Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2017;27:237–245
DOI: 10.1159/000479183

Received: April 4, 2017
Accepted: July 3, 2017
Published online: September 14, 2017

ERAP1 and *PDE8A* Are Downregulated in Cattle Protected against Bovine Tuberculosis

Federico Carlos Blanco[a] Marcelo Abel Soria[b] Laura Inés Klepp[a] Fabiana Bigi[a]

[a]Instituto de Biotecnología, CICVyA-INTA, and [b]Facultad de Agronomía, UBA, INBA-CONICET, Buenos Aires, Argentina

Keywords

Mycobacterium bovis · Transcriptomics · Cattle · Immune response

Abstract

Bovine tuberculosis (bTB) is a zoonotic disease caused by *Mycobacterium bovis* that is responsible for significant economic losses worldwide. In spite of its relevance, the limited knowledge about the host immune responses that provide effective protection against the disease has long hampered the development of an effective vaccine. The identification of host proteins with an expression that correlates with protection against bTB would contribute to the understanding of the cattle defence mechanisms against *M. bovis* infection. In this study, we found that *ERAP1* and *PDE8A* were downregulated in vaccinated cattle that were protected from experimental *M. bovis* challenge. Remarkably, both genes encode proteins that have been negatively associated with immune protection against bTB.

Introduction

Mycobacterium bovis is the etiological agent of bovine tuberculosis (bTB) that infects a wide variety of mammals. *M. bovis* is among the causal agents of human tuberculosis. Despite the enormous efforts to control the disease, bTB is still a threat to livestock production and human health in many regions of the world. The scientific community and major livestock countries are increasingly accepting the idea that vaccination of dairy cattle would be the most efficient way to control bTB [Vordermeier et al., 2016], especially in developing countries where the policy of test and slaughter is economically non-viable. Therefore, many vaccine candidates against bTB have been tested in cattle. However, the use of BSL3 containment for *M. bovis* challenge is mandatory in clinical trials with a bTB vaccine, which significantly restricts the number of vaccines to be tested. The increasing knowledge of the mechanisms involved in the interaction of *M. bovis* with cattle has allowed the identification of protective correlates of immunity against bTB that may facilitate the selection of vaccine candidates to be assayed in clinical trials [Waters et al., 2012].

Effective immune responses against tuberculosis infection rely on the development of a T helper type 1

E-Mail karger@karger.com
www.karger.com/mmb

Federico Carlos Blanco
Instituto de Biotecnología
CICVyA-INTA, N. Repetto and De Los Reseros
Hurlingham, Buenos Aires, CP 1686 (Argentina)
E-Mail blanco.federico@inta.gob.ar

(Th1)-biased cell-mediated immune response. The adaptive T cell response involves the activation of mycobacterial-specific CD4+, CD8+, and γδ T cells by dendritic cells. The T helper type 2 (Th2) response induced by *M. bovis* infection counteracts Th1, suppressing cell-mediated immunity responses and increasing humoral responses as the disease progresses [Pollock and Neill, 2002]. The Th1 response is characterised by the production of interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), and interleukin 12 (IL-12) cytokines, while the Th2 response involves the production of IL-4, IL-5, and IL-10 cytokines. The Th1/Th2 balance of the immune response determines the progress and the outcome of the tuberculosis disease. In addition, several reports have indicated that the IL-23/IL-17 balance is crucial to the regulation of the inflammatory consequences of mycobacterial infections [Khader and Cooper, 2008]. Despite the considerable advance in the comprehension of the key processes that are involved in effective immunity against tuberculosis, there are gaps in the knowledge of the protection mechanisms against bTB that remain to be filled.

Global transcriptional analysis has been used to define the repertoire of genes expressed in host-pathogen interactions in many infectious diseases. This kind of transcriptional approach has also allowed the identification of biomarkers of disease severity in cattle infected with *M. bovis*. By using a global transcriptional approach, Bhuju et al. [2012] identified for the first time correlates of protection that may be used to predict bTB vaccine success.

In this study, to contribute to bTB vaccine development and investigations of protective immune mechanisms, we evaluated the changes in the gene expression profile of bovine peripheral blood mononuclear cells (PBMCs) from vaccinated cattle that were either completely protected against *M. bovis* challenge or showed a low level of protection.

Results and Discussion

To identify host proteins with an expression level that correlates with protection against bTB, we compared the transcriptomes of *M. bovis* antigen-stimulated PBMCs of calves vaccinated with 3 attenuated *M. bovis* strains (BCG, ΔleuBCG overexpressing Ag85B, and *M. bovis* strain knockout in *mce2* operon [Blanco et al., 2013; Rizzi et al., 2012]) and identified those genes whose expression correlated with protection against *M. bovis* challenge. The study was focussed on the analysis of gene expression variability between vaccinated/protected and vaccinated/unprotected groups upon specific antigen stimulation. Therefore, gene expression analysis of both non-stimulated samples and non-vaccinated animals were not considered in this study. The PBMC samples were obtained in 2 previous vaccine trials [Blanco et al., 2013; Rizzi et al., 2012], in which a low proportion of the vaccinated calves were not fully protected against *M. bovis* challenge, showing tuberculous lesions in lungs and/or lymph nodes (LNs). We reasoned that the day before the challenge was the most favourable time point to detect a longer lasting vaccine response within the experimental schedule for these clinical trials, and for this reason animals of both vaccine trials were blood sampled 1 day before *M. bovis* challenge (60 days postvaccination [dpv]). PBMCs from blood samples were PPDB stimulated and stored in Trizol at –80°C until use. At the end of the vaccine trials the animals were grouped based on the degree of protection, regardless of the candidate vaccine inoculated. The clinical end point to define disease severity and thus protective efficacy of a vaccine was based on the presence of macroscopic lesions in lungs, lung LNs, and head LNs. Four animals with no macroscopic lesions in the lungs, lung LNs, and head LNs composed the vaccinated/protected group. Four animals with score values of 1 for lungs (1 or 2 macroscopic lesions) and 1–3 for lung LNs (6–19 lesions) composed the vaccinated/unprotected group; these scores were the highest observed among vaccinated animals (Table 1). Only PBMC samples from the 8 selected animals were used for further analysis. All non-vaccinated animals of both vaccine trials showed significant macroscopic lesions in the lungs and LNs, indicating a very good performance on the challenging procedure used to infect the animals.

Comprehensive gene expression profiles of PBMC samples from both experimental groups were generated with 8 high-density oligonucleotide bovine arrays (Affymetrix) with 24,072 probe sets. Considering a threshold of 0.05 for the adjusted *p* value, 70 unique probe sets showed a significant differential expression. A total of 46 and 24 genes were either upregulated or downregulated at 60 dpv in the vaccinated/protected group compared to the vaccinated/unprotected group (Table 2). Further inspection of these differentially expressed genes revealed that many of them are related to the phosphorylation of proteins and Golgi apparatus and organelles, as revealed from a GO term enrichment analysis (see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.159/000479183).

Despite its importance, there is only one study in the literature that identifies and characterises the genes of cat-

Table 1. Summary of the vaccine trial studies

Animal	Vaccine	Macroscopic lesions, *n*			Pathology score			Classification
		L	LLN	HLN	L	LLN	HLN	
356	*M. bovis* Δmce2	0	0	0	0	0	0	Protected
358	*M. bovis* Δmce2	0	0	0	0	0	0	Protected
361	Δ*leuD* BCG-85B	0	0	0	0	0	0	Protected
374	Δ*leuD* BCG-85B	0	0	0	0	0	0	Protected
369	BCG	2	19	0	1	1	0	Unprotected
372	Δ*leuD* BCG-85B	1	11	0	1	1	0	Unprotected
375	BCG	2	6	0	1	1	0	Unprotected
378	*M. bovis* Δmce2	1	17	0	1	3	0	Unprotected

Candidate vaccine inoculated, macroscopic lesions observed at necropsy, and classification according to protection status for cattle used in the present study. L, lungs; LLN, lung lymph nodes; HLN, head lymph nodes. Pathology scores: lung lesion scores: 0, no lesions; 1, 1–9 lesions; 2, 10–29 lesions; 3, 30–99 lesions; 4, 100–199 lesions; 5, ≥200 lesions. Total LN lesion score per animal for individual nodes: 0, no lesions; 1, 1–19 small lesions (diameter 1–4 mm); 2, ≥20 small lesions; 3, medium-sized lesions (diameter 5–9 mm); 4, large lesions (diameter ≥10 mm).

tle with an expression that correlates with protection against bTB [Bhuju et al., 2012]. This previous study identified a larger number of differentially expressed genes [Bhuju et al., 2012] compared to the results obtained here. However, the remarkable disparity between the experimental designs of their study and ours may explain the different results obtained: (i) they used massive RNA sequencing and this method has an increased specificity (low background signal) and sensitivity, a broader dynamic range, and can explore new transcripts compared to microarrays; (ii) the vaccinated/unprotected group studied here included animals with low lung pathology (few visible lesions in lungs in post-mortem examination), whereas in the Bhuju et al. [2012] study the unprotected group comprised animals with a pathology undistinguishable from unvaccinated control animals. Therefore, with fewer pathological changes between the groups, a lower number of differentially expressed genes was expected in this study. In spite of these differences, 3 genes (*PIM2*, *SGOL2*, and *SLC35A5*) changed their expression levels between vaccinated/protected and vaccinated/unprotected cattle in this study and that of Bhuju et al. [2012]. Interestingly, 2 of these differentially expressed genes are involved in cellular processes.

Among the differentially expressed genes with a higher fold change (greater than ±0.8) between conditions were genes likely involved in autophagy and apoptosis (*GBP6* and *HTRA4*), cytoskeleton rearrangement (*TTLL1* and *TNIK*), inflammatory response (*LTB*), T cell activation (*PDE8A*), antigen presentation (*ERAP1*), receptors of stress ligands in immune cells (*KLRK1*), and a lysosome protein (*AGA*). All these processes are related to the innate or adaptive immune response against tuberculosis.

GBP6 is a member of the 65-kD guanylate-binding protein (Gbp) family (Gbp1, Gbp6, Gbp7, and Gbp10). It has been postulated that this family is involved in oxidase-dependent killing and autophagy pathways in phagocytes to combat listerial or mycobacterial infection. Thus, 65-kD Gbps function to co-ordinate a potent oxidative and vesicular trafficking program to protect the host from infection [Kim et al., 2011a].

HTRA proteins belong to a unique family of multidomain serine proteases conserved from prokaryotes to humans that participate in the degradation of abnormal periplasmic proteins. In humans, HTRA participates in the regulation of cell death, cell signalling and motility, apoptosis, and other processes [Singh et al., 2015]. Interestingly, in a fusion with Rv1196 protein, an *M. tuberculosis* HtrA (called Rv0125) has shown to enhance the protection conferred by BCG in *M. tuberculosis*-infected guinea pigs [Brandt et al., 2004].

TNIK is a member of the germinal centre kinase family involved in cell spreading or migration through cytoskeleton organisation [Lee et al. 2017]. However, various evidence [Mahmoudi et al., 2009] suggests that TNIK participates in the regulation of the inflammatory response against *Salmonella* [Liu et al., 2010] and *Mycobacterium* [Neumann et al., 2010].

Lymphotoxin beta (LTB) is a type II membrane protein of the TNF family that is important for innate and adap-

Table 2. Transcriptional response of PBMCs from cattle immunised with candidate vaccines against bTB

Affitmetrix ID	log FC	Average expression	Adjusted *p* value	Entrez ID	Gene name	Genbank	Refseq	Symbol
a List of genes significantly upregulated in vaccinated/protected calves compared to vaccinated/unprotected animals; PBMCs were isolated prior to *M. bovis* infection and stimulated with bovine PPD								
Bt.18440.3.A1_at	3.442	3.717	0.015	510382	guanylate-binding protein 6-like	CK775666	XR_083420	GBP6
Bt.6803.1.A1_at	1.769	2.857	0.031	539627	TRAF2 and NCK interacting kinase	BF440275	XM_002684922	TNIK
Bt.12712.2.S1_at	1.284	7.928	0.031	540313	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	CB450619	NM_001099106	ETS1
Bt.17874.1.A1_at	1.207	1.693	0.031	514946	HtrA serine peptidase 4	CB435726	NM_001272014	HTRA4
Bt.20365.1.S1_at	1.112	6.176	0.015	508424	pim-2 oncogene	CK976360	NM_001206378	PIM2
Bt.3115.1.A1_at	1.111	2.487	0.042	511345	aspartylglucosaminidase	CK846513	NM_001075511	AGA
Bt.1565.1.S1_at	0.977	4.270	0.015	512648	elongation factor RNA polymerase II-like 3	CK776111	NM_001046126	ELL3
Bt.3780.1.S1_at	0.956	6.269	0.015	100125763	chromosome 10 open reading frame; human C5orf13	CB447606	NM_001105045	C10H5orf13
Bt.3651.2.A1_at	0.897	4.974	0.015	100270684	chromosome 15 open reading frame; human C11orf96	CB440199	NM_001145035	C15H11orf96
Bt.24545.1.S1_at	0.885	1.825	0.031	404058	killer cell lectin-like receptor subfamily K; member 1	CK952080	NM_001075139	KLRK1
Bt.21431.1.S1_at	0.839	9.147	0.039	529757	lymphotoxin beta (TNF superfamily; member 3)	CK948133	XM_002697371	LTB
Bt.2846.1.A1_at	0.730	3.879	0.031	781044	ras homolog gene family; member U	CK769446	NM_001098147	RHOU
Bt.13777.2.S1_at	0.716	8.366	0.031	100125415	GTPase; IMAP family member 7	BM446151	NM_001105034	GIMAP7
Bt.22234.1.S1_at	0.711	7.627	0.031	505901	protein kinase C; theta	BI683322	NM_001192077	PRKCQ
Bt.23131.1.S1_at	0.685	5.958	0.031	789163	family with sequence similarity 134; member C	CK961524	XM_001255597	FAM134C
Bt.27420.1.A1_at	0.657	3.232	0.025	789222	PR domain containing 2; with ZNF domain	CK848881	XM_003583252	PRDM2
Bt.26815.1.S1_at	0.645	4.605	0.042	616564	single-stranded DNA binding protein 2	CB533272	NM_001035478	SSBP2
Bt.28480.1.S1_at	0.639	4.771	0.025	506073	phospholipase A2-activating protein	BE667456	NM_001105335	PLAA
Bt.15887.2.S1_a_at	0.606	8.059	0.039	509076	zinc and ring finger 1	BE758011	NM_001192210	ZNRF1
Bt.12957.1.A1_a_at	0.603	7.140	0.031	528167	trinucleotide repeat containing 6B	CB170194	NM_001192655	TNRC6B
Bt.2196.1.A1_at	0.588	3.151	0.044	522721	zinc finger. MYM-type 3	CK773306	NM_001193249	ZMYM3
Bt.25776.1.A1_at	0.585	2.904	0.031	540812	armadillo repeat containing 12	CK773277	XM_002697212	ARMC12
Bt.12561.1.S1_at	0.575	5.525	0.031	617894	ras homolog gene family; member F (in filopodia)	CK770695	NM_001040602	RHOF
Bt.24440.1.S1_at	0.575	5.205	0.044	504668	megakaryocyte-associated tyrosine kinase	CK769212	NM_001035011	MATK
Bt.15942.1.A1_at	0.567	7.239	0.039	512524	sushi domain containing 3	CB169651	XM_002689847	SUSD3
Bt.1054.1.S1_at	0.554	6.617	0.038	522521	Rho/Rac guanine nucleotide exchange factor (GEF) 18	CK962863	XM_002688833	ARHGEF18
Bt.12039.1.S1_at	0.529	5.254	0.038	507839	protein arginine methyltransferase 2	CK775810	NM_001024495	PRMT2
Bt.17436.1.S1_at	0.505	4.908	0.031	504554	Tctex1 domain containing 2	CK769279	NM_001166555	TCTEX1D2
Bt.20360.1.A1_at	0.494	4.740	0.036	518632	NUAK family; SNF1-like kinase 2	CB460226	NM_001079597	NUAK2
Bt.22387.1.S1_a_at	0.488	5.844	0.025	513458	nei endonuclease VIII-like 1 (*E. coli*)	BM088506	NM_001014917	NEIL1
Bt.7951.1.S1_at	0.479	4.419	0.038	505097	sphingomyelin phosphodiesterase 1; acid lysosomal	CK772152	NM_001075187	SMPD1
Bt.8800.1.S1_at	0.478	4.815	0.039	444859	ST3 β-galactoside alpha-2.3-sialyltransferase 3	CK777084	NM_001002882	ST3GAL3
Bt.4865.1.S1_at	0.474	1.895	0.038	540451	elastin microfibril interfacer 1	CK960276	NM_001192434	EMILIN1
Bt.9350.1.A1_at	0.467	1.406	0.039	613472	ganglioside-induced differentiation-associated protein 1	CK975062	NM_001101222	GDAP1
Bt.25479.1.S1_at	0.456	5.745	0.044	618186	CTF8; chromosome transmission fidelity factor 8 homolog (*S. cerevisiae*)	CK770425	NM_001109804	CHTF8
Bt.20517.1.S1_at	0.451	5.641	0.038	538529	solute carrier family 25; member 28	CK771221	NM_001205552	SLC25A28
Bt.13087.1.S1_at	0.430	2.935	0.039	282881	CD6 molecule	AB042274	XM_002699258	CD6
Bt.12770.1.A1_at	0.425	1.485	0.039	282857	synuclein; alpha (non-A4 component of amyloid precursor)	CB425735	NM_001034041	SNCA
Bt.21087.1.A1_at	0.424	2.506	0.038	514216	transcription factor 19	CK772914	NM_001083684	TCF19
Bt.9505.1.A1_at	0.411	3.712	0.039	527458	kelch-like family member 26	CK776961	XM_002688529	KLHL26
Bt.27003.1.S1_at	0.403	2.810	0.044	614455	retinol saturase (all-trans-retinol 13.14-reductase)	CK946791	NM_001102279	RETSAT
Bt.28518.1.S1_at	0.399	1.123	0.044	404172	pancreatic trypsin inhibitor	CB224161	NM_001001554	PTI

Table 2 (continued)

Affitmetrix ID	log FC	Average expression	Adjusted *p* value	Entrez ID	Gene name	Genbank	Refseq	Symbol
Bt.8669.1.S1_at	0.395	1.366	0.042	785489	membrane bound O-acyltransferase domain containing 2	CB441263	XM_001253490	MBOAT2
Bt.1430.1.S1_at	0.381	7.797	0.044	282848	prefoldin subunit 5	CB533409	NM_174732	PFDN5
Bt.12071.2.S1_at	0.381	4.651	0.042	505662	sirtuin 7	CB459499	NM_001075217	SIRT7
Bt.6586.1.S1_at	0.350	3.400	0.038	789485	notch 3	AU278833	XM_003582382	NOTCH3
b Downregulated genes in PBMCs of protected animals								
Bt.21054.1.A1_at	–1.810	4.066	0.016	506787	phosphodiesterase 8A	CK773933	NM_001191455	PDE8A
Bt.3125.1.S1_at	–1.178	2.768	0.015	539530	tubulin tyrosine ligase-like family; member 1	CB464371	NM_001076171	TTLL1
Bt.28338.1.S1_at	–0.838	4.012	0.025	514617	endoplasmic reticulum aminopeptidase 1	CK954890	NM_001102003	ERAP1
Bt.6700.1.A1_at	–0.834	2.901	0.038	515917	pancreatic trypsin inhibitor-like	BP107527	XM_002692339	LOC515917
Bt.7067.1.A1_at	–0.691	4.456	0.036	100125307	methyltransferase like 21B	BF651616	NM_001103340	METTL21B
Bt.23657.1.A1_at	–0.658	2.486	0.031	506046	coiled-coil domain containing 90B	BP104854	NM_001101863	CCDC90B
BtAffx.1.20.S1_at	–0.652	2.351	0.044	614441	regulator of G-protein signaling 20	U89254	NM_001076327	RGS20
Bt.4786.1.S1_at	–0.609	9.352	0.015	281080	clathrin. heavy chain (Hc)	CB464066	NM_174023	CLTC
Bt.28187.1.S1_at	–0.608	4.686	0.034	540322	WEE1 homolog (*S. pombe*)	AW461388	NM_001101205	WEE1
Bt.22626.1.A1_at	–0.588	6.278	0.031	534739	ankyrin repeat domain 12	CB463743	XM_002697743	ANKRD12
Bt.27320.1.A1_at	–0.587	5.759	0.031	532412	shugoshin-like 2 (*S. pombe*)	CK848417	NM_001191290	SGOL2
Bt.28047.1.A1_at	–0.586	3.892	0.015	528478	protein kinase C, iota	CK976492	NM_001205955	PRKCI
Bt.17311.2.S1_at	–0.581	2.660	0.031	534054	retinitis pigmentosa 2 (X-linked recessive)	CB428565	NM_001035403	RP2
Bt.25171.1.A1_at	–0.562	5.123	0.036	510557	heat shock transcription factor 2	CK729778	NM_001083405	HSF2
Bt.8032.1.S1_at	–0.503	6.767	0.031	537619	kinectin 1 (kinesin receptor)	BE681760	NM_001102205	KTN1
Bt.4713.1.S1_at	–0.493	3.954	0.039	281416	proopiomelanocortin	NM_174151	NM_174151	POMC
Bt.17473.1.S1_at	–0.484	5.033	0.025	533764	ribulose-5-phosphate-3-epimerase	CK951838	NM_001098079	RPE
Bt.29874.1.S1_at	–0.478	5.821	0.031	406231	eukaryotic translation initiation factor 2C, 3	AY436348	NM_001001133	AGO3
Bt.17757.1.S1_at	–0.477	3.653	0.038	534488	solute carrier family 35; member A5	BM032171	NM_001076025	SLC35A5
Bt.10928.1.A1_at	–0.454	2.613	0.038	513263	retinitis pigmentosa GTPase regulator	CK966935	NM_001098383	RPGR
Bt.13620.1.A1_at	–0.441	1.724	0.040	618190	KIAA1310 ortholog	CB449832	NM_001099172	KIAA1310
Bt.18123.2.S1_at	–0.415	2.478	0.031	614920	golgin; RAB6-interacting	BG689203	NM_001167897	GORAB
Bt.20392.1.A1_at	–0.408	6.770	0.042	539052	eukaryotic translation initiation factor 3; subunit J	CB530299	NM_001076143	EIF3J
Bt.15981.1.S1_at	–0.377	1.717	0.040	337885	G protein-coupled receptor 44	AY194233	XM_001254753	GPR44

tive immune responses. LTB is predominantly expressed in B and T lymphocytes, plasma cells, and natural killer (NK) cells. A role for LTB has been suggested in resistance to *M. tuberculosis* infections [Ehlers et al., 2003].

The killer cell lectin-like receptor subfamily K member 1 (KLRK1) is a member of the C type lectin-like receptor family that is expressed on NK cells, CD8+ T cells, γδ+ T cells, and NKT cells. KLRK1 recognises a number of inducible ligands that are major histocompatibility complex (MHC) class I-like proteins. Cellular stress, such as transformation and viral or bacterial infection, including infection with *M. tuberculosis* [Das et al., 2001], can lead to the induction of KLRK1 ligands on cells. Ligand-receptor interactions can result in the activation of NK and T cells, which results in the recognition and elimination of the cell by the immune system. Some studies have shown that KLRK1 may play a role in tuberculosis. Blocking KLRK1 leads to inhibition of *M. tuberculosis*-specific CD8+ T cells in vitro [Rausch et al., 2006] while KLRK1 expression is upregulated by mycobacterial stimulation in vitro [Cliff et al., 2004].

The aspartylglucosaminidase APA is a hydrolase that catalyses the final steps in the lysosomal breakdown of glycoproteins [Enomaa et al., 1992]. Despite its lysosomal location, a role for this protein during phagocytosis of intracellular pathogens has not been reported so far.

ERAP1 encodes an endoplasmic reticulum aminopeptidase 1 that plays a central role in peptide trimming, a step required for the generation of most human leukocyte antigen (HLA) class I-binding peptides. Inhibition of ERAP1 peptide trimming is a viral strategy to manipulate the host immune response. For example, human cytomegalovirus-HCMV inhibits ERAP1 expression during viral infection [Kim et al., 2011b], which leads to the

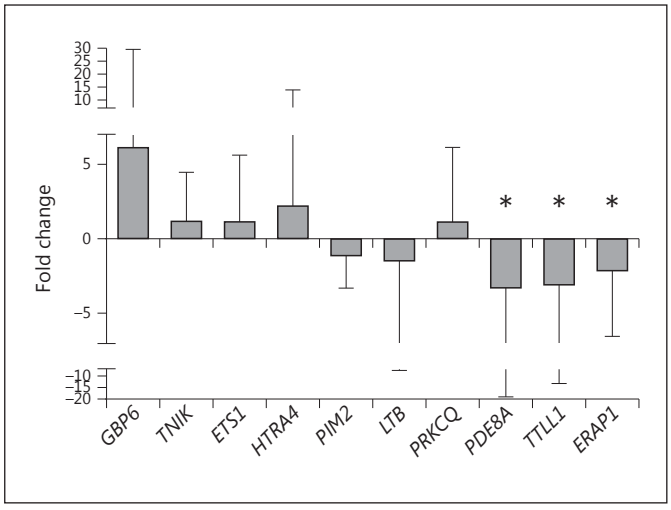

Fig. 1. Relative gene expression between PBMCs from vaccinated/unprotected animals (n = 4) and vaccinated/protected animals (n = 4) obtained by RT qPCR. The fold change was calculated using *gadph* mRNA expression as the reference gene and unprotected condition as the calibrator. Data were analysed using a random permutation test (fg statistical software, * $p < 0.05$). Bars represent the average expression ratios ± SD between the protected and unprotected conditions.

downregulation of classical MHC class I molecules and upregulation of the non-classical MHC class I molecule HLA-E [Tomasec et al., 2000]. Remarkably, responses to HLA-E binding peptides of *M. tuberculosis* by CD8 T cells with many characteristics of Th2 cells have been identified [van Meijgaarden et al., 2015]. This novel unorthodox CD8 T cell phenotype displays cytotoxic and immunoregulatory activities that can strongly inhibit the growth of *M. tuberculosis* in human macrophages [van Meijgaarden et al., 2015]. Therefore, the lower expression of *ERAP1* in protected cattle may result in a change in the immunodominance hierarchies in favour of a protective cytotoxic T lymphocyte response.

PDE8A encodes cyclic nucleotide phosphodiesterase-8. PDE families can be grouped into 3 broad categories; some are specific for cAMP hydrolysis (PDE4, PDE7, and PDE8), others are cGMP specific (PDE5, PDE6, and PDE9), and some families exhibit dual specificity (PDE1, PDE2, PDE3, PDE10, and PDE11), hydrolysing both cAMP and cGMP with different affinities. In particular, PDE8 family members are enzymes characterised by their relatively high affinity and specificity for cAMP [Soderling et al., 1998]. The expression of PDE8A was detected in the testes, in cardiomyocytes [Martinez et al., 2014], and in effector CD4+ T cells [Vang et al., 2016]. In *Bordetella pertussis*-infected monocytes, high levels of cAMP causes the inhibition of RhoA GTPase activity and subverts phagocytic functions, decreases the capacity of dendritic cells to present soluble protein antigen to CD4+ T cells, and blocks the capacity of macrophages to kill bacteria [Kamanova et al., 2008]. In this context, low transcription of *PDE8A* in dendritic cells and macrophages, and in turn high levels of cAMP in vaccinated animals, does not seem to be coherent with an effective induction of a protective immune response against *M. bovis*. However, it has been extensively demonstrated that the expression of IFN-γ by T cells in response to a microbial pathogen, including *M. tuberculosis*, is dependent on a CREB (cAMP response element-binding) protein, among other factors. CREB is particularly known to bind to the IFN-γ promoter and enhances the transcription of IFN-γ by primary human T cells that are stimulated with *M. tuberculosis* [Samten et al., 2002, 2005]. Since cAMP is necessary for T cells to produce IFN-γ in response to *M. tuberculosis* and likely to *M. bovis*, a low level of PDE8A expression in PBMC is consistent with a more effective immune response against tuberculosis with a higher production of IFN-γ.

TTLL1 is a member of a large family of proteins with a TTL homology domain that regulates the dynamics of the microtubules by catalysing the ligations of glutamate side chains of variable lengths on tubulins. Since polyglutamylation occurs in other protein substrates and may be considered as a potential regulator of diverse cellular functions [Janke et al., 2005] there is not a clear relationship between the transcription of *TTLL1* and the lack of protection against bTB.

The 10 most differentially expressed genes in the microarray experiment were assessed by RT quantitative (q) PCR (Fig. 1). The RT qPCR analysis was performed on RNA samples obtained 60 dpv from the same sets of animals used for microarrays analysis. As shown in Figure 1, *PDE8A*, *ERAP1*, and *TTLL1* expression showed significant downregulation in the protected group, which is consistent with the microarray results. The RT qPCR data for *GBP6*, *TNIK*, *ETS1*, *PRKCQ*, and *HTR4A* genes were consistent with that of the microarray experiments but the differences were not statistically significant. In contrast to the microarray results, the expression of *LTB* and *PIM2* was downregulated in the protected group, and differences were not statistically significant between the groups. Therefore, *PDE8A*, *ERAP1*, and *TTLL1* changed their expression between the groups (in microarray and RT qPCR experiments) irrespective of the vaccine used, which makes these genes good targets to be validated as bTB protective biomarkers.

Conclusions

The low expression of *ERAP1* and *PDE8A* in the context of protective immunity against tuberculosis is consistent with a possible scenario in which the non-classical MHC class I molecule HLA-E is upregulated and the production of IFN-γ is increased, respectively, representing 2 immunological events that should favour the killing of intracellular mycobacteria. In addition, the *GBP6*, *HTRA*, *TNIK*, and *KLRKI* genes, which were upregulated in the protected group, express proteins that also participate in the immune and/or cellular responses against tuberculosis. However, their differential expression could not be confirmed by RT qPCR for the first 3 genes, probably due to the limited sample sizes and the high biological variation among animals. Therefore, the differentially expressed genes identified here would clearly assist the evaluation and characterisation of candidate vaccines. However, further validation in samples from independent experiments is required.

Experimental Procedures

Vaccine Trials and Sample Collection

PBMCs were obtained from blood samples of cattle from 2 bTB vaccine trials. The animals were sampled at 60 dpv and 1 day before challenge with virulent *M. bovis* (*M. bovis* 04–303). At the end of these vaccine trials, the animals were euthanised and necropsy was conducted paying special attention to typical tuberculous lesions [Blanco et al., 2013; Rizzi et al., 2012]. Pathology scores were determined as previously published [Wedlock et al., 2008] to evaluate vaccine efficacy.

PBMCs Isolation and RNA Purification

Heparinised blood (10 mL) from each animal was used for PBMC isolation by gradient centrifugation over histopaque 1077 (Sigma Aldrich, St Louis, MO, USA) following the manufacturer's protocol. Cells were stimulated in 12-well tissue culture plates with a 20-μg/mL final concentration of PPDB (Biocor Animal Health, Omaha, NE, USA) overnight (16 h) at 37°C [Bhuju et al., 2012] in RPMI complete medium supplemented with 10% bovine foetal serum (Internegocios, Mercedes, Buenos Aires). Total RNA was purified from 1 × 10e7 PBMCs with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and RNeasy MiniElute kit (Qiagen, Hilden, Germany). The RNA quality and quantity were verified by microelectrophoresis in a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

Microarray Hybridisation

The Affymetrix GeneChip Bovine Genome Array platform (Affymetrix, Santa Clara, CA, USA) was used in this study. The array contains 24,027 probe sets representing over 23,000 transcripts from *Bos taurus* and includes approximately 19,000 annotated UniGene clusters. Each RNA sample was processed and hybridised to individual slides. Target preparation and microarray processing procedures were carried out at the Affymetrix facility of the School of Agronomy of the University of Buenos Aires, as described in the Affymetrix GeneChip expression analysis manual, and scanning was performed with a Microarray Scanner 3000 7G (Affymetrix). Eight microarrays were used in total (1 for each RNA sample).

Statistical Analysis of Microarray Data

The analysis of expression data was performed with different packages from the Bioconductor project (http://www.bioconductor.org/), an extension for bioinformatics of the R statistical language (http://www.r-project.org/). The data quality of cell intensity (CEL) files obtained from the Affymetrix chips was assessed with the affyPLM [Brettschneider et al., 2008] package. The expression data were normalised using the rma function of the package Affy [Gautier et al., 2004], which essentially reproduces the standardisation procedure of Affymetrix MicroArray Suite (MAS) software. To filter the arrays, the genefilter package was used. Only probe sets with entries in the NCBI gene database (http://www.ncbi.nlm.nih.gov/gene) were considered, and an Anova filter was applied to remove genes with little variation between treatments or high levels of noise (cut-off = 0.055). A total of 203 probe sets passed the filtering step. The detection of probe sets with differential expression between the protected and unprotected animals was performed with linear models and empirical Bayes methods to correct for multiple comparisons as implemented in the Linear Models for Microarray Data (LIMMA) package [Smyth, 2004]. The functional analysis of differentially expressed genes was performed with the DAVID tool [Huang et al., 2009].

Validation of Microarray Results by RT qPCR

In total, 200 ng of each RNA sample was employed for cDNA synthesis. Reverse transcription was made using SuperScrip reverse transcriptase II (Thermo Fisher Scientific). qPCR was performed in an Applied Biosystems Step One plus real-time thermocycler (Thermo Fisher Scientific) under standard cycling conditions. Specific oligonucleotides for 10 selected genes were designed using primer 3 [Untergasser et al., 2007] and employed at a final concentration of 300 nM. The primer sequences, PCR efficiencies, and the melting temperature of the amplicon are listed in online supplementary Table 2. Reactions were carried out using Taq platinum polymerase mix (Thermo Fisher Scientific), SYBR green I (Roche, Penzberg, Germany), 1 μL of a 2-fold dilution cDNA template and primers. All reactions were performed in duplicate, and qPCR data were analysed using the 2–ΔΔCq method with efficiency correction. The amplification curves were studied using LinReg software [Ramakers et al., 2003]. The fold change was calculated using *gapdh* as the reference gene [Robinson et al., 2007] and the unprotected condition as calibrator. A permutation test developed by Pfaffl et al. [2002] and included in the *fg statistic* software [di Rienzo et al., 2013] was used for the statistical analysis of the data. This test uses the $2^{-\Delta\Delta CT}$ algorithm and permutations between control and treatment samples as a method to analyse the relative changes in gene expression. The qPCR procedure was designed according to MIQE general recommendations [Bustin et al., 2009].

Acknowledgements

This work was funded by INTA grant PNBIO1131034 and ANPCyT grant PICT (2014 3637). B.F.C., K.L.I., and B.F. are CONICET fellows. We are grateful to Julia Sabio y García for critical reading of the manuscript.

Statement of Ethics

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which they were conducted.

Disclosure Statement

The authors declare that they have no conflicts of interest.

References

Bhuju S, Aranday-Cortes E, Villarreal-Ramos B, Xing Z, Singh M, Vordermeier HM: Global gene transcriptome analysis in vaccinated cattle revealed a dominant role of IL-22 for protection against bovine tuberculosis. PLoS Pathog 2012;8:e1003077.

Blanco FC, Bianco MV, Garbaccio S, Meikle V, Gravisaco MJ, Montenegro V, Alfonseca E, Singh M, Barandiaran S, Canal A, Vagnoni L, Buddle BM, Bigi F, Cataldi A: *Mycobacterium bovis* Δmce2 double deletion mutant protects cattle against challenge with virulent *M. bovis*. Tuberculosis 2013;93:363–372.

Brandt L, Skeiky YAW, Alderson MR, Lobet Y, Dalemans W, Turner OC, Basaraba RJ, Izzo AA, Lasco TM, Chapman PL, Reed SG, Orme IM: The protective effect of the *Mycobacterium bovis* BCG vaccine is increased by coadministration with the *Mycobacterium tuberculosis* 72-kilodalton fusion polyprotein Mtb72F in *M. tuberculosis*-infected guinea pigs. Infect Immun 2004;72:6622–6632.

Brettschneider J, Collin F, Bolstad BM, Speed TP: Quality assessment for short oligonucleotide microarray data. Technometrics 2008;50:241–264.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–622.

Cliff JM, Andrade INJ, Mistry R, Clayton CL, Lennon MG, Lewis AP, Duncan K, Lukey PT, Dockrell HM: Differential gene expression identifies novel markers of CD4+ and CD8+ T cell activation following stimulation by *Mycobacterium tuberculosis*. J Immunol 2004;173:485–493.

Das H, Groh V, Kuijl C, Sugita M, Morita CT, Spies T, Bukowski JF: MICA engagement by human Vγ2Vδ2 T cells enhances their antigen-dependent effector function. Immunity 2001;15:83–93.

di Rienzo JA, Casanoves F, Balzarini M, Gonzalez L, Tablada M, Robledo C: Infostat: Software Estadístico. Córdoba, Universidad Nacional de Córdoba, 2013. http://www.infostat.com.ar/.

Ehlers S, Hölscher C, Scheu S, Tertilt C, Hehlgans T, Suwinski J, Endres R, Pfeffer K: The lymphotoxin β receptor is critically involved in controlling infections with the intracellular pathogens *Mycobacterium tuberculosis* and *Listeria monocytogenes*. J Immunol 2003;170:5210–5218.

Enomaa N, Heiskanen T, Halila R, Sormunen R, Seppala R, Vihinen M, Peltonen L: Human aspartylglucosaminidase: a biochemical and immunocytochemical characterization of the enzyme in normal and aspartylglucosaminuria fibroblasts. Biochem J 1992;286:613–618.

Gautier L, Cope L, Bolstad BM, Irizarry RA: affy – analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 2004;20:307–315.

Huang DW, Sherman BT, Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.

Janke C, Rogowski K, Wloga D, Regnard C, Kajava AV, Strub JM, Temurak N, van Dijk J, Boucher D, van Dorsselaer A, Suryavanshi S, Gaertig J, Eddé B: Tubulin polyglutamylase enzymes are members of the TTL domain protein family. Science 2005;308:1758–1762.

Kamanova J, Kofronova O, Masin J, Genth H, Vojtova J, Linhartova I, Benada O, Just I, Sebo P: Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling. J Immunol 2008;181:5587–5597.

Khader SA, Cooper AM: IL-23 and IL-17 in tuberculosis. Cytokine 2008;41:79–83.

Kim B-H, Shenoy AR, Kumar P, Das R, Tiwari S, MacMicking JD: A family of IFN-γ-inducible 65-kD GTPases protects against bacterial infection. Science 2011a;332:717–721.

Kim S, Lee S, Shin J, Kim Y, Evnouchidou I, Kim D, Kim YK, Kim YE, Ahn JH, Riddell SR, Stratikos E, Kim VN, Ahn K: Human cytomegalovirus microRNA miR-US4-1 inhibits CD8$^+$ T cell responses by targeting the aminopeptidase ERAP1. Nat Immunol 2011b;12:984–991.

Lee Y, Jung JI, Park KY, Kim SA, Kim J: Synergistic inhibition effect of TNIK inhibitor KY-05009 and receptor tyrosine kinase inhibitor dovitinib on IL-6-induced proliferation and Wnt signaling pathway in human multiple myeloma cells. Oncotarget 2017;8:41091–41101.

Liu X, Lu R, Wu S, Sun J: Salmonella regulation of intestinal stem cells through the Wnt/β-catenin pathway. FEBS Lett 2010;584:911–916.

Mahmoudi T, Li VSW, Ng SS, Taouatas N, Vries RGJ, Mohammed S, Mohammed S, Heck AJ, Clevers H: The kinase TNIK is an essential activator of Wnt target genes. EMBO J 2009;28:3329–3340.

Martinez A, Gil C: cAMP-specific phosphodiesterase inhibitors: promising drugs for inflammatory and neurological diseases. Expert Opin Ther Pat 2014;24:1311–1321.

Neumann J, Schaale K, Farhat K, Endermann T, Ulmer AJ, Ehlers S, Reiling N: Frizzled1 is a marker of inflammatory macrophages, and its ligand Wnt3a is involved in reprogramming *Mycobacterium tuberculosis*-infected macrophages. FASEB J 2010;24:4599–4612.

Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30:e36.

Pollock JM, Neill SD: *Mycobacterium bovis* infection and tuberculosis in cattle. Vet J 2002;163:115–127.

Ramakers C, Ruijter JM, Deprez RHLL, Moorman AF: Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 2003;339:62–66.

Rausch A, Hessmann M, Hölscher A, Schreiber T, Bulfone-Paus S, Ehlers S, Hölscher C: Interleukin-15 mediates protection against experimental tuberculosis: a role for NKG2D-dependent effector mechanisms of CD8+ T cells. Eur J Immunol 2006;36:1156–1167.

Rizzi C, Bianco MV, Blanco FC, Soria M, Gravisaco MJ, Montenegro V, Vagnoni L, Buddle B, Garbaccio S, Delgado F, Leal KS, Cataldi AA, Dellagostin OA, Bigi F: Vaccination with a BCG strain overexpressing Ag85B protects cattle against *Mycobacterium bovis* challenge. PLoS One 2012;7:e51396.

Robinson TL, Sutherland IA, Sutherland J: Validation of candidate bovine reference genes for use with real-time PCR. Vet Immunol Immunopathol 2007;115:160–165.

Samten B, Ghosh P, Yi A-K, Weis SE, Lakey DL, Gonsky R, Pendurthi U, Wizel B, Zhang Y, Zhang M, Gong J, Fernandez M, Safi H, Vankayalapati R, Young HA, Barnes PF: Reduced expression of nuclear cyclic adenosine 5′-monophosphate response element-binding proteins and IFN-γ promoter function in disease due to an intracellular pathogen. J Immunol 2002;168:3520–3526.

Samten B, Howard ST, Weis SE, Wu S, Shams H, Townsend JC, Safi H, Barnes PF: Cyclic AMP response element-binding protein positively regulates production of IFN-γ by T cells in response to a microbial pathogen. J Immunol 2005;174:6357–6363.

Singh H, Zhao M, Chen Q, Wang Y, Li Y, Kaitu'U-Lino TJ, Tong S, Nie G: Human HtrA4 expression is restricted to the placenta, is significantly up-regulated in early-onset preeclampsia, and high levels of HtrA4 cause endothelial dysfunction. J Clin Endocrinol Metab 2015;100:E936–E945.

Smyth GK: Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:3.

Soderling SH, Bayuga SJ, Beavo JA: Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. Proc Natl Acad Sci USA 1998;95:8991–8996.

Tomasec P, Braud VM, Rickards C, Powell MB, McSharry BP, Gadola S, Cerundolo V, Borysiewicz LK, McMichael AJ, Wilkinson GW: Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science 2000;287:1031.

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM: Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 2007;35:W71–W74.

Van Meijgaarden KE, Haks MC, Caccamo N, Dieli F, Ottenhoff THM, Joosten SA: Human CD8$^+$ T-cells recognizing peptides from *Mycobacterium tuberculosis* (*Mtb*) presented by HLA-E have an unorthodox Th2-like, multifunctional, *Mtb* inhibitory phenotype and represent a novel human T-cell subset. PLoS Pathog 2015;11:e1004671.

Vang AG, Basole C, Dong H, Nguyen RK, Housley W, Guernsey L, Adami AJ, Thrall RS, Clark RB, Epstein PM, Brocke S: Differential expression and function of PDE8 and PDE4 in effector T cells: implications for PDE8 as a drug target in inflammation. Front Pharmacol 2016;7:259.

Vordermeier HM, Jones GJ, Buddle BM, Hewinson RG, Villarreal-Ramos B: Bovine tuberculosis in cattle: vaccines, DIVA tests, and host biomarker discovery. Annu Rev Anim Biosci 2016;4:87–109.

Waters WR, Palmer MV, Buddle BM, Vordermeier HM: Bovine tuberculosis vaccine research: historical perspectives and recent advances. Vaccine 2012;30:2611–2622.

Wedlock DN, Denis M, Painter GF, Ainge GD, Vordermeier HM, Hewinson RG, Buddle BM: Enhanced protection against bovine tuberculosis after coadministration of *Mycobacterium bovis* BCG with a mycobacterial protein vaccine-adjuvant combination but not after coadministration of adjuvant alone. Clin Vaccine Immunol 2008;15:765–772.