; $P = 6 \times 10^{-12}$; Table 1). The reduced risk, however, does not extend to the animals with LTI (OR = 0.83; $P = 0.40$; Table 1). Heterozygosity at the A625C SNP is associated with intermediate levels of IFN-γ, TNF-α, and NOS (Fig. 4). What is the biological advantage of an intermediate level of production of these mediators in the case of active tuberculosis? The short answer is that heterozygosity provides the optimal level of inflammation. The deficiency of IFN-γ, TNF-α, or NOS favors tuberculosis (4, 5). At the same time, some symptoms of the disease are known to be caused by the immune response of the host rather than by the mycobacterium (20). Episodes of disease reactivation and inflammatory syndrome related to preexisting *M. tuberculosis* (21) or *M. avium* (22) infection have been observed in HIV-coinfected patients after antiretroviral therapy. The study also displays differences in cytokine expression among animals of the same genotype with acute or latent tuberculosis. This difference is particularly evident in the case of the AA animals (Fig. 4). Whether the differences are caused by the mycobacterium or the host immune response, these results, though preliminary, point to increased levels of proinflammatory cytokine expression as potential markers of disease reactivation. The A625C polymorphism—located in intron 1 of the Myd88 gene—adds evidence to the notion that noncoding regions can influence gene expression. It is not surprising that this also occurs in the case of inflammation, which needs to be under fine and complex regulation.

In cattle, exposure to environmental mycobacteria, which occurs in the majority of the subjects, interferes with the diagnosis of *M. bovis* infection by the tuberculin skin test (TST) or the IFN-γ assay (23). Variability in the reagents, incubation time, and diagnostic cutoff levels also influence the specificity and sensitivity of these assays (24). The postmortem culture test—still the "gold standard" method (25)—was therefore preferred for the diagnosis of infection. Also, the limits of the TST and IFN-γ assays and—on the other side—the high prevalence of *M. bovis* infection among the enrolled animals (150 subjects with acute infection and as many with latent infection out of approximately 650 randomly tested animals) persuaded the authors that the number of false-positive and false-negative results would be better minimized by assuming that all controls were exposed subjects, rather than relying on the TST or the IFN-γ assay for exposure diagnosis. The authors do not claim that the method adopted here is superior to current methods in general; rather, they trust that it yields a better-defined disease spectrum and more reproducible results under a case-control design.

Tuberculosis is influenced by several genes interacting among themselves (26) and with the environment (15). The presence of the mycobacterium is necessary but not sufficient to acquire the disease, as shown by the control subjects, which, exposed to the pathogen, did not acquire the disease (Table 1); see also the work of Diamond (27). Environmental factors (climate, herd size, animal purchases, cattle movements) are known to promote bovine tuberculosis (2). Even strong genetic effects on *M. tuberculosis* can be missed when environmental effects are not taken into account (15). We claim that the unusually small OR and *P* values (OR =

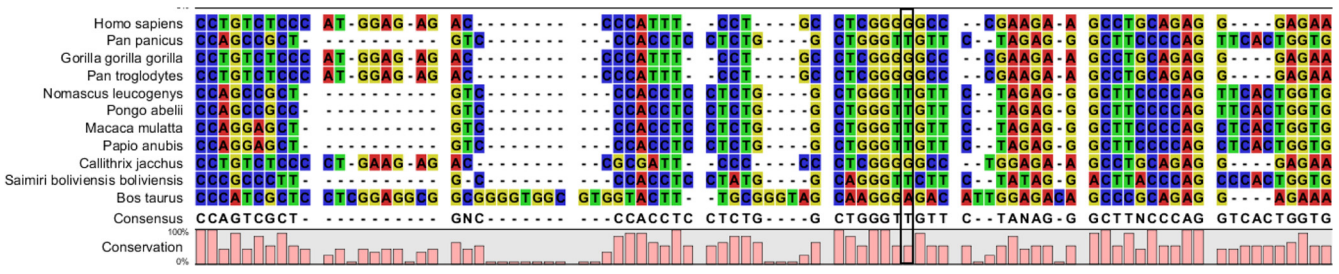

FIG 3 Alignment and conservation analysis of 11 mammalian MyD88 sequences within a region surrounding the polymorphic site under study (marked with a black line).

TABLE 2 Nucleotide motifs overrepresented in the bovine intron 1 sequence[a]

Sequence[b]	Searched consensus sequence	Counts within the *Bos taurus* genome
GGGTAGCAAGGGAGACA	GGGNVNVDDDSSHSACA	5
GGGAGACATTGGAGACA	GGGNVNVDDDSSHSACA	5
AGGGA	AGGGA	7
AGGGAGACAT	AGGGRBVCAT	3

[a] Motifs were identified using the SCOPE motif finder (http://genie.dartmouth.edu/scope/). All strands were plus strands, and 100% coverage was achieved for all sequences.
[b] The polymorphic nucleotide is underlined.

0.19; $P = 6.0 \times 10^{-12}$) reported in the present study reflect how the problems confronting the genetic analysis of this complex disease were solved. Cases were made homogeneous (active and latent tuberculosis cases were analyzed separately), and the environmental confounders were either excluded (sex and breed) or randomized (age). More importantly, control subjects were from the same source population as the cases. Controls were therefore subjects that remained infection free (negative by the bacteriological and PCR tests), though they had the same opportunity as the cases to become infected. Population stratification often has been claimed to be responsible for false-positive results in association studies, yet rarely has it been demonstrated to be the culprit (28, 29). Human studies have shown that stratification might originate when different ethnicities are mixed (30). In the present study, only one breed was studied. Furthermore, replication of the association across 2 independent population samples argues against the result being a product of population stratification.

Genetic association studies are characterized by a high rate of false-positive results (29). This condition is often due to the selection of a candidate gene without a functional relation to the disease (29, 31). In the present study, MyD88 was selected on the basis of a large body of experimental data showing that—at least in mice—this gene is critical for signaling downstream the presence of mycobacterial components and inducing the production of the innate response mediators (IFN-γ, TNF-α, and NOS) against mycobacteria (4, 5). Further, the two-stage study design allowed the reproducibility of the association to be directly proved. Replication of the results at the time that they are first described is gaining consensus as an approach for reducing the number of false-positive results (28, 32). The two-stage design was also of value to define the precise phenotype (active versus latent *M. bovis* infection) to study (Table 1). In conclusion, the high biological relevance of the gene to study, the accurate choice of diagnostic criteria, and randomization of environmental confounders were all carefully kept in mind during the present journey in the puzzling field of association studies. However, since the association is being described for the first time, the results of this study are presented as preliminary.

Lastly, the test used here to differentiate between acute and latent disease could potentially be extended to the periodic testing of cattle for tuberculosis. The count of dormant mycobacteria awakened by RpfB in milk samples would be an easy way to know the prevalence of latent tuberculosis in the population, a parameter greatly influencing the control of the pathogen.

TABLE 3 Heterozygosity at the A625C SNP and resistance to active pulmonary tuberculosis shown by binary logistic regression

Reference genotype	TB[a]	Binary logistic regression analysis result			
		Wald	*P*	e^b	H-L P^c
AA	Active	40	1.8×10^{-10}	0.19	1
	Latent	0.78	0.37	0.83	1
CC	Active	0.01	0.91	1	1
	Latent	0.68	0.40	0.74	1

[a] TB, *M. bovis* pulmonary tuberculosis infection.
[b] Odds ratios estimated by the binary analyses.
[c] The nonsignificance of the Hosmer-Lemeshow (H-L) *P* value indicates that the model predicted by the logistic regression fits the observed data.

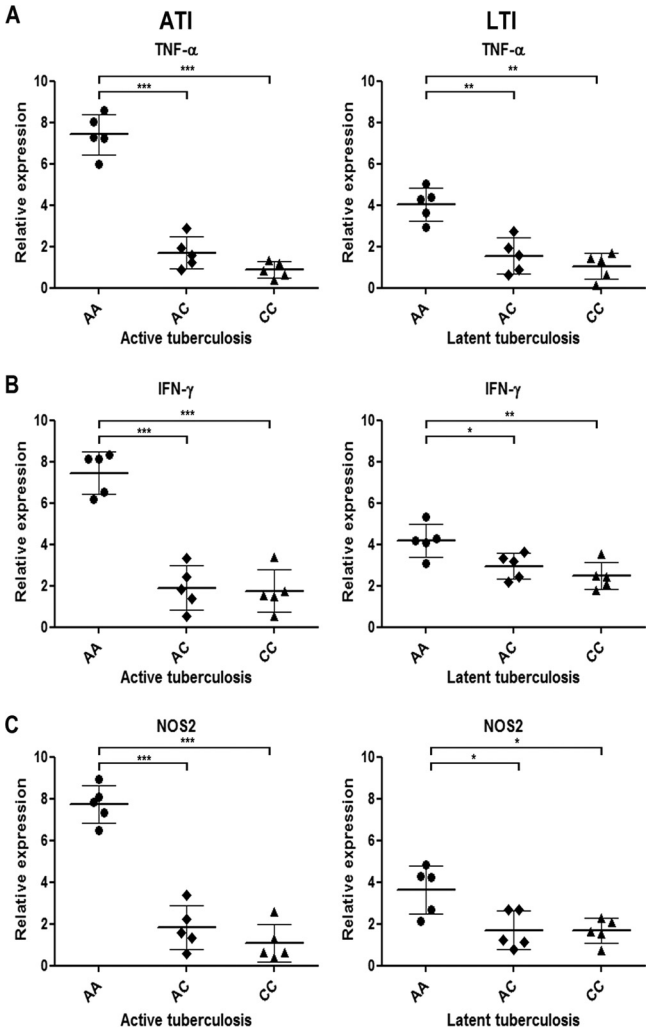

FIG 4 Proinflammatory cytokine mRNA levels measured by the TaqMan gene expression assay. Specimens were from controls or animals with active (ATI) or latent (LTI) *M. bovis* infection. Animals were grouped according to genotype (AA, AC, or CC) and class (controls, subjects with ATI or LTI). Each group consisted of 5 subjects. (A to C) Levels of TNF-α, IFN-γ, and NOS mRNA expression, respectively. Relative sample quantification was carried out by the comparative $2^{-\Delta\Delta CT}$ method. The endogenous control was the β-actin gene.

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We have no conflicts of interest to declare.

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RESEARCH ARTICLE Open Access

New perspectives for natural antimicrobial peptides: application as antinflammatory drugs in a murine model

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Abstract

Background: Antimicrobial peptides (AMPs) are an ancient group of defense molecules. AMPs are widely distributed in nature (being present in mammals, birds, amphibians, insects, plants, and microorganisms). They display bactericidal as well as immunomodulatory properties. The aim of this study was to investigate the antimicrobial and anti-inflammatory activities of a combination of two AMPs (temporin B and the royal jellein I) against *Staphylococcus epidermidis*.

Results: The temporin B (TB-KK) and the royal jelleins I, II, III chemically modified at the C terminal (RJI-C, RJII-C, RJIII-C), were tested for their activity against 10 different *Staphylococcus epidermidis* strains, alone and in combination. Of the three royal jelleins, RJI-C showed the highest activity. Moreover, the combination of RJI-C and TB-KK (MIX) displayed synergistic activity. In vitro, the MIX displayed low hemolytic activity, no NO_2^- production and the ability to curb the synthesis of the pro-inflammatory cytokines TNF-α and IFN-γ to the same extent as acetylsalicylic acid. In vivo, the MIX sterilized mice infected with *Staphylococcus epidermidis* in eleven days and inhibited the expression of genes encoding the prostaglandin-endoperoxide synthase 2 (COX-2) and CD64, two important parameters of inflammation.

Conclusion: The study shows that the MIX – a combination of two naturally occurring peptides - displays both antimicrobial and anti-inflammatory activities.

Background

Coagulase-negative staphylococci (CoNS) are highly abundant on the human skin, already a few hours after birth. The CoNS *Staphylococcus epidermidis* is an ubiquitous and permanent colonizer of human skin and the first cause of nosocomial infections [1]. Most infections with high morbidity and mortality are caused by methicillin-resistant strains of *Staphylococcus epidermidis* (MRSE) [2,3]. In addition, many MRSE strains form a capsule which favors biofilm development, where the pathogen can persist protected from antibiotics and invisible to the immune system [4,5].

New, unconventional antimicrobials are therefore urgently needed [6,7]. In this context, antimicrobial peptides (AMPs), in their natural form or after chemical modification, display interesting features as candidates to become new antimicrobials. They have a broad spectrum of activity against Gram-positive and Gram-negative bacteria, can be easily synthesized in laboratory and have limited toxicity for eukaryotic cells [8,9]. As innate immune components, AMPs lack specificity and immune memory, with the consequence that the pathogens rarely develop resistance to them [10]. Importantly, AMPs rapidly intercept and kill pathogens [11]. AMPs differ each other by size, sequence and secondary structure (α-helix or β-sheet) [12]. Most of them are hydrophobic and amphipathic [13]. AMPs can exert their activity by disrupting the membrane [14] or passing through the bacterial membrane [15]. Molecules belonging to the former class of AMPs permeabilize the membrane phospholipids bilayer and kill the bacterial cell; those belonging to the latter class pass through the bacterial membrane and

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interacts with variable intracellular components, much as traditional antibiotics. AMPs, in addition to the antimicrobial activity, display also immune-modulatory properties (such as chemiotaxis, which contributes to bacterial elimination) and interact with natural and adaptive immunity [16,17]. Thus, in view of the above properties, AMPs represent one of the most promising future strategies for combating infections and microbial drug resistance. The present study describes two chemically modified AMPs - an analogue of the temporin B (TB-KK) secreted by the granular glands of the European red frog (*Rana temporaria*) [18] and an analogue of the royal jellein I (RJI-C) secreted by the mandible and hypopharyngeal glands of honeybees (*Apis mellifera*) [9,19]. These two peptides behave differently towards the bacterial membrane. RJI-C folds into beta sheets and aggregates onto the membrane; TB-KK folds into an alpha helix and does not aggregate onto the membrane [8,9].

Recent data demonstrate that hydrophobic peptides, when mixed with peptides possessing a net positive charge, give origin to a mixture with potential antibacterial activity [20,21]; second, that the combination of antimicrobial peptides derived from different organisms are highly active against Gram positive bacteria [9]. In agreement with these results, here we show that a mixture of TB-KK and RJI-C – two AMPs derived from different sources - displays strong antimicrobial activity against Gram-positive bacteria - modulates pro-inflammatory cytokines and nitric oxide production, in vitro and in vivo. The two peptides, following chemical modification, potentially can be made available in large quantities and in a homogeneous and highly pure form.

Results

Characterization of *Staphylococcus epidermidis* strains

To establish the clonal origin of the *Staphylococcus epidermidis* strains used in the study, the strains (10) were characterized phenotypically - with respect to their antibiotic resistance pattern and molecularly with respect to their Restriction Endonucleases Analysis (Pulse Field Gel Electrophoresis - REA-PFGE) pattern. All strains resulted resistant to aztreonam (30 μg; ATM30), bacitracin (10 μg; B2), cloxacillin (1 μg; CX1) and metronidazole (80 μg; M80) and sensitive to imipenem (10 μg; IPM10). The remaining 25 antibiotics displayed a strain specific pattern (Table 1). Also, with one exception (the strain SE), the strains displayed all different macro-restriction patterns, when analyzed by Sma I REA-PAGE (Figure 1). Thus, the strains used in this study belong to different clonal lineages.

In vitro antimicrobial activity of TB-KK and RJI-C

To evaluate the antimicrobial activity of RJI-C, RJII-C, RJIII-C and TB-KK (Table 2) these AMPs were tested in vitro [8,9], individually and in combination, against 10 *Staphylococcus epidermidis* strains. Among the three royal jelleins, RJI-C showed the highest activity (MIC: 30 μg/ml) (Table 3). Tested in various combination (RJI-C at 20 μg/ml and RJII-C at 5–20 μg/ml ; RJI-C at 20 μg/ml and RJIII-C at 5–20 μg/ml; RJII-C at 20 μg/ml and RJIII-C at 5–20 μg/ml), the royal jelleins did not display synergistic effects. Only RJI-C was thus tested for synergism with TB-KK. The combination of the two antimicrobials – RJI-C at 9 μg/ml and TB-KK at 6 μg/ml (MIX) – displayed a fractional inhibitory concentration index ≤ 0.5, which is evidence of synergism [20] (Table 3). The strains of *Staphylococcus epidermidis* were all sensitive to the MIX, but not its components (Table 4). This conclusion is supported by the larger inhibition ring of the MIX, compared to that of the individual components (Figure 2A).

Interestingly, the antibacterial activity of the MIX against probiotics bacteria (*Lactobacillus plantarum*, *Lactobacillus Paracasei*, *Bifidobacterium animalis*) was five-fold lower than that of gentamicin (Table 5).

In vitro hemolytic and cytotoxic activities of the MIX

To test the cytotoxic activity of the MIX, we used the hemolytic and the LC50 assays. The MIX lysed less than 12% of the murine erythrocytes (data not shown) and the LC50 value was 143,8 mg/ml versus 58.5 μg/ml of TB-KK and 64.6 μg/ml of RJI-C (Additional file 1: Table S1). The MIX was not toxic towards the macrophage J774 cells, which remained vital at 72 hours (Figure 2B).

In vitro the MIX does not induce synthesis of NO_2^-

The MIX (RJI-C at 9 μg/ml and TB-KK at 6 μg/ml) did not induce NO_2^- synthesis in J774 cells. Rather, when these cells were stimulated with LPS (10 μg/ml/well for 3 hours) and then treated with the RJ-IC, TB-KK and MIX reduced NO_2^- synthesis (Table 6), one of the parameters to determine the cellular toxicity.

In vitro anti-inflammatory activity of the MIX

To investigate whether the MIX, in addition to the antimicrobial activity, also displays anti-inflammatory activity, J774 cells (10^6 cells/well) were stimulated with either LPS or LTA (0.1, 1 or 10 μg/ml) for 3 hours. The results show that LPS stimulates inflammation in the J774 cells better than LTA (Figure 3A). Later, J774 cells were treated with gentamicin (5 μg/ml), acetylsalicylic acid (ASA, 5 μg /ml) or MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml) for 3 hours. In the absence of the agent causing inflammation (LPS), the MIX, gentamicin and ASA do not induce inflammation (Figure 3B). In J774 cells (10^6 cells/well) stimulated with LPS for 3 hours, the MIX curbs the synthesis of the pro-inflammatory cytokines TNF-α

Table 1 Results of antibiotic susceptibility tests of ten *Staphylococcus epidermidis* strains

	Antibiotics tested																												
Strain	**FD10**	**P120**	**AMX25**	**AM10**	**ATM30**	**B2**	**CB100**	**CD30**	**FOX30**	**CAZ30**	**A30**	**CX1**	**K15**	**FF50**	**GM10**	**IPM10**	**MY2**	**M80**	**MZ75**	**NET30**	**FM300**	**NB30**	**T30**	**P10**	**PIP100**	**RF30**	**SP100**	**RL100**	**TE30**	**VA30**
SE	R	R	R	R	R	R	R	R	I	S	I	R	R	S	R	S	R	R	R	R	R	R	I	R	R	R	R	R	R	R
3/28	R	I	S	S	R	R	R	S	S	I	I	R	R	R	R	S	R	R	S	I	S	S	S	R	S	S	S	R	I	I
2/2	S	R	R	R	R	R	R	R	R	I	S	R	R	R	I	S	R	R	R	I	S	S	S	R	R	I	R	R	I	I
5/6	I	R	I	R	R	R	I	R	I	S	I	R	R	R	I	S	I	R	R	I	S	S	S	R	R	S	I	R	I	I
5/8	S	R	I	R	R	R	I	R	I	I	R	R	R	S	R	S	R	R	R	I	S	S	R	R	R	S	S	R	S	S
12/14	S	R	R	R	R	R	I	R	S	S	R	R	R	R	R	S	S	R	R	I	S	S	R	R	R	R	I	R	R	I
9/1	S	R	R	R	R	R	I	R	S	I	S	R	S	S	I	S	S	R	R	S	S	S	S	R	R	S	I	R	S	I
10/28	S	I	R	R	R	R	R	R	S	I	R	R	R	S	R	S	S	R	R	I	S	S	R	R	R	S	I	R	I	I
12/26	S	R	R	R	R	R	R	R	S	I	S	R	S	S	R	S	S	R	R	I	S	S	S	R	R	S	I	R	S	I
5/25	S	R	R	R	R	R	R	R	S	I	S	R	R	I	I	S	R	R	R	I	S	S	S	R	R	S	I	R	S	S

R= strain resistant to the antibiotic.
S= strain sensitive to the antibiotic.
I= intermediate strains sensitive to the antibiotic.

and IFN-γ more efficiently than gentamicin and at the same extent of the ASA (Figure 3C).

These experiments demonstrate that the MIX exerts anti-inflammatory as well as antimicrobial activities, while the single components of the MIX have no anti-inflammatory activity (Additional file 2: Figure S1). Since COX-2 is a well-established parameter of inflammation [22] , the J774 cells were stimulated with LPS (10 μg/ml) and 1 hour later treated with the MIX, RJII-C (non-active peptide), acetylsalicylic acid (ASA), gentamicin or vehicle (PBS) for 3 hours. The level of the COX-2 protein was then detected by western blot. The MIX-treated cells, displayed a COX-2 protein level comparable to that of the cells treated with ASA or gentamicin, and much lower than that of the cells treated with RJII-C or the vehicle (Figure 3D). The above results demonstrate that the MIX curbs inflammation to the same extent as ASA [23].

In vivo anti-inflammatory activity of the MIX in mice stimulated with LPS

To investigate further the property of the MIX to curb inflammation in vivo, LPS (250 μg, ~10 mg/Kg) was administrated to four groups of mice (3 mice/group). After 3 hours, the groups were treated respectively with the MIX (RJI-C 9 μg/mouse + TB-KK 6 μg/mouse), gentamicin (5 μg in 100 μl/mouse) or ASA (5 μg in 100 μl/mouse). The last group received 100 μl of saline buffer as control. After 3 hours, the mice that received the MIX showed a reduced level of both the pro-inflammatory cytokines TNF-α and IFN-γ, when compared to gentamicin-treated group, but an higher expression level of IFN- γ, when compared to the ASA group (Figure 3E). In conclusion,

Table 2 Peptide sequences and mass analysis of the royal jelleins (RJ) and temporin (TB) used in the study

Peptide	Sequence	Calc. mass (DA)	Meas. mass (DA)
RJI-C	PFKIDIHLGGY-NH_2	1230.46	1231.02
RJII-C	TPFKISIHLGGY-NH_2	1331.56	1331.90
RJIII-C	EPFKISIHLGGY-NH_2	1359.57	1360.10
TB	YLLPIVGNLLKSLL-NH2	1391.80	1391.20
TB-KK.	KKYLLPI VGNLLKSLL-NH2	2295.40	2294.30

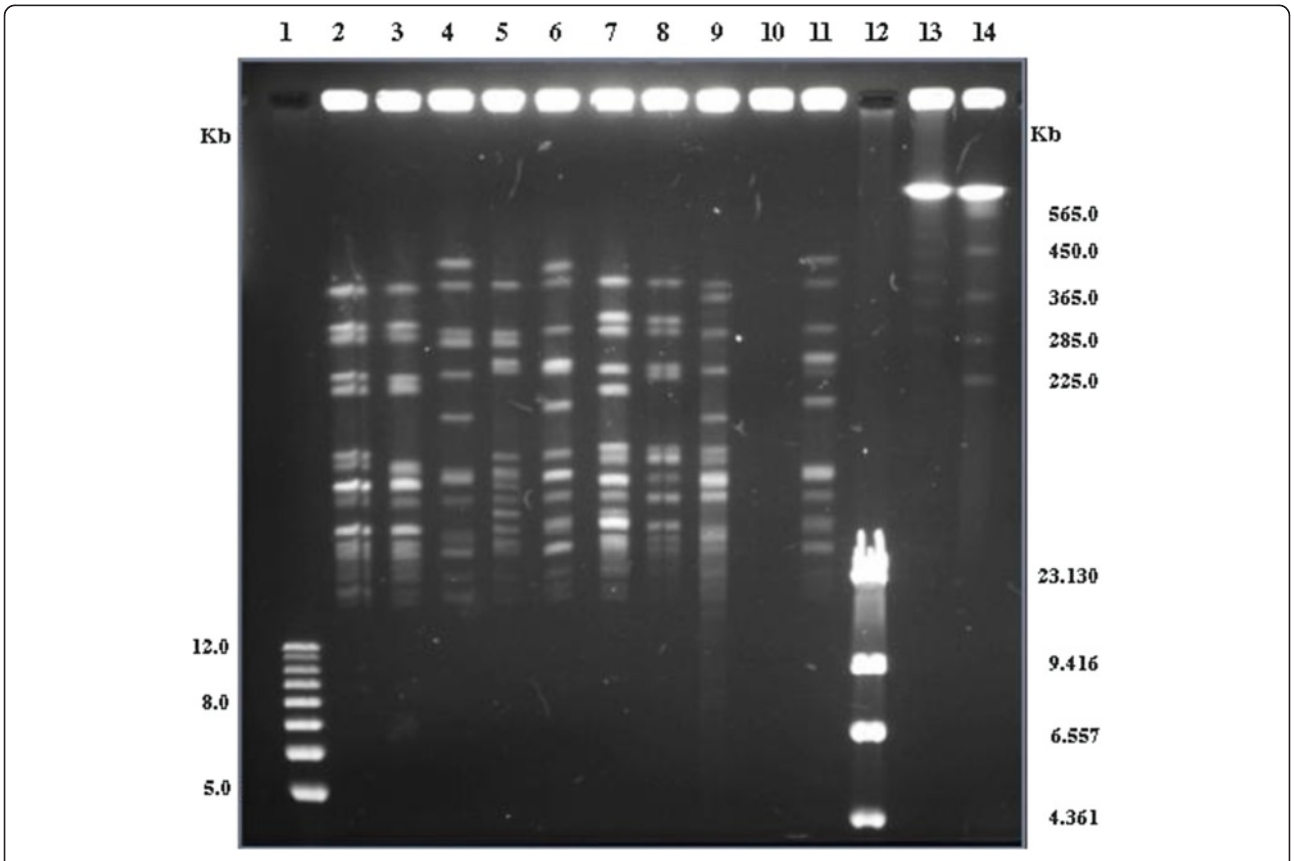

Figure 1 Sma I REA (Restriction Endonucleases Analysis)-PFGE patterns of Staphylococcus epidermidis strains: 1) 1Kb plus DNA Ladder (Invitrogen); 2) strain 5/25; 3) strain9/1; 4) strain 2/2; 5) strain 10/28; 6) strain 12/14; 7) strain 5/8; 8) strain 12/26; 9) strain 5/6; 10) strain SE (untypable); 11) strain 3/28; 12) Lambda DNA - Hind III Digested (Invitrogen); 13) DNA Size Standards - Lambda Ladder (Bio-Rad); 14) PFGE marker, 0.225–2.2 Mb S. cerevisiae chromosomal DNA (Bio-Rad).

Table 3 The FIC index against Staphylococcus epidermidis strains: ≤ 0.5, synergy ; >0.5, no interaction

Antimicrobial peptides	MIC_{100}	Fic index
RJI-C	30 μg/ml (24 μM)	
RJII-C	200 μg/ml (150 μM)	
RJIII-C	300 μg/ml (220 μM)	
TB-KK	7 μg/ml (3 μM)	
Gentamicin	5 μg/ml (10 μM)	
RJI-C + TB-KK	9 μg/ml + 6 μg/ml (7.3 μM + 2.6 μM)	0.5

the MIX performs better than gentamicin, but worse than ASA.

In vivo antimicrobial efficacy of the MIX given intravenously at 12 hours post infection

To evaluate the efficacy of the MIX to contrast microbial infection, four groups of mice (15 mice/group) were infected with lethal dose (10^8 CFU/mouse) of *Staphylococcus epidermidis* (SE). This strain was chosen since it is resistant to the majority of the antibiotics tested (Table 1).

One group did not receive any treatment (control group); a second group received sterile PBS (100 μl/mouse) (placebo group – data not shown); the third group received the MIX (RJI-C 9 μg/mouse + TB-KK 6 μg/mouse); the fourth group received gentamicin (5 μg/mouse). PBS, MIX and gentamicin were administered intravenously at 3 hours post infection. In both, placebo and control groups, the bacterial load of kidneys and spleens increased progressively, while it decreased in the groups treated with gentamicin or the MIX (Additional file 3: Figure S2). Upon treatment of the mice with the MIX, the acute phase proteins, which represent important markers of inflammation [24], were evaluated (Additional file 4: Table S2). The SAA (Serum amyloid A), haptoglobin and fibrinogen were within normal ranges in the mice treated with the MIX or with gentamicin, while significantly high in the control mice (infected but not treated) (Additional file 4: Table S2).

In vivo anti-inflammatory efficacy of the MIX given intravenously at 12 hours post infection

The four groups of mice described before have been used also to evaluate the anti-inflammatory activity of the MIX. For this purpose, the expression levels of the TNF-α , IFN-γ, IL-10 cytokine genes were measured at 3, 6 and 9 hours after treatment in the kidney samples (Figure 4A-C, respectively). In the group treated with the MIX, the TNF-α and IFN-γ were under expressed (at 6, 9 hours from treatment), as compared to the group treated with gentamicin (Figure 4A-C). This result suggests that the MIX controls inflammation better than gentamicin.

Also CD64 and COX-2 markers of inflammation in vivo were evaluated. Blood samples were collected 3, 6, or 9 hours after the treatments. CD64 was measured by flow cytometry (Figure 5A). Six and nine hours after the treatment with gentamicin or the MIX, the mice displayed a decreased expression of the CD64 marker (Figure 5A). The level of COX-2, was evaluated by RT-PCR on the mRNA extracted from kidney samples. In control mice displayed a significantly higher expression level of COX-2, compared to the mice treated with MIX or gentamicin. In the control mice COX-2 peaked 3 hours after the treatment. In the mice treated with gentamicin or the MIX, COX-2 expression level returned to the normal level nine hours after the treatment (Figure 5B).

To verify whether the MIX affected granulocytic infiltration in the kidneys of infected mice, hematoxylin-eosin staining was performed. As expected, kidneys of control mice displayed granulocytic infiltration within the lumen of the cortical convoluted tubules and hence lymphocytic infiltration, vessel activation and glomerular hyperplasia (Figure 6 panel 1, 5). Instead, kidneys of MIX-treated mice showed a dramatic reduction in the number of granulocytic cells localized in the cortical

Table 4 Antimicrobial activity of the MIX and its components against different strains of *Staphylococcus epidermidis*

Strains	% inhibition of bacterial growth RJI-C 9 μg/ml (7.3 μM)	% inhibition of bacterial growth TB-KK 6 μg/ml (2.6 μM)	% inhibition of bacterial growth RJI-C 9 μg/ml + TB-KK 6 μg/ml (RJI-C 7.3 μM + TB-KK 2.6 μM) (MIX)
3/28	17 ± 2	19 ± 2	91 ± 1
2/2	18 ± 1	23 ± 0.5	96 ± 2
5/6	4 ± 3	10 ± 1	100 ± 0
5/8	12 ± 2	21 ± 2	95 ± 2
12/14	11 ± 0.5	20 ± 3	92 ± 1
9/1	18 ± 0	26 ± 2	96 ± 2
10/28	0	4 ± 1	100 ± 0
12/26	19 ± 2	14 ± 2	100 ± 0
5/25	15 ± 1	21 ± 1	90 ± 2

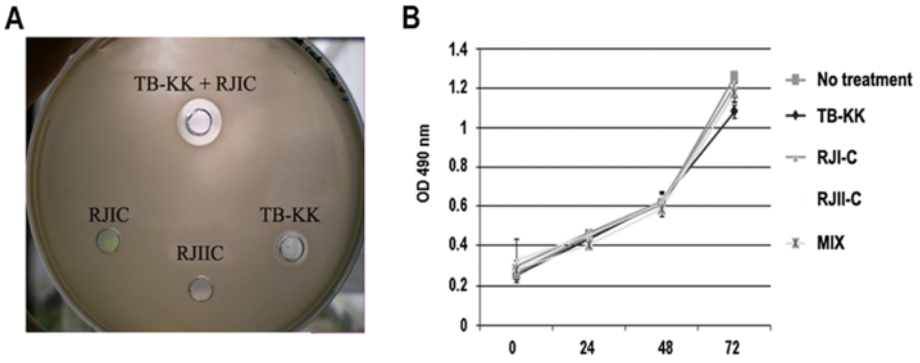

Figure 2 (A) Antimicrobial activity of the single peptides (RJI-C 9 μg/ml; RJII-C 15 μg/ml; TB-KK 6 μg/ml) and of MIX (RJI-C at 9 μg/ml and TB-KK at 6 μg/ml) are shown as inhibition zone assay. A larger zone of inhibition is evident around the MIX compared to the single components. (**B**) J774 cell line treated with the single peptides (RJI-C 9 μg/ml; RJII-C 15 μg/ml; TB-KK 6 μg/ml) or the MIX (RJI-C at 9 μg/ml and TB-KK at 6 μg/ml) maintain the same growth rate compare to the untreated control.

convoluted tubules, less glomerular hyperplasia, and no lymphocyte infiltration (Figure 6 panel 2–4).

In vivo antimicrobial efficacy of the MIX for the period of 12 days

To test the antimicrobial activity of the MIX in vivo for a longer period, mice were infected with a sub-lethal dose (10^7 CFU/mouse) of *Staphylococcus epidermidis* and then treated with the MIX. Four groups of mice (24 mice/group) were infected with the bacterial strain (SE). One group of mice did not receive any treatment (control group); a second group received sterile PBS (100 μl/mouse) (placebo group); the third group received the MIX (RJI-C: 9 μg/mouse + TB-KK: 6 μg/mouse); the fourth group received gentamicin (5 μg/mouse). PBS, MIX and gentamicin were administered intravenously in three boosts 3, 6 and 9 days post infection. In the placebo and the control groups, the bacterial load of kidneys and spleens (the target organs of the pathogen) increased progressively, while the load was significantly lower in the groups treated with gentamicin or the MIX. Eleven days after the infection, the mice treated with gentamicin were still infected, while those treated with the MIX were already sterile (Figure 7A-B).

Table 5 Antimicrobial activity of the MIX or gentamicin on probiotic bacteria

Strains	MIX RJI-C 9 μg/ml +TB-KK 6 μg/ml (RJI-C 7.3 μM +TB-KK 2.6 μM)	Gentamicin 5 μg/ml (10 μM)
Bifidobacterium animalis	29% ± 3	96% ± 4
Lactobacillum plantarum	23% ± 2	97% ± 4
Lactobacillum paracasei	25% ± 2	96% ± 3

Four days after the infection, in addition to spleen and kidneys (10^6 CFU/gr and 10^7 CFU/gr respectively), the bacterium was also detected (at a threshold level: 10^2 CFU/g) in the liver (data not shown). Thus, the MIX is slightly more effective than gentamicin (Figure 7A-B). In all four groups, bacteria were no longer detected in the blood circulation within 2 h from infection (Additional file 5: Figure S3).

In vivo anti-inflammatory efficacy of the MIX for the period of 12 days

To evaluate the anti-inflammatory activity of the MIX, the expression levels of the TNF-α, IFN-γ, IL-10 cytokines genes were measured in the kidneys. The experiment was carried out on the same four groups of mice described in the previous paragraph. For this purpose, the expression levels of the cytokines were measured 24 and 48 hours after each treatment with MIX (or 4, 5, 7, 8,10 and 11 days post infection). In the group treated with the MIX, compared to the group treated with gentamicin, the TNF-α and IFN-γ levels were under expressed (at 7 days) while the IL-10 levels were over expressed (at 10 days) (Figure 7C). This result suggests that the MIX controls inflammation better than gentamicin.

Discussion

Recently we demonstrated that new antimicrobials are more effective than traditional antibiotics against *Staphylococcus epidermidis* [25,26]. The present study extends these results, providing evidence that the MIX – a mixture of a royal jellein modified at the C-terminal (RJI-C) and an analogue of temporin B (TB-KK) – is a valid alternative to the use of gentamicin against skin infections caused by *Staphylococcus epidermidis*.

In vivo, endogenous antimicrobial peptides (such as human defensins and cathelecidins) are known to be pleiotropic: they act as antimicrobials [27]; neutralize

Table 6 NO_2^- production of J774 cells: Mouse macrophages untreated, treated with RJI-C, TB-KK or the MIX, stimulated with LPS, stimulated with LPS and treated with RJI-C, TB-KK or the MIX

Treatment	Time of incubation (h)		
	24	48	72
No treatment	0.25 ± 0.04	0.69 ± 0.02	0.92 ± 0.2
RJI-C (15 μg/ml) (12 μM)	0.42 ± 0.03	0.75 ± 0.01	1.02 ± 0.3
TB-KK (15 μg/ml) (6.5 μM)	0.82 ± 0.05	1.25 ± 0.2	1.34 ± 0.2
MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml) (RJI-C 7.3 μM + TB-KK 2.6 μM)	0.72 ± 0.3	0.85 ± 0.3	1.06 ± 0.2
LPS (10 μg/ml)	2.93 ± 0.2	10.96 ± 0.4	12.16 ± 0.5
LPS + RJI-C (15 μg/ml) (12 μM)	2.85 ± 0.3	8.42 ± 0.1	10.21 ± 0.2
LPS + TB-KK (15 μg/ml) (6.5 μM)	3.12 ± 0.6	9.75 ± 0.1	11.45 ± 0.2
LPS + MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml) (RJI-C 7.3 μM + TB-KK 2.6 μM)	2.63 ± 0.4	7.25 ± 0.3	8.26 ± 0.1

Data are expressed as micromoles of NO2- for 106 input cells, and are means ± standard deviation of three different experiments each performed in triplicate.

bacterial components (LTA and LPS), which otherwise would induce an excess of inflammation and tissue damage [28,29]; attract inflammatory cells to the wound site and promote wound healing.

The two exogenous components of the MIX also behave in a pleiotropic fashion: they control the bacterial load (Figure 7A-B and Additional file 3: Figure S2), inhibit the synthesis of pro-inflammatory cytokines (Figures 4 and 7C) and control the expression of COX-2 (Figures 3D and 5B), the acute phase proteins (Additional file 4: Table S2) and the expression of the CD64 receptor (Figure 5A). At the histological level, the MIX reduces kidney lymphocyte infiltration (Figure 6).

Mice infected with a sub-lethal dose of *Staphylococcus epidermidis* and three days later treated with the MIX (RJI-C: 9 μg/mouse + TB-KK: 6 μg/mouse), within 11 days from treatment, displayed sterile kidneys and spleen – the organs targeted by the bacterial strain used in this study (Figure 7A-B). Samples collected at 15 min intervals from infection showed that bacteria leave the blood circulation within 2 h (Additional file 5: Figure S3). These results are clinically relevant since they suggest that the MIX can potentially be used in humans, where infection is generally caused by a small initial inoculum and treatment is therefore initiated several days after infection (Figure 7A-B).

The MIX is not toxic for eukaryotic cells, in vitro and in vivo (Figure 2B); its components act synergistically (Figure 2A) and becomes moderately hemolytic (12%). In addition, the MIX reduces the synthesis of NO_2^- in cells infected with *Staphylococcus epidermidis* (Table 6). These additional properties make the MIX a candidate for a new generation drug.

In vitro and in vivo experiments demonstrate that the MIX down regulates the level of the pro-inflammatory cytokines TNF–α and IFN-γ while enhancing the expression of the anti-inflammatory cytokine IL-10. This effect is comparable to that of gentamicin, a well-known antimicrobial drug. These results confirm that the MIX, in addition to an antibacterial activity, also exerts – in vivo and in vitro - an anti-inflammatory activity.

The intestinal flora represents a defense barrier against pathogens [30]. We therefore also investigated whether the MIX spared probiotic bacterial species in vitro. While gentamicin killed the totality of the probiotics tested (*Lactobacillus plantarum*, *Lactobacillus Paracasei*, *Bifidus animalis*), the MIX killed a minority of each bacterial species (29%-23%-25%, respectively) (Table 5).

The influence of the MIX on the major cell signaling pathways was also studied. CD64 and COX-2 warn about the cell exposure to inflammatory stimuli [31,32]. The MIX reduced the expression level of COX-2 (Figures 3D and 5B) and CD64 (Figure 5A), proofing that the MIX exerts also anti-inflammatory activity. The CD64 levels are high in the mice infected. In the mice infected and then treated with MIX at both 3, 6 and 9 hours from treatment, levels of CD64 are reduced (Figure 5A). This last result provides evidence that the MIX has effects on mechanisms of both innate and adaptive immunity.

Conclusions

This study provided evidence which suggests an analogy between endogenous AMP and the MIX, consisting of exogenous and chemically modified AMPs. Both display a two-fold role, rapidly recognizing the presence of a pathogen and preventing an excess of inflammation.

Methods

Bacteria

List and origin of *Staphylococcus epidermidis* used in this study is reported in Additional file 6: Table S3. All strains were isolated from patients hospitalized at the Medical School of the University of Naples Federico II. All strains were molecular identified by means of *kat*

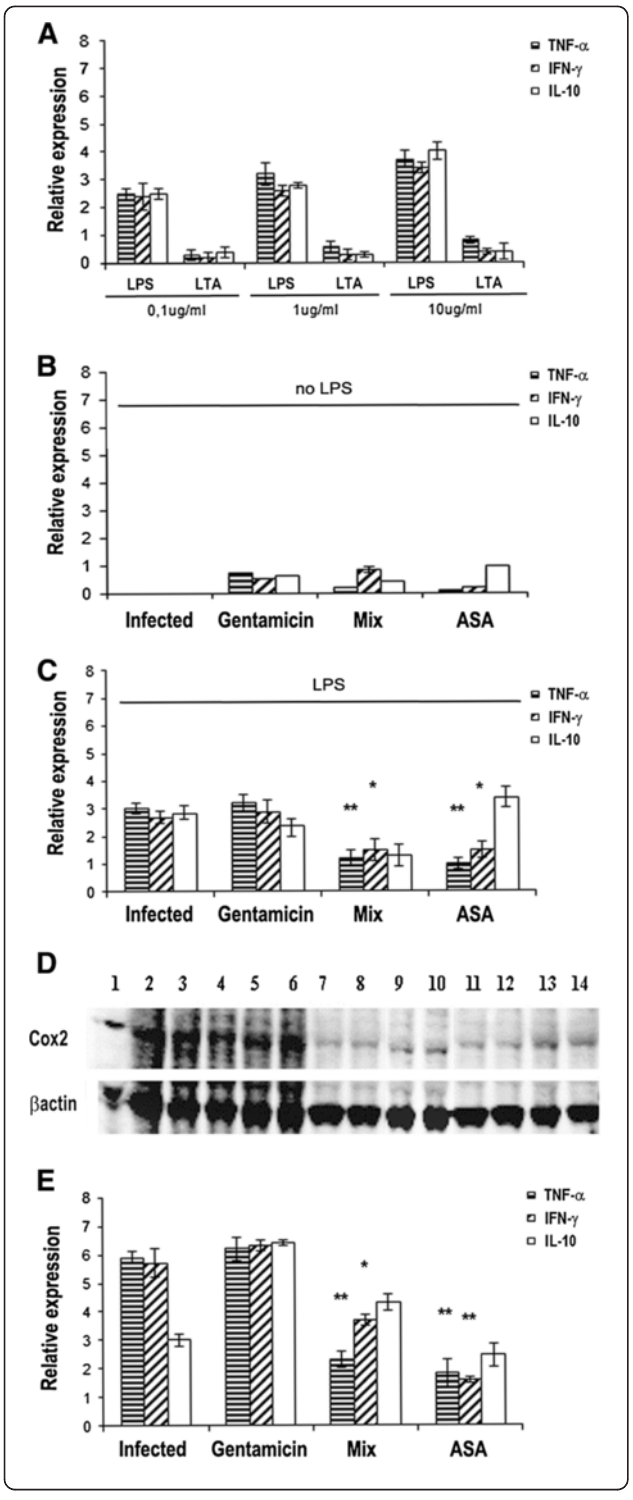

Figure 3 (A) TNF-α, IFN-γ, IL-10 mRNA expression levels in J774 cells stimulated with LPS or LTA (0,1,1 or 10 μg/ml) for 3 hours. (**B**) J774 cells treated with gentamicin (5 μg/ml) or MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml) or ASA (5 μg/ml) for 3 hours. (**C**) J774 cells stimulated with LPS (10 μg/ml) for 3 hours and treated with gentamicin (5 μg/ml) or MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml) or ASA (5 μg/ml) for further 3 hours. (**D**) Western blot analysis of COX-2 in J774 cell line. **Lane 1**–**3**: J774 cells + LPS(10 μg/ml); **Lane 4**–**6**: J774 cells + LPS (10 μg/ml) + inactive peptide (RJII-C 15 μg/ml); **Lane 7**–**9**: J774 cells + LPS (10 μg/ml) + ASA (5 μg/ml); **Lane 10**–**12**: J774 cells + LPS (10 μg/ml) + MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml; **Lane 13**–**14**: J774 cells + LPS (10 μg/ml) + gentamicin (5 μg/ml). (**E**) TNF-α, IFN-γ, IL-10 mRNA expression levels in kidney of mice (3mice/group) stimulated with LPS (250 μg, ~10 mg/Kg) for 3 hours; stimulated with LPS (250 μg, ~10 mg/Kg) for 3 hours and treated with gentamicin (5 μg/mouse) or MIX (RJI-C 9 μg/mouse + TB-KK 6 μg/mouse) or ASA (5 μg/mouse) for 3 hours. Values were normalized with GAPDH and compared to untreated control. *P <0.05, **p < 0.01; ***p < 0.001, Student's *t* test gentamicin vs MIX and gentamicin vs ASA.

A-RFLP analysis technique described by Blaiotta et al. [33].

The study does not investigate clinical aspects of the disease, nor it uses human specimen. The study therefore does not require the Ethic Committee approval.

Antibiotic susceptibility of *Staphylococcus epidermidis* strains

The antibiotic-susceptibility profile of strains was tested using the disk diffusion method on Mueller-Hinton agar, according to the NCCLS guidelines (2002) [34]. The antibiotics used and their concentrations were as follows: amoxicillin (25 μg; AMX25), ampicillin (10 μg; AM10), aztreonam (30 μg; ATM30), bacitracin (10 μg; B2), carbenicillin (100 μg; CB100), ceftazidime (30 μg; CAZ30), cefoxidin (30 μg; FOX30), cephaloridine (30 μg; CD30), cloxacillin (1 μg; CX1), erythromycin (15 μg; E15), fosfomycin (50 μg; FF50), fusidic acid (10 μg; FD10), gentamicin (10 μg; GM10), imipenem (10 μg; IPM10), lincomycin (2 μg; MY2), metronidazole (80 μg; M80), mezlocillin (75 μg; MZ75), netilmycin (30 μg; NET30), nitrofurantoin (300 μg; FM300), novobiocin (30 μg, NB30), oxytetracycline (30 μg, T30), penicillin-G (10 μg; P10), piperacillin (100 μg, PIP100), rifampicin (30 μg; RF30), chlorotetracycline (30 μg; A30), spiramycin (100 μg; SP100), sulfamethoxazole (100 μg; SP100), tetracycline (30 μg; TE30), and vancomycin (30 μg; VA30). All antibiotics were provided by BioMérieux SA, (Marcy l'Etoile, France).

Pulsed-field electrophoresis of *Staphylococcus epidermidis* strains

The procedure adopted was that described [35]. Briefly, inserts of intact DNA were digested in 200 μl of appropriate buffer supplemented with 40 U of *Sma* I (Promega, Milan). Pulsed field gel electrophoresis (PFGE) of the restriction digests was performed by using the CHEF system (Bio-Rad Laboratories, Hercules, CA, USA) with 1% (wt/vol) agarose gels and 0.5 x TBE as running buffer, at 10°C. Restriction fragments were resolved in a single run, at constant voltage of 6 V cm^2 and an orientation angle of 120° between electric fields, by a single phase procedure for 24 h with a pulse ramping between 1 and 50s.

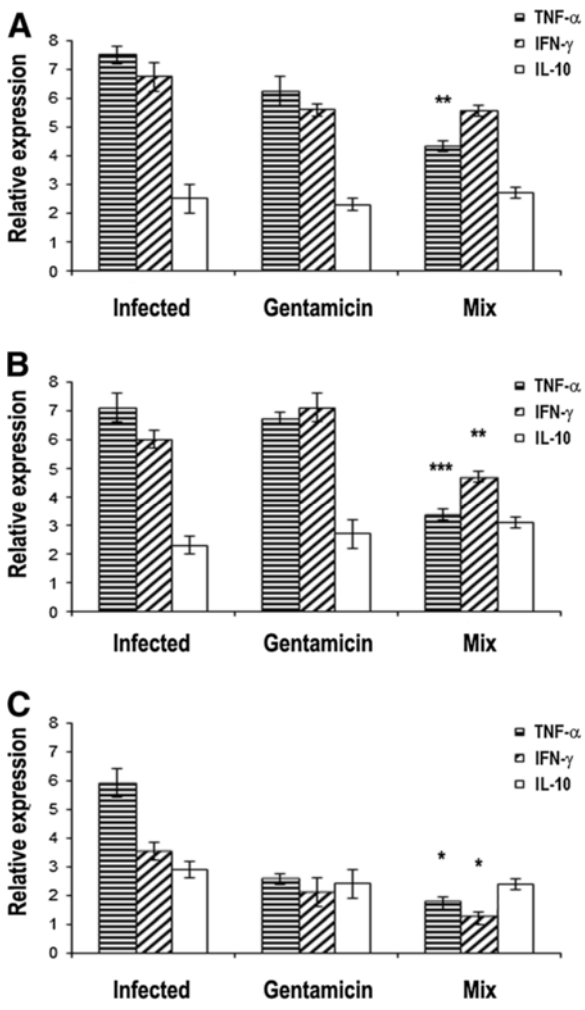

Figure 4 (A-C) TNF-α, IFN-γ, IL-10 mRNA expression levels in infected mice with Staphylococcus epidermidis (108 CFU/mouse) or infected with Staphylococcus epidermidis (108 CFU/mouse) and treated with the MIX (RJI-C at 9 μg/mouse and TB-KK at 6 μg/mouse) or gentamicin (5 μg/mouse) at 3(A), 6 (B) and 9 (C) hours after treatment. Values were normalized with GAPDH and compared to untreated control. *P <0.05, **p < 0.01; ***p < 0.001, Student's *t* test gentamicin vs MIX.

Antibacterial activity of AMPs

Antibacterial activity of the peptides used in this work was evaluated as described previously [8]. A potential synergism (FIC) between TB-KK and RJI-C (MIX) was evaluated by adding combinations of two peptides in a serial two-fold dilutions (RJI-C 5–100 μg, 40 μl/well; TB-KK 5–100 μg, 40 μl/well;) to wells containing 10^5 CFU/well in 60 μl [8]. The fractional inhibitory concentration (FIC) index for combinations of two peptides was calculated according to the equation: FIC index = FICA + FICB = A/MICA + B/MICB , where A and B are the MICs of drug A and drug B in the combination, MICA and MICB are the MICs of drug A and drug B alone, and FICA and FICB are the FICs of drug A and drug B. The FIC indices were interpreted as follows: ≤0.5, synergy; 0.51–4.0, no interaction; > 4.0, antagonism [23].

The growth inhibition percentages of *Staphylococcus epidermidis* and probiotic strains were assessed under the same conditions.

Inhibition zone assay and test of the haemolytic activity of the antimicrobials

The MIX (RJI-C at 9 μg/ml and TB-KK at 6 μg/ml) was tested for its haemolytic activity using mouse red blood cells and for inhibition zone assay test [8]. The MIX was tested for its haemolytic activity using mouse red blood cells. The blood was collected from the tail of the animals and centrifuged ($4x10^2$ g for 3 min). The erythrocytes were washed with saline, suspended at $3x10^6$ erythrocytes/ml, mixed with the peptide combination (RJI-C 9 μg and TB-KK 6 μg in 100 μl saline) and incubated for 1 h at 37°C. The haemolytic activity was measured according to the formula $OD_{peptide}$ - $OD_{negative\ control}/OD_{positive\ control}$ - $OD_{negative\ control}$ X 100 where the negative control (0% haemolysis) was represented by erythrocytes suspended in saline and the positive control (100% haemolysis) was represented by the erythrocytes lysed with 1% triton X100 [36].

The LC50 values relative to the two peptides and the MIX were calculated as described [37].

Cell culture

J774 murine macrophages from the American Tissue Culture Collection (ATCC, Rockville, MD,USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Cambrex Bio Science, Verviers, Belgium). Culture media contained 10% fetal bovine serum (FBS, Sigma, Milan, Italy), 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Paisley, Scotland). Cells were seeded on 96-well plates (Falcon, Milan) for the MTT Assay, and on 24-well plates (Falcon, Milan) for NO_2^- measurements, fluorescence microscopy analysis, and RT-PCR assays. Cell monolayers were grown to adherence before the experiments were started.

Mice

Experiments were carried out on female BALB/c mice (aged 8 to 10 weeks) at the animal facility of the University of Naples. Bacteria (10^7 or 10^8 CFU/mouse) were inoculated by intravenous routes (i.v.). LPS (250 μg, ~10 mg/Kg) (Sigma-Aldrich Milan), or an equivalent volume of sterile 0,9% saline vehicle (250 μl) was administered intraperitoneally. Blood samples were drawn from the tail vein using 0.5 ml syringes. Spleen and kidney were collected at several time points (4,5,7,8,10, 11and 12 days) after the mice infection with a sub-lethal dose of *Staphylococcus epidermidis* (10^7 CFU/mouse). However the same organs were also collected at 3, 6, 9

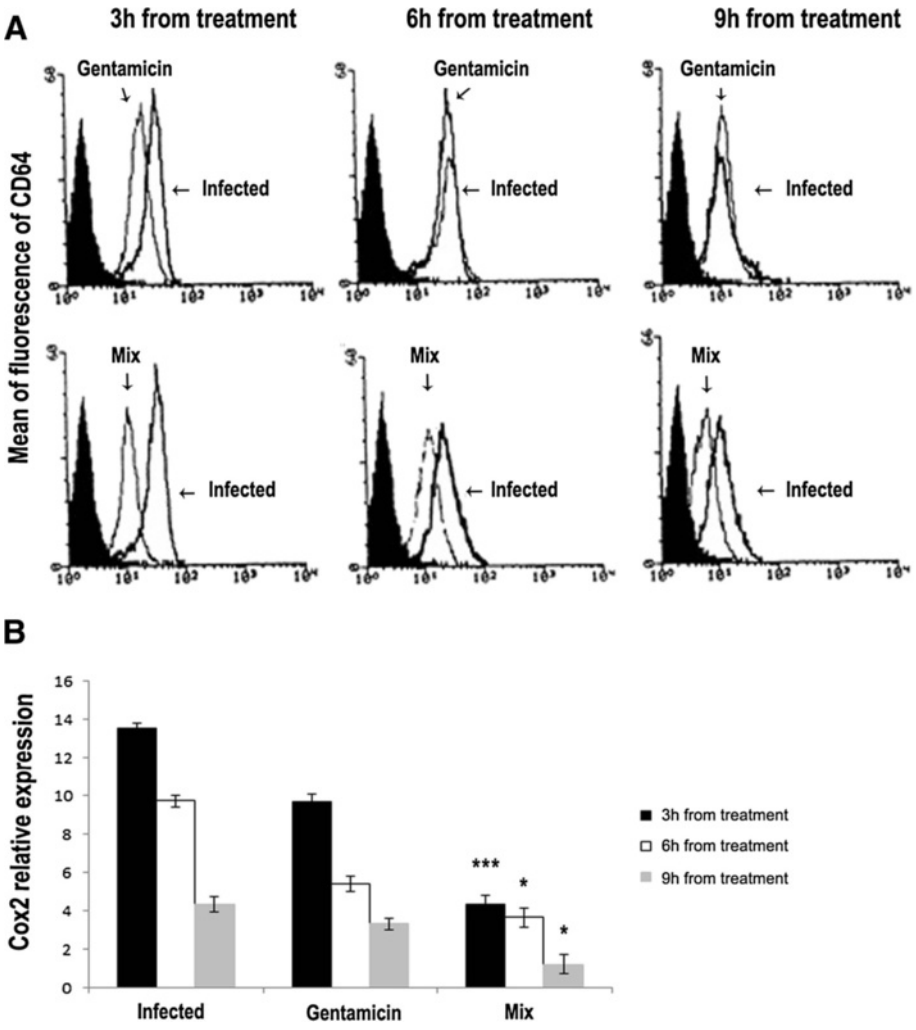

Figure 5 (A) Using flow cytometry, CD64 levels were measured at 3, 6 and 9 hours after treatment in blood samples from mice infected with Staphylococcus epidermidis (108 CFU/mouse), from mice infected with Staphylococcus.epidermidis (108 CFU/mouse) and treated either with MIX (RJI-C at 9 μg/mouse and TB-KK at 6 μg/mouse) or with gentamicin (5 μg/mouse). (**B**) mRNA expression level of COX-2, measured in kidneys of *Staphylococcus.epidermidis* (10^8 CFU/mouse) infected mice and in kidneys of *Staphylococcus epidermidis* (10^8 CFU/mouse) infected mice and treated with MIX (RJI-C at 9 μg/mouse and TB-KK at 6 μg/mouse) or gentamicin (5 μg/mouse) . *p <0.05, **p < 0.01; ***p < 0.001, Student's *t* test gentamicin vs MIX.

and 12 hours after infection with a lethal dose of *Staphylococcus epidermidis* (10^8 CFU/mouse). Spleens and kidneys were dissected and weighed. One g of each sample was homogenized in 1 ml saline and serially diluted in saline.

Colony forming units (CFU) were evaluated by the plate count assay. Animal experiments were approved by the Animal Care Committee of the University of Naples.

Measurement of cell viability

Analysis of cell viability was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay system (MTS assay) (Promega, Madison,WI, USA). J774 cells were seeded at 2500 cells per well in a 96-well plate and incubated at 37°C, in a humidified atmosphere with 5% CO_2. TB-KK 15 μg/ml, RJI-C 15 μg/ml, MIX (TB-KK 6 μg/ml + RJI-C 9 μg/ml) or RJII-C (Control 15 μg/ml) were added to the medium immediately after cell adhesion. At each time point 20 μl of CellTiter 96® AQueous One Solution reagent was added to each well, according to the manufacturer's instructions. Absorbance was recorded at 490 nm after 2 h using an EnVision 2102 multilabel reader (PerkinElmer, Waltham, USA).

Nitrite formation in J774 cells stimulated with LPS and treated with RJI-C, TB-KK, and the MIX

Nitrite accumulation (NO_2^-, μmol/10^6 cells) in the cell culture medium was determined by the Griess reaction [38].

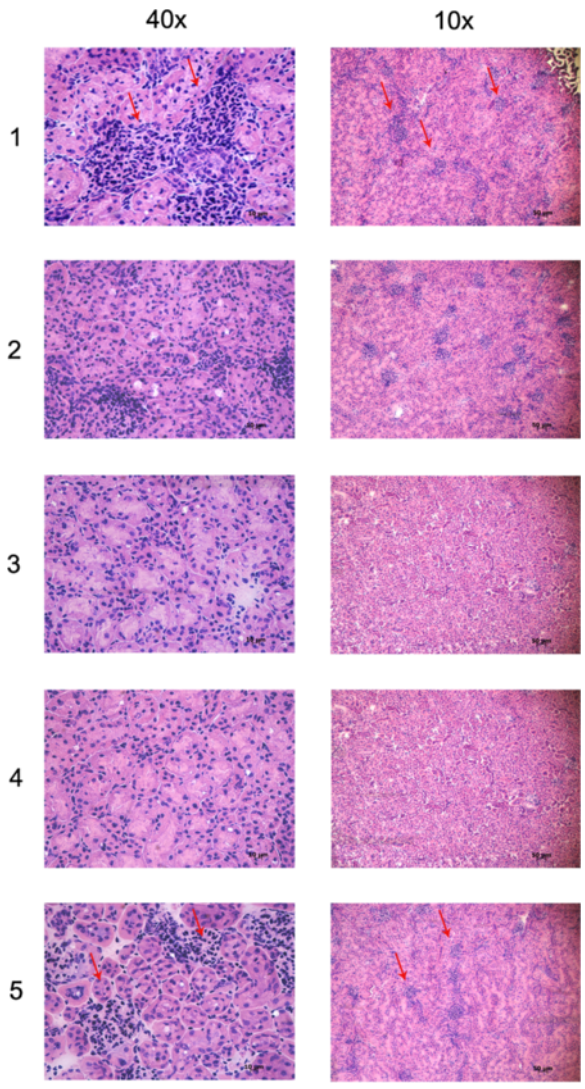

Figure 6 Haematoxylin eosin staining. Kidney sections from *Staphylococcus epidermidis* (10^8 CFU/mouse) infected mice after 3 or 9 hours (panel 1and 5); kidney sections from *Staphylococcus epidermidis* (10^8 CFU/mouse) infected mice after 3 hours and treated with MIX (RJI-C at 9 μg/mouse and TB-KK at 6 μg/mouse) for 3, 6 and 9 hours (panel 2–4).

Western Blot Analysis COX-2

Cell lysates for Western blotting were prepared by washing cells twice with ice-cold phosphate-buffered saline followed by cell lysis in 500 μl of Fastprep lysis buffer (1X protease inhibitor cocktail tablet (Roche EDTA free) resuspended in 1X PBS) on ice and lysed 20s at 6.5 intensity, 2X intervalling with 5–10 minutes on ice. Cell lysates were centrifuged for 10 min at 7800 *g* at 4°C, and the supernatants were collected and stored at −80°C until analysis. Lysate protein concentrations were measured using the Bio-Rad protein assay method, as described in the manufacturer's instructions. Cell lysate volumes corresponding to 20 μg of total protein were diluted 1:1 in Laemmli buffer (Bio-Rad) and boiled for 5 min prior to electrophoresis on a 10% acrylamide gel. The resolved proteins were electroblotted on PVDF membrane (Bio-Rad) by the Bio-Rad semidry transfer method, according to the manufacturer's instructions. Membranes were stained with PonceauRed to verify uniform protein transfer, and then blocked with blocking buffer (1X TBS, 0.1% Tween-20, 5% w/v non-fat dry milk) for 1 h at RT. Blocked membranes were incubated overnight at 4°C with COX-2 mouse monoclonal antibody (diluted 1/2000), β-actin mouse monoclonal antibody (diluted 1/10,000). Blots were washed three times in TBS-Tween before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse IgG diluted 1/5000) for 1 h at room temperature.

After three washes with TBS-Tween, the signal was developed using standard procedure. Gel image was acquired in Fujifilm LAS-3000 Chemiluminescence system (Fujifilm Life science).

Real time PCR of pro-inflammatory

Total RNA was isolated from the tissue and the cell line after treatment by using Trizol reagent (Invitrogen, Milan, Italy). RNA was suspended in RNase-DNAse free distilled water, assessed for concentration (by measuring the absorbance at 260 nm) and purity (by ascertaining that the A260/A280 ratio was .1.9). RNA (1 μg) was then treated with 1U RNAse-free DNAse (Promega, Madison, WI). DNA contamination of RNA samples was excluded by PCR with primers specific for the gapdh gene. Reverse transcription was carried out with ImProm-II reverse transcriptase (Promega, Madison, WI) and oligo (dT). Real-time PCR was performed on 50 ng cDNA, using 1x master mix SYBRGreen (Applied Biosystem, Milan) in a StepOne Applied Biosystem instrument (Applied Biosystem, Milan). Reactions were performed in 20 μl in triplicate. The primer list is reported in Additional file 7: Table S4.

ELISA test of pro-inflammatory cytokines

In addition, the ELISA test was used to measure the anti-inflammatory activity of the MIX and its components : RJI-C 9 μg/mL e TB-KK 6 μg/mL.

Briefly, J774 cells (10^6 cells/well) were stimulated with LPS (10 μg/ml; 1 hour), treated with RJI-C 9 μg/ml or TB-KK 6 μg/ml or MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml) in presence or absence of LPS (10 μg/ml). The supernatants from these cells (100 μl/well) were transferred into the wells of a plate previously coated with mouse anti-human TNF-α (BD Pharmingen; 50 μl diluted 2 x 10-3/well) or mouse anti-human IFN-γ (Biosciences, 50 μl diluted 2 x 10-3/well) along with a second dose of anti IFN- γ or TNF- α, HRP-labelled rabbit anti mouse IgG diluted 10^{-3} (100 μl/well) and TMB peroxidase

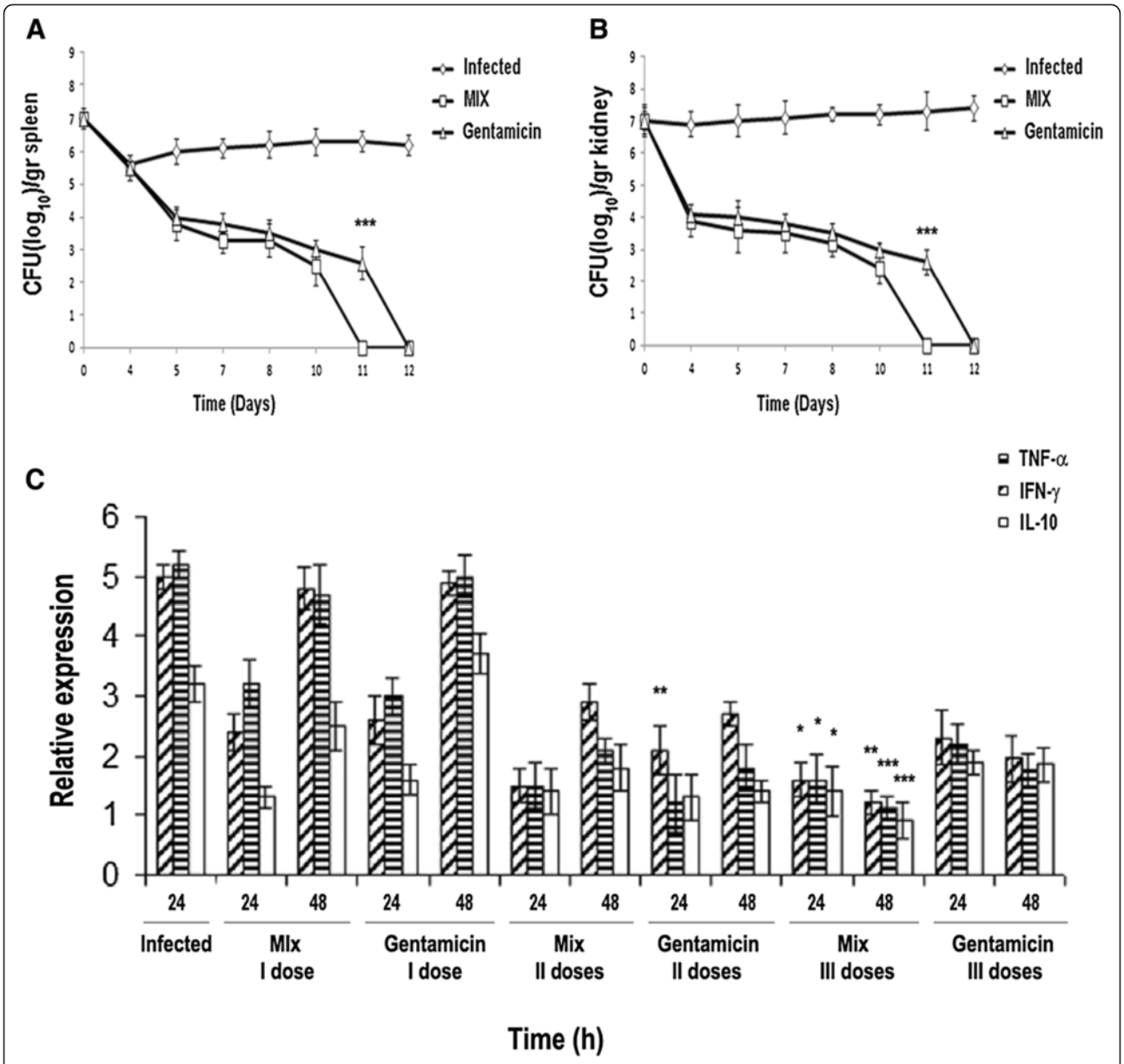

Figure 7 (A-B) Bacterial load in spleen and kidneys of animals (24/groups) infected with Staphylococcus epidermidis (107 CFU/mouse; rumble line); infected with Staphylococcus epidermidis(107 CFU/mouse) and treated with the MIX (RJI-C at 9 μg/mouse and TB-KK at 6 μg/mouse; square line) or gentamicin (5 μg/mouse; triangle line) *P <0.05, **p < 0.01; ***p < 0.001, Student's t test gentamicin vs MIX. (**C**) TNF-α, IFN-γ, IL-10 mRNA expression levels were quantified, at the indicated time points, in mice infected with *Staphylococcus epidermidis* (10^7 CFU/mouse) or infected with *Staphylococcus epidermidis* (10^7 CFU/mouse) and treated with three different doses (I,II,III) of the MIX (RJI-C at 9 μg in 100 μl/mouse and TB-KK at 6 μg in 100 μl/mouse) or gentamicin (5 μg in 100 μl/mouse). Values were normalized with GAPDH and compared to untreated control. *P <0.05, **p < 0.01; ***p < 0.001, Student's *t* test gentamicin vs MIX.

substrate (BIORAD; 100 μL/well), in the order. The optical density of each well was read at 405 nm using a microplate reader (Bio-Rad, Japan). Triplicate positive and negative controls were included in each plate [39].

Cytofluorimetric analysis

CD64 expression in total White Blood Cells was analyzed using a Flow cytometry EPICS Elite (Beckman Coulter, Fullerton, CA). Daily instrument quality control including fluorescence standardization, linearity assessment, and spectral compensation were performed to ensure identical operation from day to day. At least 10.000 events for each sample was analyzed and the data were saved for later analysis on EXPO32 software (Beckman Coulter). Data analysis was performed by using electronic gating on the basis of FSC and SSC excluded

cellular debris and nonviable cells. PE-coniugated anti-mouse CD64 expression was measured using a $\log_{10}$ scale. Briefly, 50 ul of whole blood was incubated for 10 minutes at room temperature with saturating amounts of phycoeritrine- conjugated anti-CD64 murine monoclonal antibody (Becton Dickinson) followed by red blood cell lysis with an ammonium chloride–based red cell lysis solution (Beckman Coulter, Fullerton, CA). Samples were then washed once and resuspended with phosphate-buffered saline at pH 7.4, to a volume of 1 mL.

Other methods

The kidney was fixed in 10% buffered formalin, sectioned (10 μm) and stained with hematoxylin-eosin according to standard protocols. Bacterial counts and cytokine levels were analyzed using Student's *t* test.

Additional files

Additional file 1: Table S1. Lethal concentration (LC_{50}) of Temporin B –KK, Royal jelleins-IC, MIX through their hemolytic activity on mouse erythrocytes.

Additional file 2: Figure S1. Anti-inflammatory activity. The levels of IFN- γ and TNF- α were determined by a sandwich ELISA test in J774 cells untreated; J774 cells infected with *S. epidermidis* for 1 h; J774 cells stimulated with RJI-C (9 μg/ml) for1 h; J774 cells stimulated with TB-KK. (6 μg/ml) for1 h; J774 cells stimulated with MIX (RJI-C 9 μg/ml + TB-KK 6 μg/ml) for1 h; J774 cells infected with *S. epidermidis* for 1 h and stimulated with MIX for 1 h. Results from two representative experiments are presented as mean value ± S.D. *P <0.05, **p < 0.01; ***p < 0.001, Student's *t* test *S. epidermidis* vs *S. epidermidis* + MIX.

Additional file 3: Figure S2 (A-B). Bacterial load in spleen and kidneys of *S. epidermidis* infected mice (rumble line) and subsequently treated with the MIX (square line) or gentamicin (triangle line). Data are representative of 15 animals/group. Student's *t* test gentamicin vs MIX not significant.

Additional file 4: Table S2. Acute phase proteins. Acute phase proteins from blood samples of mice infected with *Staphylococcus epidermidis* and treated with MIX or with Gentamicin.

Additional file 5: Figure S3. Time course (30, 60, 90 and 120 minutes) of bacterial load in blood of *S. epidermidis* infected mice.

Additional file 6: Table S3: Origin of S. epidermidis strains.

Additional file 7: Table S4: Sequences of the primers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RC designed the study and wrote the paper. AR and CA designed and synthesized the peptides. GB carried out the antibiotic resistance test. NN, RCM and MI carried out cell culture and in vivo tests. FDC, AF and FC carried out biochemical, statistical and in vivo tests. All authors read and approved the final manuscript.

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