Veterinary Immunology and Immunopathology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice

Virginie Roupie[a], Sophie Viart[b], Baptiste Leroy[b], Marta Romano[a], Nicolas Trinchero[c], Marc Govaerts[d], Jean-Jacques Letesson[c], Ruddy Wattiez[b], Kris Huygen[a,*]

[a] *Scientific Service Immunology, Scientific Institute of Public Health WIV-ISP (Site Ukkel), 642 Engelandstraat, 1180 Brussels, Belgium*
[b] *Department of Proteomics and Microbiology, University of Mons, Place du parc 20, 7000 Mons, Belgium*
[c] *URBM, Narilis, Facultés Universitaires Notre Dame de la Paix, 5000 Namur, Belgium*
[d] *Veterinary and Agrochemical Research Institute VAR-CODA-CERVA, 1180 Brussels, Belgium*

ARTICLE INFO

Article history:
Received 14 September 2011
Received in revised form 17 October 2011
Accepted 21 October 2011

Keywords:
Paratuberculosis
Johne's disease
DNA vaccines
Vaccination

ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (*Map*), the etiological agent of chronic enteritis of the small intestine in domestic and wild ruminants, causes substantial losses to livestock industry. Control of this disease is seriously hampered by the lack of adequate diagnostic tools and vaccines. Here we report on the immunogenicity of eight *Map* specific antigens, i.e. MAP1693c, Ag3, MAP2677c (identified by post-genomic and immunoproteomic analysis of *Map* secretome) and Ag5, Ag6, MAP1637c, MAP0388 and MAP3743 (identified by bioinformatic *in silico* screening of the *Map* genome). Strong, antigen-specific IFN-γ responses were induced in mice vaccinated with plasmid DNA encoding MAP1693c, MAP1637c, MAP0388 and MAP3743. In contrast, T cell responses in *Map* infected mice were directed preferentially against Ag5 and to a lesser extent against MAP3743. None of the tested DNA vaccines conferred protection against subsequent challenge with *Map*.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the etiological agent of paratuberculosis also known as Johne's disease (JD). *Map* causes a chronic and incurable infection of the small intestine of ruminants leading to diarrhea, weight loss and death in both captive and free ranging ruminants. Disease is prevalent worldwide (Harris and Barletta, 2001; Nielsen and Toft, 2009) and causes heavy economic losses due to reduced milk production (Smith et al., 2009), veterinary expenses, reduced slaughter value, infertility and premature killing of animals especially in the dairy industry (McKenna et al., 2006; Ott et al., 1999).

Histopathological and clinical similarities between JD in cattle and Crohn's disease (CD) in humans lie at the basis of the controversy on the implication of *Map* in the development CD. However, it is still unclear whether *Map* is an actual causal agent or just associated with CD (Economou and Pappas, 2008; Feller et al., 2007; Uzoigwe et al., 2007).

It is generally accepted that IFN-γ producing T cells of the Th1 subset are responsible for restricting bacterial multiplication in the early stage of *Map* infection. The immune responses generated in the course of disease progression are somewhat less well characterized but immune suppression induced by parturition and lactation is an important factor (Karcher et al., 2008). The development of the clinical phase associated with an increase in fecal *Map* excretion and reduced systemic IFN-γ responses, has been correlated with the advent of a Th2 or regulatory (IL-10 and TGF-β) immune response, and an increase in antibody production (Stabel, 2006). Recently, Robinson et al. (2011) analyzed the expression of a range of cytokines

* Corresponding author at: Service Immunology, WIV-ISP (Site Ukkel), 642 Engelandstraat, B1180 Brussels, Belgium. Tel.: +32 2 373 33 70; fax: +32 2 373 33 67.
E-mail address: kris.huygen@wiv-isp.be (K. Huygen).

0165-2427/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2011.10.012

using quantitative mRNA PCR in jejunal lymph nodes of experimentally challenged red deer. Whereas no difference in expression was found in mucosal responses between control, and minimally diseased animals, severely diseased animals showed an elevated pro-inflammatory cytokine expression profile, characterized by elevated mRNA levels of gamma interferon, interleukin-1α and IL-17. In contrast, transcripts indicative of an anti-inflammatory Th2 and Treg response, e.g. IL-4, GATA-3, TGF-β and Foxp3 were found to be significantly reduced in the latter group. Unlike the other Th1 cytokine IFN-γ, expression of IL-2 was diminished in severely diseased animals, which might explain the reduced systemic T cell responses generally observed during late-stage disease (Robinson et al., 2011). Likewise, in infected cattle, expression of IFN-γ, IL-22, and IL-17 was increased in ileocecal lymph nodes from experimentally and naturally infected animals as compared to expression in ileocecal lymph nodes from negative control animals (Allen et al., 2011).

The first generation vaccines against paratuberculosis consist of whole bacteria, live or inactivated, formulated in mineral oil. However, their use does not protect against infection and they interfere with the skin test used in the screening for bovine tuberculosis due to *Mycobacterium bovis*, and with the indirect antibody and IFN-γ ELISA tests used for the diagnosis of paratuberculosis (Rosseels and Huygen, 2008). Alternatives to these whole-cell based vaccines are the so-called sub-unit vaccines, made up of peptides or proteins (Perrie et al., 2008) or of plasmid DNA (Romano and Huygen, 2009). Identifying immunodominant protein antigens that induce a strong Th1 type immune response and confer protection in challenge models (mice and target species) is critical to the development of sub-unit vaccines (Rosseels and Huygen, 2008).

Here, we have studied the vaccine potential of eight *Map* antigens selected for *Map* specificity by two approaches. Three antigens, i.e. Ag3, MAP2677c and MAP1693c were previously identified by post-genomic and immunoproteomic analysis of the *Map* secretome (Leroy et al., 2007) and five other antigens, i.e. Ag5, Ag6, MAP1637c, MAP0388 and MAP3743 were identified by *in silico* screening of the *Map* genome for absence in other mycobacterial genomes (Leroy et al., 2009). Immunogenicity of the eight *Map* antigens was analyzed in plasmid DNA vaccinated mice and in mice infected with *Map* and *M. bovis*. Moreover, we analyzed their protective potential as DNA vaccines in mice challenged with luminescent *Map* by quantifying bacterial replication in spleen and liver by luminometry, previously shown to be a valid alternative to cumbersome colony forming unit (CFU) plating (Rosseels et al., 2006b; Roupie et al., 2008a, b).

2. Materials and methods

2.1. Antigen selection

MAP1693c, Ag3 and MAP2677c belong to a group of 25 *Map* specific antigenic proteins identified previously in culture filtrate of *Map* (Leroy et al., 2007). Combination of three of them, MAP1693c, MAP2677c and MAP4308c, was shown to compete in performance with available commercial assays. MAP1693c has also been identified by Cho et al. (2007, 2006) to be a good candidate for an indirect antibody detection ELISA. The selection of Ag5, Ag6, MAP1637c, MAP0388 and MAP3743 was based on an *in silico* analysis of *Map* genome performed in 2003 (Leroy et al., 2009). Briefly, genomic sequences with no significant homologies in other mycobacterial genomes as analyzed by BLASTn and BLASTp/nr were identified and three different T cell epitope prediction programs for MHC class II (Feller and de la Cruz, 1991) and MHC class I (Parker et al., 1994; Rammensee et al., 1999) were used to select five antigens, predicted to be strongly immunogenic, among eighty-seven *in silico* predicted *MAP*-specific ORF.

2.2. Blast analysis

BLASTp analysis for homology of protein sequence was updated in May 2011 against all mycobacterial genomes available on the National Centre for Biotechnology Information (NCBI) web site: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (Altschul et al., 1997, 2005). To check for conserved protein domains, CDD (Conserved Domain Data base) was used at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (Marchler-Bauer et al., 2011). Details on sequence homology and putative function of the 8 antigens is given in Table 1.

2.3. Mice

Female BALB/c and C57BL/6 mice were bred in the Animal Facilities of the WIV-ISP (Site Ukkel), Belgium, from breeding couples originally obtained from JANVIER SAS in Le Genest Saint Isle, France. All animals were 6–8 weeks old at the start of the experiments. We have previously shown that these two mouse strains are susceptible to intravenous *Map* infection and that multiplication of *Map* can be monitored in spleen and liver (Roupie et al., 2008b).

2.4. Bacteria and crude antigens

Culture filtrate (CF-P) of *M. avium* subsp. *paratuberculosis* (*Map*) ATCC 19698 was prepared from a culture growing as a surface pellicle on synthetic Sauton medium supplemented with mycobactin J (2 μg/ml) (Synbiotics Europe) at 39 °C for 4 weeks as described previously (Rosseels et al., 2006a). Culture filtrate of *M. bovis* strain AN5 (CF-B) was obtained from cultures grown as surface pellicle for 2 weeks at 37° on synthetic Sauton medium. A *M. bovis* Purified Protein Derivative (PPD-B) preparation for intradermal use (Bovituber) was purchased from Synbiotics Europe and dialysed against PBS for phenol removal. Johnin (PPD-P) was prepared from 6 to 8 week old cultures of strain *Map* ATCC 19698 according to a standard protocol (Magnusson and Bentzon, 1958).

2.5. Preparation of genomic DNA from M. avium subsp. paratuberculosis ATCC 19698

Genomic DNA of *M. avium* subsp. *paratuberculosis* ATCC 19698 was prepared as described by Tanghe et al. (2001)

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

Table 1
Presentation of the eight candidates: MAP numbers, molecular weight (kDa), genomic coordinates, the similarities and putative function determined by BLASTP and CDD, the similarities with *Mycobacterium avium* subp. *avium* and with other mycobacteria. The Expect value (*E*) is given in brackets.

Identification	Genes	MW (kDa)	Coordinates	Similarity/putative function[b]	*M. avium* subsp. *avium*	Other mycobacteria
Immuno proteomics	MAP1693c	18.3	1853529–1852963	Peptidyl-prolyl cis–trans isomerase (accession cl11587)	Hypothetical protein MaviaA2_11126 ($E = 2e^{-70}$)	*M. parascrofulaceum* ($E = 2e^{-61}$), *M. marinum* ($E = 2e^{-61}$), *M. intracellulare* ($E = 1e^{-60}$), *M. ulcerans* ($E = 1e^{-58}$), *M* sp. *MCS*, *KMS* and *JLS* ($E = 2e^{-55}$), *M. abscessus* ($E = 6e^{-55}$), *M. vanbaalenii* PYR-1 ($E = 1e^{-53}$), *M. smegmatis* ($E = 9e^{-53}$), *M. gilvum* ($E = 9e^{-50}$),
	Ag3[a]	26.1	3550028–3550747	None	None	None
	MAP2677c	14.5	3013753–3014193[c]	Glyoxalase family (accession cl11587)	Hypothetical protein MaviaA2_05535 ($E = 7e^{-70}$)	*M. smegmatis* ($E = 7e^{-}60$), *M. parascrofulaceum* ($E = 2e^{-58}$), *M. intracellulare* ($E = 4e^{-58}$), *M* sp. *MCS*, *KMS* and *JLS* ($E = 1e^{-06}$), *M. vanbaalenii* PYR-1 ($E = 9e^{-05}$)
	Ag5[a]	25	2383507–2384184	Hypothetical protein Mvan_3607	None	*M. vanbaalenii* ($E = 3e^{-30}$)
In silico	Ag6[a]	15	886138–886530	None	None	None
	MAP1637c	52	1791069–1792508	UbiD superfamily. (accession cl00311)	Hypothetical protein MaviaA2_11436 ($E = 0$)	*M. intracellulare* ($E = 1e^{-42}$), *M. abscessus* ($E = 2e^{-27}$)
	MAP0388	43.8	410761–411995[c]	Dyp-type peroxidase family (accession cl01607)	None	*M. vanbaalenii* PYR-1 ($E = 1e^{-180}$), *M.* sp. *MCS* ($E = 3e^{-176}$), *M.* sp. *JLS* ($E = 4e^{-176}$), *M. smegmatis* ($E = 1e^{-174}$), *M.* sp. *Spyr1* ($E = 9e^{-172}$), *M. gilvum* ($E = 9e^{-172}$),
	MAP3743	36.5	4178173–4179219	Saccharopine dehydrogenase (COG1748), putative lipoprotein	None	None

[a] Ag3, Ag5 and Ag6 are on the antisense strand on the genome. MAP3199, a member of the YceI-like family highly conserved among mycobacteria, is annotated on the genome sense strand and located on the reverse complementary strand of Ag3.
[b] Analysis by BLASTP and motif CDD analysis.
[c] MAP2677c and MAP0388 were respectively cloned with 42 and 45 additional nucleotides in the 5′ region of these genes and taking account for BLAST values. The coordinates indicated in this table include this additional sequence.

for *M. ulcerans* and Roupie et al. (2008a) for *Map*. DNA was stored at −20 °C until use.

2.6. Construction of the eight plasmid DNA vaccines

We first modified pV1J.ns-tPA vector into pV1J.ns-tPA-His, by inserting a hexa-histidine tag coding sequence in 3′ of the *Bgl*II restriction site of the pV1J.ns-tPA vector (Merck Research Laboratories, PA, USA) (Shiver et al., 1995). The description of this modification by the cloning of Ag5 (seq_2383507_2384184) into the pV1J.ns-tPA was described before (Roupie et al., 2008a). The resulting pV1J.ns-tPA-His-Ag5 construct was digested with *Bgl*II/*Eco*RI, purified on agarose gel to separate the vector backbone from the Ag5 sequence, and used for the cloning of the seven other antigens. Genes were cloned as *Bgl*II/*Eco*RI or *Bgl*II/*Mun*I-compatible *Eco*RI fragments in pV1J.ns-tPA-his. Briefly, genes were amplified by PCR (Expand Polymerase; Roche) using *M. avium* subsp. *paratuberculosis* ATCC 19698 genomic DNA as a template and using primers described in Table 2 (Proligo, Sigma) designed from the sequence of *Map* K-10 (Li et al., 2005). After T4 ligation and transformation into DH5-α cells, clones were screened on LB-kanamycin medium and plasmids were checked by restriction digestion and sequencing (StarSEQ GmbH). Finally, sufficient amounts of these DNA plasmids for vaccination experiments were purified using the PureLink™ HiPure Plasmid DNA Gigaprep kit (Invitrogen).

2.7. Cloning, expression and purification of recombinant proteins

The genes coding for histidine-tagged proteins were cloned in *Escherichia coli* expression plasmid pQE-80L (Qiagen). Briefly, sequences were amplified by PCR (Expand

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

Table 2
Primers used for the cloning of eight genes in DNA vaccine plasmid pV1J.ns-tPA-His and in the recombinant protein expression plasmid pQE-80L. Restriction enzymes used are indicated. Sense primers are listed first and antisense primers second, within primer pairs. All restriction sites are shown in bold except for *BamH*I shown in bold and underlined, the histidine-tag sequence in italic, and the STOP codon highlighted.

Genes	Primers for cloning in pV1J.ns-tPA-His	Enzyme used
MAP1693c	5'-TATAT**AGATCT**TGACGGCTGTGAACTCCGT-3' 5'-TATA**GAATTC**GGTCGTGGCGCCGAGGAT-3'	*Bgl* II *EcoR* I
Ag3	5'-GGA**AGATCT**TGGTGCCCGCATCACCCGTTC-3' 5'-TATA**GAATTC**GATTCGCCACGACAGTTGGG-3'	*Bgl* II *EcoR* I
MAP2677c	5'-GGA**AGATCT**GCTTGGGCGACACCACA-3' 5'-TATA**GAATTC**TACTTTGAACTTGGCCCGC-3'	*Bgl* II *EcoR* I
Ag5	5'-TAT**AGATCT**TGGATGGGCTCCGGC-3' 5'-TAT**GGATCC***CTA*GTGATGGTGATGGTGATG**GAATTC**CTTCT CACCGAATTTCGAAAGAATATC-3'.	*Bgl* II BamH I *EcoR* I
Ag6	5'-GGA**AGATCT**TACTTGTCCATCGAGCATCGGA-3' 5'-TATA**GAATTC**ACGTCCAGCGTGGCGGT-3'	*Bgl* II *EcoR* I
MAP1637c	5'-GGA**AGATCT**TGCGCGATTACATCCAAACGCTG-3' 5'-TATA**GAATTC**CAACGGCAACGCGGCCT-3'	*Bgl* II *EcoR* I
MAP0388	5'-GGA**AGATCT**TGCAACCGCCGGGGCGGGCGTGGCT-3' 5'-TATA**CAATTG**TGACCGTTCGCCGAGCGCCGCGAGGTAT-3'	*Bgl* II *Mun* I
MAP3743	5'-GGA**AGATCT**TGGGTGGTGCGCACGTGAGGA-3' 5'-TATA**GAATTC**CGGTACGGTCGCGGCCAGGA-3'	*Bgl* II *EcoR* I
Genes	**Primers for cloning in pQE-80L**	**Enzyme used**
MAP1693c	5'-TATAT**AGATCT**GTGACGGCTGTGAACTCCGTCCG-3' 5'-TATA**AAGCTT**CTAGGTCGTGGCGCCGAGGAT-3'	*Bgl* II *Hind*III
Ag3	5'-GGA**AGATCT**GTGCCCGCATCACCC-3' 5'-TAT**AAGCTT**CAGATTCGCCACGACAGTTGG-3'	*Bgl* II *Hind*III
MAP2677c	5'-GGA**AGATCT**TGCTTGGGCGACACCACA-3' 5'-TATA**AAGCTT**TTATACTTTGAACTTGGCCC-3'	*Bgl* II *Hind*III
Ag5	5'-TAT**AGATCT**TTGGATGGGCTCCGGCAGCTT-3' 5'-TAT**AAGCTT**CTACTTCTCACCGAATTTCG -3'	*Bgl* II *Hind*III
Ag6	5'-ATAT**GGATCC**CTTCTTGTCCATCGAGCATCGGA-3' 5'-TAT**AAGCTT**CTAACGTCCAGCGTGGCGGT -3'	*BamH*I *Hind*III
MAP1637c	5'-TAT**AGAGC**TCGTGGCTGTATTTCGCGATTTGCGCGATT -3' 5'-TATA**CTGCAG**CTACAACGGCAACGCGGCCTGCC -3'	*Sac* I *Pst*I
MAP0388	5'-GGA**AGATCT**CAACCGCCGGGGCGGGCGTGGCTG-3' 5'-TATA**AAGCTT**CTATGACCGTTCGCCGAGCGCC-3'	*Bgl* II *Hind*III
MAP3743	5'-GGA**AGATCT**TTGGGTGGTGCGCACGTGAGGA -3' 5'-TATA**AAGCTT**CTACGGTACGGTCGCGGCCAGGA -3'	*Bgl* II *Hind*III

High Fidelity PCR System, Roche), from the corresponding pV1J.ns-tPA-His constructs. Primers used for cloning are shown in Table 2.

The amplified sequences were digested with *BamH*I/*Hind*III, *Bgl*II/*Hind*III, or *Sac*I/*Pst*I (only for MAP1637c), purified on agarose (QIAkit PCR Purification kit, Qiagen) and T4 ligated into pQE-80L expression vector digested with *BamH*I/*Hind*III or *Sac*I/*Pst*I. After ligation and transformation into Top-10F′ chemically competent *E. coli* cells (Invitrogen), clones were screened on LB-ampicillin medium (100 μg/ml) and plasmid was checked by restriction digestion and sequencing. Recombinant proteins, expressed in Top-10F′ *E. coli* cells as his-tagged proteins and induced by IPTG, were purified by affinity chromatography on immobilized nickel-chelate (Ni-NTA) columns, as described previously (Rosseels et al., 2006a).

2.8. Vaccination

BALB/c and C57BL/6 mice were vaccinated four times at 3 weeks intervals with pV1J.ns-tPA-His plasmid encoding each of eight antigens or empty vector as negative control. For DNA immunizations, mice were sedated with ketamine/xylazine and injected intramuscularly in both quadriceps muscles with 2×50 μg of pDNA. As a positive control, mice were vaccinated intravenously with 2×10^6 CFU of freshly prepared *M. bovis* BCG GL2, grown as a surface pellicle on synthetic Sauton medium for 14 days.

2.9. Mycobacterium avium subsp. paratuberculosis infection

Luminescent *Map*-S23 (Foley-Thomas et al., 1995), obtained by transformation with the shuttle plasmid pSMT1 (Rosseels et al., 2006b; Snewin et al., 1999), was grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J (Allied Monitor Laboratories Inc., 2 μg/ml) and hygromycin (100 μg/ml), to an $O.D_{600\,nm}$ ranging between 0.6 and 0.8. Bacteria were centrifuged for 30 min at $2150 \times g$ and suspended in PBS to a concentration of 10^7 CFU/ml (8.5×10^6 RLU/ml). Mice were infected

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

intravenously in a lateral tail vein with 0.2 ml of bacteria and sacrificed 5 weeks post-infection.

2.10. M. bovis infection

Mice were inoculated intravenously with 1.3×10^6 CFU of *M. bovis* AN5 grown for 2 weeks as a surface pellicle on synthetic Sauton medium at 37 °C (stored as frozen stocks at −80°). Animals were sacrificed 5 weeks post-infection.

2.11. Antibody ELISA

Sera from C57BL/6 and BALB/c mice were collected by tail bleeding three weeks after the last DNA immunization or 5 weeks after *Map* or *M. bovis* infection. Levels of antigen-specific total immunoglobulin G (IgG), IgG1, IgG2a and IgG2b antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) on individual sera. The corresponding recombinant protein was used for coating (500 ng/well). Antibody isotypes were detected using peroxidase-labeled rat anti-mouse immunoglobulin IgG, IgG1, IgG2a, IgG2b (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium) and orthophenylenediamine (Sigma) for revelation. Data are presented as the optical density at 490 nm ($O.D_{490\,nm}$) for a serum dilution of 1:400 for vaccinated mice, and of 1:100 for infected mice.

2.12. Cytokine production

DNA vaccinated mice were sacrificed 3 weeks after the last immunization, optimal time for measuring immunogenicity in DNA vaccinated mice. *M. bovis* and *M. paratuberculosis* infected mice were sacrificed 5 weeks after infection. Spleens from three to four individual mice per group were removed aseptically and homogenized by gentle disruption in a Dounce homogenizer, and cells were adjusted to 4×10^6 white blood cells/ml in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, penicillin, streptomycin and fungizone. In experiments in which responses after infection were analyzed, indomethacin (1 μg/ml; Sigma) was added to complete RPMI-1640 culture medium.

Cells were stimulated with purified recombinant antigens (5 μg/ml), with Purified Protein Derivative (10 μg/ml) from *M. bovis* (PPD-B) and from *Map* (PPD-P) and with culture filtrate (10 μg/ml) from *M. bovis* AN5 (CF-B) and from *Map* (CF-P), incubated at 37 °C in round-bottom, 96-well microwell plates in a humidified CO_2 incubator. Culture supernatants were harvested after 24 h for interleukin-2 (IL-2) assays and after 72 h for IFN-γ assays, when peak values of the respective cytokines can be measured. Supernatants were stored frozen at −20 °C until testing.

2.13. IFN-γ ELISA

IFN-γ activity was quantified by sandwich ELISA using coating antibody R4-6A2 and biotinylated detection antibody XMG1.2 (both BD Pharmingen). The detection limit of the IFN-γ ELISA is 5 pg/ml. Antigen-specific cytokine levels were considered positive when values were at least fivefold higher than those of unstimulated cells.

2.14. IL-2 ELISA

IL-2 activity was quantified by sandwich ELISA using coating antibody anti-mouse interleukine-2 (14-7022, eBioscience) and biotinylated detection antibody anti-mouse IL-2 (JES6-5H4, 13-7021, eBioscience). The detection limit of the IL-2 ELISA is 5 pg/ml. Antigen-specific cytokine levels were considered positive when values were at least fivefold higher than those of unstimulated cells.

2.15. Mycobacterium avium subsp. paratuberculosis challenge

Mice were challenged with luminescent *Map* 6 weeks after the fourth DNA immunization or 12 weeks after BCG vaccination, using the same protocol as described higher for the *Map* infection experiment. Eight weeks after challenge, mice were sacrificed and the number of bioluminescent bacteria was determined in spleen and liver homogenates. Fresh 1-ml spleen or liver homogenates were tested in duplicate after erythrocyte lysis with respectively 40 μl or 80 μl of zap-oglobin™ lytic reagent (Beckman Coulter) to minimize quenching. Spleen homogenates were centrifuged at $2150 \times g$ for 15 min and liver homogenates for 30 min. Pellets were suspended in 1 ml PBS and tested for luminescence in a Lumat LB 9507 Luminometer (Berthold Technologies) as flash emission (15 s integration time) using 1% *n*-decanal (Sigma) in ethanol as substrate.

For statistical analysis (one way ANOVA, Tukey's Multiple Comparison Test, CI 95%), results obtained in Relative Light Units (RLU)/spleen were converted to $\log_{10}$ values (Rosseels et al., 2006b).

2.16. Counting the number of colony forming units (CFU)

The CFU number of the *Map* stock was determined by plating serial tenfold dilutions in duplicate on 9 cm Petri dishes on Middlebrook 7H11-OADC agar supplemented with mycobactin J. Petri dishes were sealed in plastic bags and incubated at 39 °C for 8 weeks, before counting the colonies visually.

3. Results

3.1. Homology of the eight selected antigens

Predicted function, predicted molecular weight and sequence homologies in BLASTp to *M. avium* subsp. *avium* and other mycobacteria of the eight selected antigens is summarized in Table 1. MAP1693c and MAP2677c show homology with *M. avium* subsp. *avium* and some other mycobacteria, but not with *M. bovis* (respectively *E*-values = 9.9 and 0.009), in line with their initial selection based on recognition by sera from *Map* infected but not from *M. bovis* infected cattle. Ag3 (CF036) shows no homology with any known mycobacterial peptide sequence and remains apparently unannotated in all mycobacterial

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

genomes despite the fact that it has been identified in culture filtrate of *Map* by Leroy et al. (2007).

MAP1637c, initially selected in 2003 *in silico* for being absent from *M. avium* subsp. *avium*, has been found to be identical with the hypothetical protein MaviaA2_11436 (*E*-value = 0) of *M. avium* subsp. *avium* ATCC 25291 for which only contigs are available as of now (see: http://www.xbase.ac.uk/taxon/Mycobacterium; Chaudhuri et al., 2008). A very poor homology is found with a metallophosphoesterase MAV3455 from *M. avium* subsp. *avium* 104 (*E*-value = 0.009, higher that the cut-off 10^{-3} used for selection in 2003). Ag6 and MAP3743 show no homology with any of the known mycobacterial sequences, whereas Ag5 and MAP0388 show some homologies with other mycobacteria. None of the *in silico* selected proteins has a significant *E*-value score with *M. bovis*.

3.2. Th1-type cytokine secretion in response to eight Map antigens in plasmid DNA-vaccinated C57BL/6 mice and BALB/c mice

C57BL/6 mice produced strong antigen specific IFN-γ responses following vaccination with DNA encoding MAP1693c (31,993 ± 15,482 pg/ml) and MAP1637c (44,653 ± 18,749 pg/ml) (Fig. 1A). Weaker responses were found with DNA encoding MAP0388. The strongest antigen-specific IL-2 level was detected in response to MAP1637c (Fig. 1B). Only weak antigen-specific Th1 cytokine responses were induced by vaccination with the other five plasmids.

DNA-vaccinated BALB/c mice produced very strong antigen-specific IFN-γ responses to MAP1637c and MAP3743 (Fig. 1C) and somewhat weaker responses to MAP1693c. Antigen-specific IL-2 levels were overall very low in DNA-vaccinated BALB/c mice (Fig. 1D).

3.3. Specific antibody response in plasmid DNA-vaccinated C57BL/6 and BALB/c mice

As shown in Fig. 2, significant levels of antigen-specific antibody were induced in C57BL/6 mice in response to MAP1693c and to a lesser extent to MAP2677c. DNA vaccinated BALB/c mice showed strong antibody responses to MAP1693c and MAP2677c and lower antibody responses against three *in silico* identified antigens, i.e. Ag5, Ag6 and MAP1637c. Production of IgG2a and IgG2b antibodies reflected the Th1 type cytokine profile induced by these DNA vaccines.

3.4. Immune responses in BALB/c and C57BL/6 mice infected with M. avium subsp. paratuberculosis S-23 or M. bovis AN5

We next compared the immunogenicity and specificity of the eight *Map* proteins in mice experimentally infected with *Map* or *M. bovis*. As shown in Fig. 3, *Map* infected C57BL/6 mice produced IFN-γ levels of comparable magnitude (between 10,000 and 15,000 pg/ml) upon stimulation with culture filtrates and PPDs from both *Map* and *M. bovis* (Fig. 3A). On the other hand, *M. bovis* infection of C57BL/6 mice induced a very robust IFN-γ response to CF-B and PPD-B from *M. bovis* (192,491 and 233,452 pg/ml respectively) which was much higher than the response to CF-P and PPD-P from *Map* (16,632 and 21,923 pg/ml respectively). *Map* infection of BALB/c mice also stimulated strong IFN-γ responses to culture filtrate and PPD from both *Map* and *M. bovis* (between 20,000 and 40,000 pg/ml) (Fig. 3B). As for C57BL/6 mice, *M. bovis* infection of BALB/c mice stimulated a stronger IFN-γ response against culture filtrate and PPD of *M. bovis* than against CF-P and PPD-P of *Map*, but the overall magnitude of the response was lower than in *M. bovis* infected C57BL/6 mice (15,000–20,000 pg/ml).

Analysis of the IFN-γ response in infected animals against the selected eight *Map* antigens revealed a different immunogenicity profile than the one observed in DNA vaccinated mice, with the *in silico* identified Ag5 and MAP3743 being the most strongly recognized both in C57BL/6 (Fig. 3C) and BALB/c (Fig. 3D) mice infected with *Map*. Despite their predicted specificity for *Map*, Ag5 and MAP3743 were recognized with the same magnitude by *M. bovis* and *Map* infected C57BL/6 mice. Alternatively, *Map* infected BALB/c mice reacted more strongly to Ag5 and MAP3743 than *M. bovis* infected mice.

Although these two antigens were selected *in silico* for absence in *M. bovis* genome, it is possible that short H-2^b restricted peptide sequences would be shared between the two mycobacterial species (on the same or even on other proteins), and this could explain the cross-reactive responses observed against Ag5 and MAP3743 in C57BL/6 mice. A detailed epitope mapping would be needed to answer this question.

3.5. Specific antibody production in C57BL/6 and BALB/c mice infected with M. avium subsp. paratuberculosis S-23 or M. bovis AN5

In contrast to the strong IFN-γ responses detected in *Map* infected C57BL/6 and BALB/c mice against *Map* culture filtrate, antibody levels against *Map* culture filtrate were below detection level in these mice (Fig. 4). *M. bovis* infection of C57BL/6 and particularly BALB/c mice however stimulated significant antibody production against whole culture filtrate of *M. bovis* (CF-B). Weak Ag6 and MAP1637c specific antibodies were detected in *M. bovis* infected BALB/c mice, whereas MAP2677c was strongly recognized by serum of *M. bovis* infected BALB/c mice, but also to a weaker extent by sera of naïve and *Map* infected BALB/c mice (Fig. 4).

3.6. Protective efficacy of eight encoded antigens in plasmid DNA-vaccinated C57BL/6 and BALB/c mice

Immunization with none of the eight plasmid DNA could protect BALB/c or C57BL/6 mice from an experimental challenge with *Map* (administered 6 weeks after the last immunization), as analyzed at 8 weeks post-challenge and compared to mice vaccinated with the empty vector (Table 3). In contrast, and confirming previous findings, vaccination with *M. bovis* BCG conferred significant protection against *Map* ($p < 0.001$), as indicated by a tenfold reduction in RLU numbers in liver of both mouse strains and in spleen of C57BL/6 mice. In spleen of BCG vaccinated

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

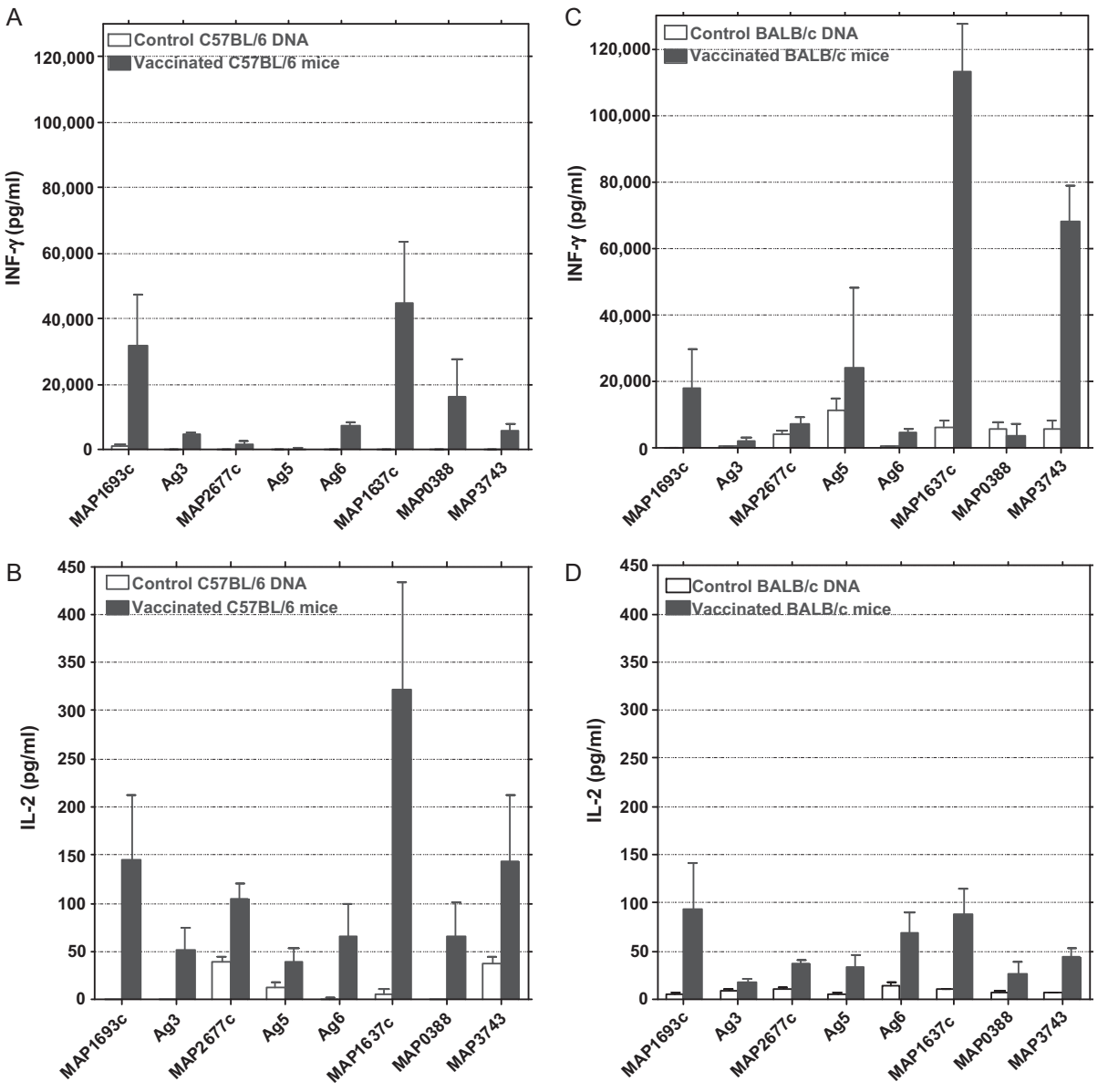

Fig. 1. IFN-γ (panels A and C) and IL-2 (panels B and D) levels in respective 72-h and 24-h spleen cell culture supernatants from C57BL/6 (A and B) and BALB/c (C and D). Mice were vaccinated with empty plasmid DNA (control) or plasmid DNA encoding the 8 *Map* antigens and stimulated with the corresponding antigen (5 μg/ml) 3 weeks after the fourth DNA vaccination. Results represent mean ± SEM (error bars) values of three to four mice tested individually in each group.

BALB/c mice, the bacterial numbers were only very modestly (twofold) reduced.

4. Discussion

Paratuberculosis is spread worldwide and causes heavy economic losses particularly in the dairy sector. Several countries have initiated control programs, aiming to reduce transmission, progression to clinical phase, or shedding. However, control of Johne's disease is hampered by increasing trade (McKenna et al., 2006) and the poor performance of diagnostic tests (Nielsen and Toft, 2008). A subunit vaccine composed of one or several *Map*-specific and protective antigens, which would prevent infection or – perhaps more realistically – prevent progression to clinical disease and which would not interfere with the diagnosis of bovine tuberculosis or JD, would be an invaluable tool for the control of this disease (Huygen et al., 2010).

Most of the new – possibly protective – *Map* antigens have been identified by genomic and immunoproteomic approaches and the majority of them have been evaluated within the framework of serodiagnosis or cell-mediated (IFN-γ) diagnostic tests (for a comprehensive review see Mikkelsen et al., 2011).

However, few of these antigens have been tested so far as vaccine candidates, particularly in target species

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

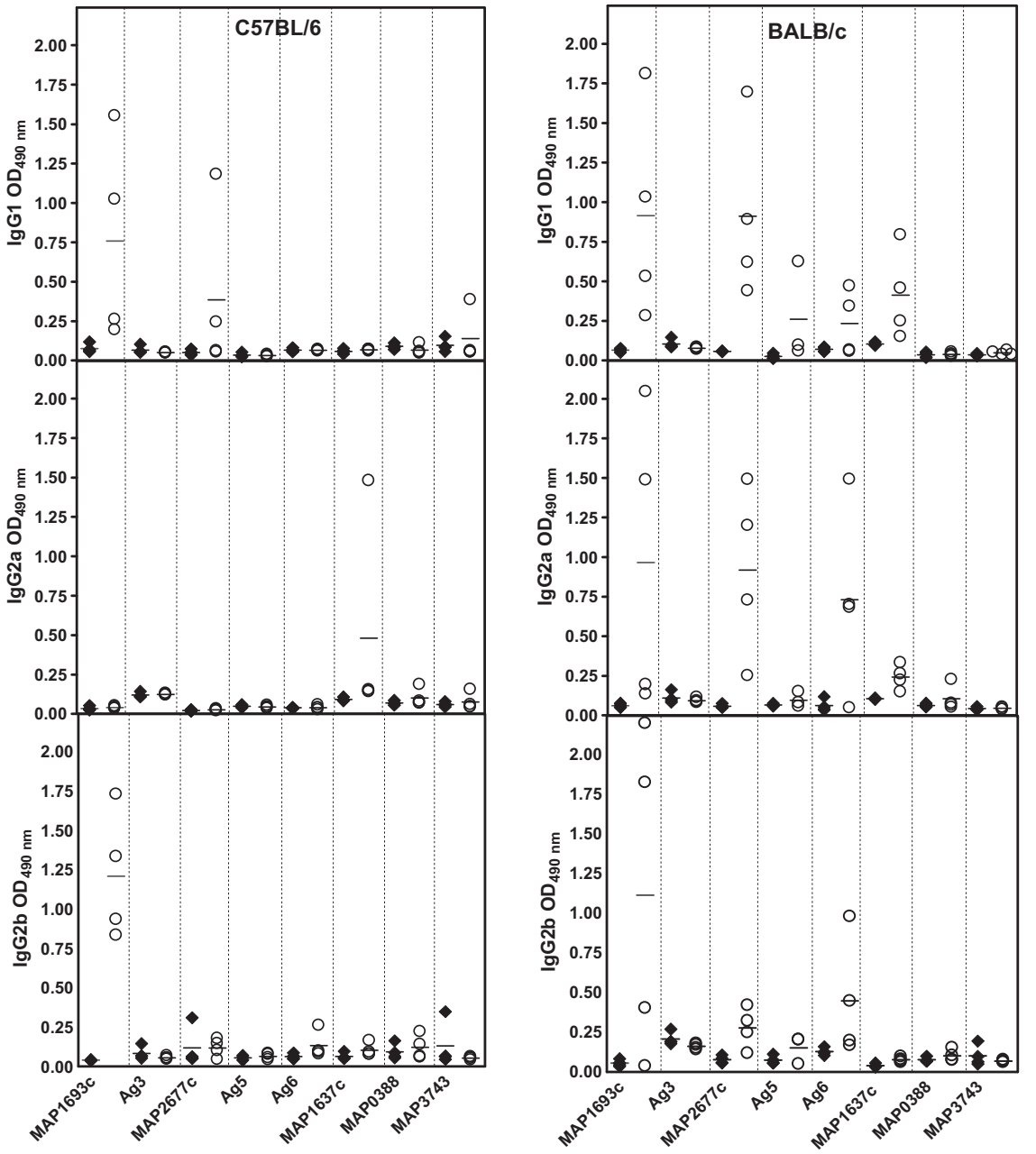

Fig. 2. MAP1693c, Ag3, MAP2677c, Ag5, Ag6, MAP1637c, MAP0388 and MAP3743 specific total IgG1 (top), IgG2a (center) and IgG2b (bottom) antibody responses in sera from C57BL/6 (left) or BALB/c (right) vaccinated with control DNA (black diamond), and with DNA-corresponding *Map* antigens (open circle). Sera were collected 3 weeks after the last immunization and results show individual OD_{490} levels of ELISA performed at a 1:400 serum dilution.

(Rosseels and Huygen, 2008). Up to now, sub-unit protein vaccines composed of Hsp70 (Koets et al., 2006), MAP74F (Chen et al., 2008) or a mix of Ag85A, Ag85B, Ag85C and SOD (Kathaperumal et al., 2008) have been reported. Ag85A, Hsp65 (Sechi et al., 2006), a mix of Ag85A, Ag85B, Ag85C, SOD and MAP2121c (Park et al., 2008), MAP0586c and MAP4308c (Roupie et al., 2008a) have been tested as DNA vaccines and Huntley et al. (2005) reported on the DNA based screening of an expression library. Recombinant viral vectors have been used to study the vaccine potential of MAP1589c/AphC, MAP1234/Gsd, MAP244c and 1235/Mpa gene (Bull et al., 2007).

Here we have analyzed the immunogenicity and vaccine potential of eight new *Map* proteins, using the technique of DNA vaccination, which is an easy and a powerful tool for the screening of the vaccine potential of protein antigens. DNA vaccines are effective in inducing long-lasting cellular and humoral immune responses and DNA vaccines encoding mycobacterial antigens are known to be potent inducers of $CD4^+$ Th1-type and $CD8^+$ cytotoxic T

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

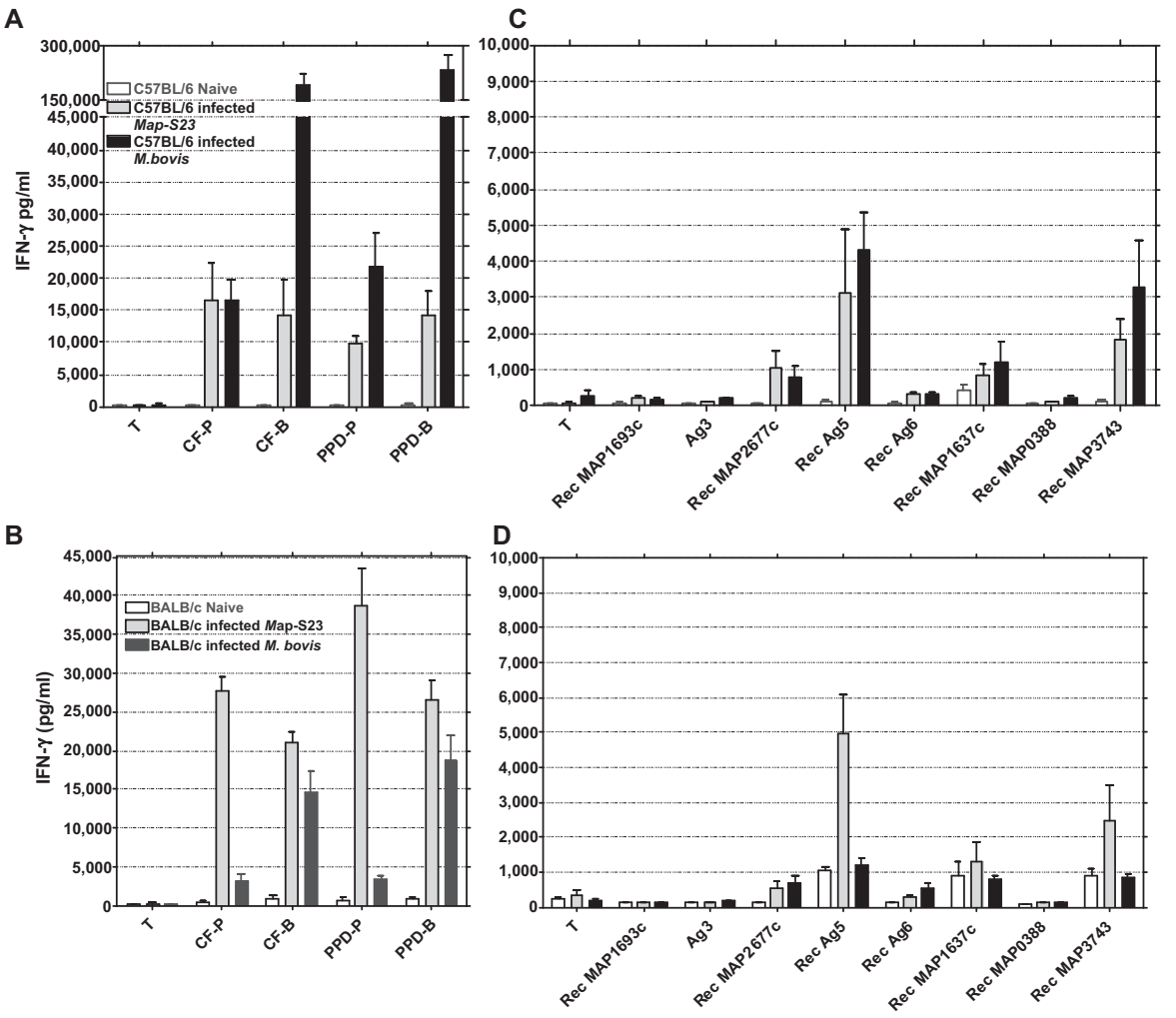

Fig. 3. IFN-γ levels as measured in 72-h spleen cell culture supernatants, from C57BL/6 (top) or BALB/c (bottom) mice either non-infected, infected with *Mycobacterium avium* subsp. *paratuberculosis* S23 (10^6 CFU) or *M. bovis* AN5 (2×10^6 CFU) and non-stimulated (T) or stimulated with the recombinant protein (5 μg/ml) of MAP1693c, Ag3, MAP2677c, Ag5, Ag6, MAP1637c, MAP0388 and MAP3743. Results represent mean ± SEM (error bars) values of three to four mice tested individually in each group.

cell-mediated immune responses (Romano and Huygen, 2009).

In the present study, strong, antigen-specific IFN-γ responses were detected in DNA vaccinated mice against MAP1693c identified in the *Map* secretome and against three of the five *in silico* selected antigens, i.e. MAP1637c, MAP0388 and MAP3743. Strongest antibody responses were detected in DNA vaccinated mice against two of the three secretome proteins, i.e. MAP1693c and MAP2677c. The latter antigen was also recognized by sera from naïve and *Map* infected BALB/c mice. Weaker antibody responses were induced against the *in silico* predicted proteins, which may be related to the final selection step we used, which was based on T cell epitope predictions.

In contrast to the immune response induced in DNA vaccinated mice, T cells from *Map* infected mice reacted very strongly against Ag5, the antigen that was not or only very weakly immunogenic in DNA vaccinated mice. *Map* infected mice also reacted against MAP3743 and against MAP1637c. However, responses against MAP1637c were not *Map* specific, as T cells from naïve, uninfected mice also recognized this protein. Despite being predicted initially as *Map* specific, BLASTP analysis revealed that MAP1637c sequence was identical with an hypothetical protein MaviaA2_11436 (*E*-value = 0) from *M. avium* subsp. *avium*. ATCC 25291. Therefore, recognition of MAP1637c by T cells of naïve mice is possibly related to exposure of the mice to environmental mycobacteria (Le Dantec et al., 2002), perhaps *M. avium* subsp. *avium* in the drinking water (Hilborn et al., 2006, 2008).

DNA vaccines composed of one or several antigens offer an interesting alternative to live attenuated or whole killed bacterial vaccines. Up till now, four veterinary DNA vaccines against equine West Nile virus, salmon infectious haematopoietic necrosis virus, *Mycoplasma hyopneumoniae* disease in pigs and melanoma in dogs have been

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

Table 3
Bacterial replication in spleen from C57BL/6 and BALB/c mice vaccinated 4 times with control DNA, DNA-Ag3, DNA-MAP2677c, DNA-Ag5, DNA-Ag6, DNA-MAP1637c, DNA-MAP0388, DNA-MAP3743, DNA-MAP1693c and with *Mycobacterium bovis* BCG (used as positive control), and infected with bioluminescent *M. avium* subsp. *paratuberculosis* ATCC 19698 8 weeks before. Spleens and livers from individual infected mice were homogenized, and the number of bacteria/organ was enumerated by luminometry (results in RLU converted to $\log_{10}$).

	C57BL/6 RLU/spleen	C57BL/6 RLU/liver	BALB/c RLU/spleen	BALB/c RLU/liver
Control DNA	4.33 ± 0.08 (5)	4.76 ± 0.09 (5)	4.28 ± 0.29 (4)	5.30 ± 0.35 (4)
Control *M. bovis* BCG	3.58 ± 0.10 (4)***	3.83 ± 0.13 (4)***	3.90 ± 0.07 (4)*	4.24 ± 0.05 (4)***
DNA-Ag3	4.63 ± 0.26 (5)ns	4.70 ± 0.19 (5)ns	4.33 ± 0.06 (4)ns	5.53 ± 0.11 (5)ns
DNA-MAP2677c	4.52 ± 0.21 (5)ns	4.49 ± 0.20 (5)ns	4.55 ± 0.15 (4)ns	5.35 ± 0.14 (4)ns
DNA-Ag5	4.56 ± 0.17 (5)ns	4.62 ± 0.17 (5)ns	4.37 ± 0.07 (5)ns	5.38 ± 0.03 (5)ns
Control DNA	5.03 ± 0.12 (4)	4.84 ± 0.09 (4)	4.31 ± 0.15 (4)	5.41 ± 0.07 (4)
Control *M. bovis* BCG	3.64 ± 0.22 (4)***	3.85 ± 0.12 (4)***	3.96 ± 0.24 (5)ns	4.47 ± 0.13 (5)***
DNA-Ag6	4.67 ± 0.64 (4)ns	4.62 ± 0.41 (4)ns	4.43 ± 0.09 (4)ns	5.50 ± 0.18 (4)ns
DNA-MAP1637c	5.04 ± 0.57 (5)ns	4.64 ± 0.45 (5)ns	4.30 ± 0.12 (4)ns	5.37 ± 0.10 (4)ns
DNA-MAP0388	4.96 ± 0.11 (5)ns	4.68 ± 0.09 (5)ns	4.31 ± 0.24 (4)ns	5.30 ± 0.22 (4)ns
DNA-MAP3743	4.79 ± 0.26 (5)ns	4.75 ± 0.11 (5)ns	4.36 ± 0.11 (4)ns	5.26 ± 0.32 (4)ns
DNA-MAP1693c	5.05 ± 0.16 (5)ns	4.70 ± 0.25 (5)ns	4.19 ± 0.17 (5)ns	5.43 ± 0.10 (5)ns

Data represent mean ± S.D. values of 4–5 mice (numbers given in parentheses). Statistical analysis was performed using GraphPad Prism 4: one-way-ANOVA Tukey (confidence intervals: 95%).
ns, not significantly different as compared to $\log_{10}$ RLU values in control DNA vaccinated mice.
*** $p < 0.001$ as compared to $\log_{10}$ RLU values in control DNA vaccinated mice.
* $p < 0.05$ as compared to $\log_{10}$ RLU values in control DNA vaccinated mice.

licensed for commercial use in Canada and US. These licensures are an important validation of the DNA vaccine platform showing its commercial potential and feasibility at a large scale (Kutzler and Weiner, 2008).

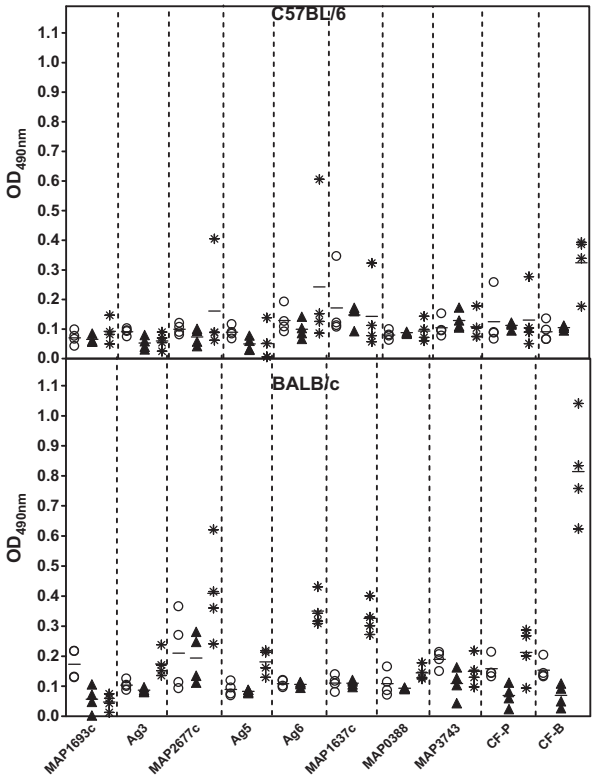

Fig. 4. MAP1693c, Ag3, MAP2677c, Ag5, Ag6, MAP1637c, MAP0388 and MAP3743 specific total IgG antibodies in sera from C57BL/6 (top) or BALB/c (bottom) non infected (open circles), infected with *Map*-S23 (black triangles) or with *M. bovis* (stars). Sera were collected 5 weeks after infection and results show individual OD 492 nm levels of ELISA performed at a 1:100 serum dilution.

Although strong Th1 type immune responses could be induced by some of the DNA vaccines in this study, none of them protected mice against infection with *Map* ATCC 19698. Of all eight antigens tested, only one, i.e. MAP3743 was recognized effectively by both T cells from DNA immunized and *Map* infected mice. In contrast to some of the other antigens, no specific antibodies could be detected in mice vaccinated with MAP3743 DNA. We postulate that differences in immunogenicity profile may explain the negative findings for these eight antigens, T cells induced by the strongest DNA vaccines being unable to exert a protective role because infected cells do not present the immunodominant epitopes of the encoded antigens. Our previous findings on the protective efficacy of a DNA vaccine encoding MAP0586c, i.e. an antigen that is very immunogenic following plasmid vaccination and *Map* infection, are in favor of this hypothesis (Roupie et al., 2008a).

Acknowledgements

V. Roupie held a FRIA bursary (2004–2007) and is supported since 1/02/2008 by Contractueel Onderzoek FOD-Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu Convention RT-07/6 PARATUB. S. Viart holds a FRIA bursary (2008–2011). This work was partially financed through PARADIAG grant from Walloon Region (to J.-J.L. and R.W.). We are very grateful to F. Jurion and C. Van den Poel for excellent technical assistance.

References

Allen, A.J., Park, K.T., Barrington, G.M., Lahmers, K.K., Abdellrazeq, G.S., Rihan, H.M., Sreevatsan, S., Davies, C., Hamilton, M.J., Davis, W.C., 2011. Experimental infection of a bovine model with human isolates of *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Immunol. Immunopathol. 141, 258–266.
Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schaffer, A.A., Yu, Y.K., 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J 272, 5101–5109.

Bull, T.J., Gilbert, S.C., Sridhar, S., Linedale, R., Dierkes, N., Sidi-Boumedine, K., Hermon-Taylor, J., 2007. A novel multi-antigen virally vectored vaccine against *Mycobacterium avium* subspecies *paratuberculosis*. PLoS ONE 2, e1229.

Chaudhuri, R.R., Loman, N.J., Snyder, L.A., Bailey, C.M., Stekel, D.J., Pallen, M.J., 2008. xBASE2: a comprehensive resource for comparative bacterial genomics. Nucleic Acids Res. 36, D543–D546.

Chen, L.H., Kathaperumal, K., Huang, C.J., McDonough, S.P., Stehman, S., Akey, B., Huntley, J., Bannantine, J.P., Chang, C.F., Chang, Y.F., 2008. Immune responses in mice to *Mycobacterium avium* subsp. *paratuberculosis* following vaccination with a novel 74F recombinant polyprotein. Vaccine 26, 1253–1262.

Cho, D., Shin, S.J., Talaat, A.M., Collins, M.T., 2007. Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. Protein Expr. Purif. 53, 411–420.

Cho, D., Sung, N., Collins, M.T., 2006. Identification of proteins of potential diagnostic value for bovine paratuberculosis. Proteomics 6, 5785–5794.

Economou, M., Pappas, G., 2008. New global map of Crohn's disease: genetic, environmental, and socioeconomic correlations. Inflamm. Bowel Dis. 14, 709–720.

Feller, D.C., de la Cruz, V.F., 1991. Identifying antigenic T-cell sites. Nature 349, 720–721.

Feller, M., Huwiler, K., Stephan, R., Altpeter, E., Shang, A., Furrer, H., Pfyffer, G.E., Jemmi, T., Baumgartner, A., Egger, M., 2007. *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. Lancet Infect. Dis. 7, 607–613.

Foley-Thomas, E.M., Whipple, D.L., Bermudez, L.E., Barletta, R.G., 1995. Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. Microbiology 141 (Pt 5), 1173–1181.

Harris, N.B., Barletta, R.G., 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. 14, 489–512.

Hilborn, E.D., Covert, T.C., Yakrus, M.A., Harris, S.I., Donnelly, S.F., Rice, E.W., Toney, S., Bailey, S.A., Stelma Jr., G.N., 2006. Persistence of nontuberculous mycobacteria in a drinking water system after addition of filtration treatment. Appl. Environ. Microbiol. 72, 5864–5869.

Hilborn, E.D., Yakrus, M.A., Covert, T.C., Harris, S.I., Donnelly, S.F., Schmitt, M.T., Toney, S., Bailey, S.A., Stelma Jr., G.N., 2008. Molecular comparison of *Mycobacterium avium* isolates from clinical and environmental sources. Appl. Environ. Microbiol. 74, 4966–4968.

Huntley, J.F., Stabel, J.R., Paustian, M.L., Reinhardt, T.A., Bannantine, J.P., 2005. Expression library immunization confers protection against *Mycobacterium avium* subsp. *paratuberculosis* infection. Infect. Immun. 73, 6877–6884.

Huygen, K., Bull, T., Collins, M.T., 2010. Development of new, paratuberculosis vaccines. In: Behr, M.A., Collins, M.T. (Eds.), Paratuberculosis: Organism, Disease, Control. CABI Publishing, pp. 353–368.

Karcher, E.L., Beitz, D.C., Stabel, J.R., 2008. Modulation of cytokine gene expression and secretion during the periparturient period in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Immunol. Immunopathol. 123, 277–288.

Kathaperumal, K., Park, S.U., McDonough, S., Stehman, S., Akey, B., Huntley, J., Wong, S., Chang, C.F., Chang, Y.F., 2008. Vaccination with recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins induces differential immune responses and protects calves against infection by oral challenge. Vaccine 26, 1652–1663.

Koets, A., Hoek, A., Langelaar, M., Overdijk, M., Santema, W., Franken, P., Eden, W., Rutten, V., 2006. Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. Vaccine 24, 2550–2559.

Kutzler, M.A., Weiner, D.B., 2008. DNA vaccines: ready for prime time? Nat. Rev. Genet. 9, 776–788.

Le Dantec, C., Duguet, J.P., Montiel, A., Dumoutier, N., Dubrou, S., Vincent, V., 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl. Environ. Microbiol. 68, 5318–5325.

Leroy, B., Roupie, V., Noel-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K., Wattiez, R., 2007. Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. Proteomics 7, 1164–1176.

Leroy, B., Viart, S., Trinchero, N., Roupie, V., Govaerts, M., Letesson, J.J., Huygen, K., Wattiez, R., 2009. Use of *Mycobacterium avium* subsp. *paratuberculosis* specific coding sequences for serodiagnosis of bovine paratuberculosis. Vet. Microbiol. 135, 313–319.

Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S., Kapur, V., 2005. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. Proc. Natl. Acad. Sci. U.S.A. 102, 12344–12349.

Magnusson, M., Bentzon, M.W., 1958. Preparation of purified tuberculin RT 23. Bull. World Health Organ. 19, 829–843.

Marchler-Bauer, A., Lu, S., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki, C.J., Lu, F., Marchler, G.H., Mullokandov, M., Omelchenko, M.V., Robertson, C.L., Song, J.S., Thanki, N., Yamashita, R.A., Zhang, D., Zhang, N., Zheng, C., Bryant, S.H., 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res. 39, D225–D229.

McKenna, S.L., Keefe, G.P., Tiwari, A., VanLeeuwen, J., Barkema, H.W., 2006. Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. Can. Vet. J. 47, 1089–1095.

Mikkelsen, H., Aagaard, C., Nielsen, S.S., Jungersen, G., 2011. Review of *Mycobacterium avium* subsp. *paratuberculosis* antigen candidates with diagnostic potential. Vet. Microbiol..

Nielsen, S.S., Toft, N., 2008. Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. Vet. Microbiol. 129, 217–235.

Nielsen, S.S., Toft, N., 2009. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev. Vet. Med. 88, 1–14.

Ott, S.L., Wells, S.J., Wagner, B.A., 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40, 179–192.

Park, S.U., Kathaperumal, K., McDonough, S., Akey, B., Huntley, J., Bannantine, J.P., Chang, Y.F., 2008. Immunization with a DNA vaccine cocktail induces a Th1 response and protects mice against *Mycobacterium avium* subsp. *paratuberculosis* challenge. Vaccine 26, 4329–4337.

Parker, K.C., Bednarek, M.A., Coligan, J.E., 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152, 163–175.

Perrie, Y., Mohammed, A.R., Kirby, D.J., McNeil, S.E., Bramwell, V.W., 2008. Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. Int. J. Pharm. 364, 272–280.

Rammensee, H., Bachmann, J., Emmerich, N.P., Bachor, O.A., Stevanovic, S., 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50, 213–219.

Robinson, M.W., O'Brien, R., Mackintosh, C.G., Clark, R.G., Griffin, J.F., 2011. Immunoregulatory cytokines are associated with protection from immunopathology following *Mycobacterium avium* subspecies *paratuberculosis* infection in red deer. Infect. Immun..

Romano, M., Huygen, K., 2009. DNA vaccines against mycobacterial diseases. Expert Rev. Vaccines 8, 1237–1250.

Rosseels, V., Huygen, K., 2008. Vaccination against paratuberculosis. Expert Rev. Vaccines 7, 817–832.

Rosseels, V., Marche, S., Roupie, V., Govaerts, M., Godfroid, J., Walravens, K., Huygen, K., 2006a. Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. Infect. Immun. 74, 202–212.

Rosseels, V., Roupie, V., Zinniel, D., Barletta, R.G., Huygen, K., 2006b. Development of luminescent *Mycobacterium avium* subsp. *paratuberculosis* for rapid screening of vaccine candidates in mice. Infect. Immun. 74, 3684–3686.

Roupie, V., Leroy, B., Rosseels, V., Piersoel, V., Noel-Georis, I., Romano, M., Govaerts, M., Letesson, J.J., Wattiez, R., Huygen, K., 2008a. Immunogenicity and protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c of *Mycobacterium avium* subsp. *paratuberculosis* secretome. Vaccine 26, 4783–4794.

Roupie, V., Rosseels, V., Piersoel, V., Zinniel, D.K., Barletta, R.G., Huygen, K., 2008b. Genetic resistance of mice to *Mycobacterium paratuberculosis* is influenced by *Slc11a1* at the early but not at the late stage of infection. Infect. Immun. 76, 2099–2105.

Sechi, L.A., Mara, L., Cappai, P., Frothingam, R., Ortu, S., Leoni, A., Ahmed, N., Zanetti, S., 2006. Immunization with DNA vaccines encoding different mycobacterial antigens elicits a Th1 type immune response in lambs and protects against *Mycobacterium avium* subspecies *paratuberculosis* infection. Vaccine 24, 229–235.

Shiver, J.W., Perry, H.C., Davies, M.E., Liu, M.A., 1995. Immune responses to HIV gp120 elicited by DNA vaccination. In: Chanock, R.M., Brown, F., Ginsberg, H.S., Norrby, E. (Eds.), Vaccines 95. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 95–98.

Smith, R.L., Grohn, Y.T., Pradhan, A.K., Whitlock, R.H., Van Kessel, J.S., Smith, J.M., Wolfgang, D.R., Schukken, Y.H., 2009. A longitudinal study on the impact of Johne's disease status on milk production in individual cows. J. Dairy Sci. 92, 2653–2661.

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012

Snewin, V.A., Gares, M.P., Gaora, P.O., Hasan, Z., Brown, I.N., Young, D.B., 1999. Assessment of immunity to mycobacterial infection with luciferase reporter constructs. Infect. Immun. 67, 4586–4593.
Stabel, J.R., 2006. Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal. Anim. Health Res. Rev. 7, 61–70.
Tanghe, A., Content, J., Van Vooren, J.P., Portaels, F., Huygen, K., 2001. Protective efficacy of a DNA vaccine encoding antigen 85A from *Mycobacterium bovis* BCG against Buruli ulcer. Infect. Immun. 69, 5403–5411.
Uzoigwe, J.C., Khaitsa, M.L., Gibbs, P.S., 2007. Epidemiological evidence for *Mycobacterium avium* subspecies *paratuberculosis* as a cause of Crohn's disease. Epidemiol. Infect. 135, 1057–1068.

Please cite this article in press as: Roupie, V., et al., Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and *Map* infected mice. Vet. Immunol. Immunopathol. (2011), doi:10.1016/j.vetimm.2011.10.012