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Immunogenicity and protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c of *Mycobacterium avium* subsp. *paratuberculosis* secretome

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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, vaccines and therapies. In this study, we have evaluated the vaccine potential of two MAP proteins, i.e. MAP0586c and MAP4308c, previously identified by postgenomic and immunoproteomic analysis of MAP secretome as novel serodiagnostic antigens. Immunizations of BALB/c and C57BL/6 mice with plasmid DNA encoding MAP0586c and MAP4308c induced strong Th1 type immune responses to both antigens, whereas antibody responses were only induced upon immunization with DNA encoding MAP4308c. Homologous boosting of DNA vaccinated mice with recombinant protein resulted in strong antibody responses against both proteins. Using synthetic overlapping peptides, immunodominant H-2^d and H-2^b restricted Th1 T cell epitopes were identified. Finally, MAP infected mice generated strong MAP0586c-specific T cell responses and MAP0586c DNA vaccination could protect BALB/c but not C57BL/6 mice against MAP challenge mice to the same extent as the *Mycobacterium bovis* BCG vaccine, indicating that this putative transglycosylase is an interesting vaccine candidate that warrants further investigation.

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1. Introduction

Johne's disease or paratuberculosis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was first described as an atypical case of intestinal bovine tuberculosis by Johne and Frothingham [1]. MAP is the etiological agent of severe chronic enteritis of the small intestine in domesticated and wild ruminants. Disease is prevalent worldwide but mainly in temperate and humid areas [2,3] and causes substantial financial losses in livestock industry (particularly the dairy sector), due to rapid weight loss, reduced milk production, premature culling and reduced fertility of affected animals [4,5].

Moreover MAP may be implicated in Crohn's disease, a human disease with similar histopathological findings and symptoms as those observed in Johne's disease in cattle. The association between MAP and Crohn's disease has been questioned for a long time, but recent improvements in isolation and genomic techniques do seem to indicate an association of *M. paratuberculosis* as either a causative agent or as an opportunistic infection with Crohn's disease [6–9].

Existing MAP vaccines are composed of whole MAP bacteria (killed or attenuated) formulated in mineral oil adjuvants. These vaccines confer a partial protection against MAP, containing the excretion of bacteria in feces and reducing the number of diseased animals. However, their use does not protect against the infection and additionally, they interfere with the tuberculin skin-test used for the diagnosis of bovine tuberculosis and with the indirect tests used for the diagnosis of paratuberculosis (anti-

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body and IFN- γ ELISA). Moreover, they can induce an undesirable granulomatous reaction at the injection site [10,11] and their administration is not without risk for the veterinary practitioner [12]. Therefore, the development of sub-unit based vaccines based on immunodominant MAP-specific antigens, which would not interfere with existing diagnostic tests for bovine tuberculosis or paratuberculosis, could contribute actively to the control of Johne's disease.

IFN- γ is the pivotal Th1 type cytokine involved in the protective immune response against mycobacterial diseases in general [13]. It is well established that the initial stage of an infection with MAP is also controlled by a Th1-type immune response and that progression towards disease goes along with a loss of this Th1 response and the advent of a Th2/possibly regulatory type immune response (IL-4, IL-5 and IL-10) as well as an increase in antibody production [14]. More specifically, it has been shown that bovine monocytes can be protected *in vitro* against MAP by treatment with IFN- γ [15].

Plasmid DNA vaccination is a powerful and easy tool for the high throughput screening of the vaccine potential of protein antigens and DNA vaccines are particularly promising for the prevention of infections caused by intracellular pathogens, precisely because of the ease with which they induce strong Th1 biased cellular immune responses without the use of additional adjuvants. The protective efficacy of DNA vaccines has been extensively studied for *M. tuberculosis*, but a number of studies have also reported on their potential use against other mycobacteria such as *Mycobacterium leprae*, *Mycobacterium bovis*, *Mycobacterium ulcerans* and *M. avium* subsp. *avium* [16,17]. Whereas DNA vaccines are very immunogenic in small rodents, their potency in larger animals and humans is considerably lower. Optimized codon usage, *in vivo* electroporation and formulation of plasmid in adjuvants are but a few of the optimization approaches that may eventually help to overcome this hurdle [18].

Recently, we carried out a large-scale postgenomic analysis of MAP proteins, to identify MAP-specific antigens that could improve the serodiagnosis of bovine paratuberculosis. Performing systematic proteomic identification and immunoproteomic analysis of MAP culture filtrates with sera from MAP and *M. bovis* infected cattle we have identified 25 candidate antigens. Five of these, i.e. MAP0586c, MAP1693c, MAP2677c, MAP3199 and MAP4308c were tested on an extended panel of field sera in an antibody ELISA assay for their diagnostic potential and the combination of three of them (MAP1693c, MAP2677c and MAP4308c) competed in performance with available commercial assays, reaching a test sensitivity of 94.7% and a specificity of 97.9% [19]. These five antigens were also evaluated for their vaccine potential as plasmid DNA vaccines in an experimental mouse model. We present here the results obtained for two of them, namely MAP0586c (NP.959520.1) and MAP4308c (NP.963242.1). Sequence analysis has identified a possible lytic transglycosylase SLT domain in MAP0586c protein and a class I fructose-bisphosphate aldolase activity for MAP4308c protein.

BALB/c and C57BL/6 mice were immunized with either plasmid DNA alone or immunized with a DNA prime-protein boost protocol. Vaccinated mice were challenged by the intravenous route with luminescent MAP and bacterial replication in the spleen was monitored by luminometry. We have previously reported that luminometry is a valuable alternative for cumbersome colony forming unit (CFU) plating and we have used this technique to demonstrate the vaccine potential of an irradiated MAP strain [20]. Finally, the immunodominance and species-specificity of these two antigens was compared in mice infected intravenously with either MAP or virulent *M. bovis*.

2. Materials and methods

2.1. Mice

Female BALB/c and C57BL/6 (C57BL/6) mice were bred in the Animal Facilities of the IPH-Pasteur Institute in Brussels, Belgium, from breeding couples originally obtained from Bantin & Kingman (United Kingdom). All animals were 6–8 weeks old at the start of the experiments. We have recently shown that these two mouse strains are susceptible to intravenous MAP infection and that multiplication of luminescent MAP in spleen and liver can be monitored by luminometry [21].

2.2. Bacteria and antigens

M. avium subsp. *paratuberculosis* ATCC 19698 was purchased from the American Tissue Culture Collection and was grown at 39 °C for 4 weeks as a surface pellicle on synthetic Sauton medium supplemented with mycobactin J (2 μ g/ml) (Allied Laboratories Inc. Synbiotics Europe, Lyon, France) as described previously [22]. Culture filtrate of MAP (CF-P) was separated from the bacteria; CF proteins were precipitated by ammonium sulphate and extensively dialyzed against phosphate-buffered saline (PBS). CF-B of *M. bovis* (strain AN5) was obtained from *M. bovis* cultures grown as surface pellicle for 2 weeks at 37 °C on synthetic Sauton medium. Purified Protein Derivative of bovine tuberculin (PPD-B) from *M. bovis* Vallée was kindly donated to us by the late Dr. J. Nyabenda (IPH-Pasteur Institute Brussels). Johnin (PPD-P) was prepared from 8-week-old cultures of strain ATCC 19698 as described previously [23].

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

Genomic DNA of MAP ATCC 19698 was prepared as described by Tanghe et al. for *M. ulcerans* [24]. Briefly, bacteria were lysed with lysozyme, pronase, and SDS. After phenol–chloroform–iso-amyl alcohol extraction and RNaseA treatment, DNA was precipitated with ethanol, and the pellet was suspended in Tris–EDTA buffer. DNA was analyzed by agarose gel electrophoresis, and the purity was evaluated by spectrophotometry. DNA was kept at –20 °C until use.

2.4. Modification of pV1J.ns-tPA vector into pV1J.ns-tPA-his vector

In order to have an easy means for controlling protein expression by the plasmid DNA vaccines, we first modified pV1J.ns-tPA vector into pV1J.ns-tPA-his, by inserting a hexahistidine tag coding sequence in 3' of the BglII restriction site of the pV1J.ns-tPA vector (Merck Research Laboratories, PA, USA) [25]. We amplified a first gene (seq.2383507.2384184, not used further in this study) from *M. avium* subsp. *paratuberculosis* ATCC 19698 genomic DNA using a forward primer (5'-tat**AGATCT**tggatgggctccggc-3') comprising the BglII restriction site (in bold) and a reverse primer of 63 nucleotides. The latter included two restriction sites: the BglII compatible BamHI (bold) site, used to clone this sequence in pV1J.ns-tPA and the EcoRI (bold underlined) site between the histidine-tag sequence (italic) and the 3' sequence of this gene. The STOP codon (italic underlined) was introduced between the BamHI site and the sequence coding for the histidine-tag (5'-TAT**GGATCCT**AGTGATGGTGATGGT-GAT**GGAATTC**CTTCTACCGAATTTCCGAAGAATATC-3'). The gene was amplified by PCR (Expand High Fidelity PCR System, Roche) with 30 cycles of amplification. PCR fragment was purified on column ("QIAquick PCR Purification", Qiagen, Venlo, Belgium) and digested with BglII/BamHI (Roche, Vilvoorde, Belgium). After

purification on column (Qiagen), PCR fragment was ligated into pV1.Jns-tPA vector digested with BglII (Roche) and dephosphorylated with Shrimp phosphatase (Roche) with T4 DNA ligase (Fermentas, St. Leon-Rot, Germany). Ligation was used to transform DH5- α chemically competent *E. coli* cells (Invitrogen, Merelbeke, Belgium) and positive clones were screened on LB-kanamycin medium (50 μ g/ml). Resulting plasmid was checked by restriction digestion and sequencing. Resulting pV1.Jns-tPA-his digested with BglII/EcoRI (Roche), dephosphorylated with the Shrimp phosphatase (Roche) and purified on agarose gel using “QIAquick Gel Extraction Kit” (Qiagen) was used in the subsequent cloning steps of this study.

2.5. Construction and preparation of DNA vaccines encoding MAP0586c and MAP4308c

MAP0586c (NP.959520.1) and MAP4308c (NP.963242.1) genes were cloned as BglII/EcoRI fragments in pV1.Jns-tPA-his. Briefly, MAP0586c and MAP4308c genes were amplified by PCR (Expand Polymerase; Roche) using MAP ATCC 19698 genomic DNA as a template and using primers (Prologo) designed from the sequence of MAP K-10. The primers used were 5'-GGAAGATCTTGTGCGGTGTGCGCGTACGGG-3' (forward MAP4308c), 5'-ATAGAATTCGCGCGCGACCGAGGCGTCGTA-3' (reverse MAP4308c), 5'-GGAAGATCTTGTGAGCAATCGGCGCACC-3' (forward MAP0586c), 5'-TATAGAATTCCTGCGGTGCGCGGCACCTAGTCCG-3' (reverse MAP0586c).

PCR fragments were purified on column, digested with BglII/EcoRI (Roche, Vilvoorde, Belgium) and ligated into BglII/EcoRI digested pV1.Jns-tPA-his. After ligation (T4 DNA ligase, Fermentas) and transformation into DH5- α chemical competent *E. coli* cells (Invitrogen, Merelbeke, Belgium), positive clones were screened on LB-kanamycin medium (50 μ g/ml) and plasmid was checked by restriction digestion and sequencing. Finally, DNA plasmids were purified in sufficient amounts for vaccination experiments using the PureLink™ HiPure Plasmid DNA Gigaprep kit (Invitrogen).

2.6. Cloning, expression and purification of recombinant MAP0586c and MAP4308c proteins

The genes coding for histidine-tagged MAP0586c and MAP4308c were cloned in *E. coli* expression plasmid pQE-80L (Qiagen, Venlo, Belgium). Briefly, MAP0586c and MAP4308c were amplified by PCR (Expand High Fidelity PCR System, Roche), from pV1.Jns-tPA-his-MAP0586c and pV1.Jns-tPA-his-MAP4308c, respectively. The primers used were 5'-GGAAGATCTGTGAGCAATCGGCGCACCGCA-3' (forward MAP0586c), 5'-TATAAGCTTCACTGCGGTGCGCGCCACCTAGTCCG-3' (reverse MAP0586c), 5'-TATAGGATCTGCGGTGTGCGCGTACGG-3' (forward MAP4308c), 5'-TATAAGCTTCACTGCGGTGCGCGCACCGAGGCGTCGTA-3' (reverse MAP4308c) and were designed from the sequence of MAP K-10.

The amplified sequences were digested with BamHI/HindIII (MAP4308c) and with BglII/HindIII (MAP0586c), purified on agarose gel (QIAkit PCR kit, Qiagen) and ligated into pQE-80L (Qiagen) expression vector predigested with BamHI/HindIII. After ligation (T4 DNA ligase, Fermentas, St. Leon-Rot, Germany) and transformation into Top-10F chemical competent *E. coli* cells (Invitrogen Merelbeke, Belgium), positive clones were screened on LB-ampicillin medium (100 μ g/ml) and plasmid was checked by restriction digestion and sequencing. Recombinant MAP0586c and MAP4308c were expressed as his-tagged protein in Top-10F *E. coli* after IPTG induction and purified by affinity chromatography on immobilized nickel-chelate (Ni-NTA) column as described before [22].

Table 1

Protocol used for vaccination with DNA encoding MAP0586c or MAP4308c and boosting with the homologous recombinant proteins

Week	0	3	6	12
4x V1.Jns-tPA-his-MAP0586c	X	X	X	X
3x V1.Jns-tPA-his-MAP0586c/1x rMAP0586c boost	X	X	X	X
V1.Jns-tPA	X	X	X	X
<i>M. bovis</i> BCG vaccine			X	
3x V1.Jns-tPA-his-MAP4308c	X	X	X	
2x V1.Jns-tPA-his-MAP4308c/1x rMAP4308c boost	X	X	X	
V1.Jns-tPA	X	X	X	
<i>M. bovis</i> BCG vaccine	X			

2.7. Peptide synthesis

Peptides spanning the MAP0586c (313 aa) sequence were synthesized as 20-mer peptides overlapping by 10 residues. Peptides spanning the MAP4308c (312 aa) sequence were synthesized as 15-mer peptides overlapping by 9 residues. Peptides were obtained as a POL PEPscreen custom peptide library from ProlImmune Ltd. (Oxford OX4 4GA, UK). With respect to MAP4308c, our sequence has a total of 312 aa as compared to the 309 aa reported for the annotated NP.963242 sequence. Indeed, in the NH₂ terminal position, our protein encodes for a MCGVPVRAM sequence whereas the NP.963242 sequence starts with MPVRAM. The reason for this difference is not clear for the moment, but it is probably caused by the fact that we identified the MAP4308c protein by proteomics on the basis of the sequence of only some peptides and way before the final annotation was available.

2.8. Transfection of eukaryotic cells and Western blot analysis

The ability of pV1.Jns-tPA-his-MAP0586c and V1.Jns-tPA-his-MAP4308c to express their respective encoded proteins in eukaryotic cells was tested by transient transfection of BHK-21 cells (Baby Hamster Kidney 21-C13, ATCC CCL-10, Bio Whittaker, Europe) using Lipofectamine™ Reagent (Invitrogen Merelbeke, Belgium) [26,27]. BHK-21 cells were cultured to a confluence of 50–80% in Dulbecco's modified minimal essential medium (D-MEM) (Invitrogen), supplemented with 10% fetal calf serum (FCS) (Greiner bio-one, Wemmel, Belgium) in six-well plates. 2 μ g of plasmid DNA was used to transfect the cells. After 48 h, cells were harvested by vigorous pipetting in PBS, washed and cells were lysed in 60 mM Tris-HCl pH 6.8, 10% glycerol, 1% sodium dodecyl sulphate (SDS) and β -mercapto-ethanol buffer. Cell lysates were analysed by Western blot for the presence of MAP0586c and MAP4308c. Briefly, protein extracts were separated using 12% sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred onto a nitrocellulose membrane (Trans-Blot® transfer medium, Bio-Rad, Hercules, CA). Incubation of membrane with primary mouse anti-histidine tag antibodies (MCA 1396, Serotec, Düsseldorf, Germany) was followed by incubation with alkaline phosphatase-conjugated polyclonal rabbit anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark). Revelation was performed using α -chloronaphtol (Bio-rad) in presence of H₂O₂ for 10 min.

2.9. Vaccination

Mice were vaccinated three times (MAP4308c) or four times (MAP0586c) according to the protocol given in Table 1. For DNA immunizations, mice were sedated with ketamine/xylazine and injected intramuscularly in both quadriceps muscles with 2 \times 50 μ g of pDNA. For protein boosting (last immunization only), mice were injected subcutaneously (s.c.) in the back with 20 μ g of

purified protein emulsified in incomplete Freund adjuvant (IFA) (Sigma–Aldrich, Bornem, Belgium).

Mice were vaccinated intravenously with 0.5 mg (ca. 2×10^6 CFU) of freshly prepared *M. bovis* BCG GL2, grown as a surface pellicle on synthetic Sauton medium for 14 days.

2.10. *M. avium* subsp. *paratuberculosis* infection

MAP S-23 [28] was grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J 2 µg/ml (Allied Laboratories Inc. Synbiotics Europe, Lyon, France) and hygromycin (100 µg/ml) (Roche, Vilvoorde, Belgium), to an optical density ranging between 0.6 and 0.8. Bacteria were centrifuged for 30 min at 537 g and suspended in PBS to a concentration of 10^7 CFU/ml. Mice were infected intravenously in a lateral tail vein with 0.2 ml of bacteria and sacrificed 5 and 10 weeks post-infection.

2.11. *M. bovis* infection

Mice were inoculated intravenously with 0.05 mg (ca. 1.3×10^6 CFU) of *M. bovis* AN5 (kindly provided by the late Dr. J. Nyabenda, WIV-Pasteur Institute) from a stock kept frozen at -80°C which had been grown for 2 weeks as a surface pellicle on synthetic Sauton medium at 37°C . Animals were sacrificed 5 and 10 weeks post-infection.

2.11.1. Antibody ELISA

Sera from C57BL/6 and BALB/c vaccinated or infected mice were collected by tail bleeding respectively 3 weeks after the last immunization or 10 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 isotype was determined using peroxidase-labeled rat anti-mouse immunoglobulin IgG, IgG1, IgG2a, IgG2b (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium) and orthophenyldiamine (Sigma–Aldrich, Bornem, Belgium) for revelation. Data are presented as the optical density at 490 nm (OD_{490}) for a serum dilution of 1:1600.

2.11.2. Cytokine production

DNA vaccinated mice were sacrificed 3 weeks after the last immunization, *M. bovis* AN5 and MAP S23 infected mice were sacrificed 5 and 10 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–Aldrich, Bornem, Belgium) was added to RPMI-complete culture medium.

Cells were stimulated with purified recombinant antigens (5 µg/ml) or synthetic peptides (10 µg/ml) and incubated at 37°C in round-bottom, 96-well microtiter plates in a humidified CO_2 incubator. Cytokine responses against whole protein were tested on spleens from individual mice. For peptide mapping, spleens of 3–4 mice were pooled. 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.

2.12. IL-2 bio-assay

IL-2 activity was measured using a bio-assay on IL-2 dependent CTLL-2 cells, as described before [29]. Each sample was tested in duplicate. IL-2 levels are expressed as mean counts per minute (cpm) of incorporated [^3H] thymidine. A typical international standard curve of this assay has been published before [30].

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, Erembodegem, Belgium). The detection limit of the IFN-γ ELISA is 5 pg/ml. Antigen-specific cytokine levels were considered positive when values were at least five-fold higher than those of unstimulated cells.

2.14. IFN-γ enzyme-linked immunospot (ELISPOT) assay

Antigen-specific spleen cell IFN-gamma secretion was also assayed by ELISPOT as described earlier [27,31]. Briefly, 96-well flat-bottomed nitrocellulose plates (MAHA S4510, Millipore, Billerica, MA) were incubated overnight at 4°C with 50 µl of capture purified anti-mouse IFN-γ (15 µg/ml; BD Pharmingen, Erembodegem, Belgium) in phosphate-buffered saline (PBS) and then saturated with 200 µl/well of RPMI-complete medium 2 h at 37°C . 50 µl of spleen lymphocytes (pool of four mice per group) were added at a cell concentration of $20 \times 10^6 \text{ ml}^{-1}$ in the presence or absence of proteins (5 µg/ml) and plates were incubated for 48 h at 37°C , 5% CO_2 . After extensive washing, plates were incubated 2 h at 37°C , 5% CO_2 with 50 µl of biotinylated rat anti-mouse IFN-γ (2 µg/ml) (BD Pharmingen), washed and incubated for 45 min at 37°C , 5% CO_2 with alkaline phosphatase labelled ExtrAvidine (Sigma–Aldrich, Bornem, Belgium). After washing, spots were revealed with Bio-Rad (Hercules, CA) alkaline phosphatase conjugate substrate kit, following the manufacturer's instructions and plates were analysed on a Bioreader–3000 LC (BioSys, Germany). Results are shown as mean spot-forming cells (SFC) per million lymphocytes.

2.15. *M. avium* subsp. *paratuberculosis* challenge

DNA vaccinated C57BL/6 and BALB/c mice were challenged 6 weeks after the last immunization. Luminescent MAP ATCC 19698 [32] was grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J (2 µg/ml, Allied Laboratories Inc, Synbiotics Europe) and hygromycin (100 µg/ml, Roche, Vilvoorde, Belgium), to an optical density ranging between 0.6 and 0.8. Bacteria were centrifuged for 30 min at 2000 rpm, suspended in PBS to a concentration of 10^7 CFU/ml (8.5×10^6 RLU/ml). Luminescence was measured in a Lumat LB 9507 Luminometer (Berthold Technologies) as flash emission (15 s integration time) using 1% *n*-decanal (Sigma–Aldrich, Bornem, Belgium) in ethanol as substrate. In this assay, only live bacteria are enumerated, because emission of light is dependent on the presence of reduced flavin mononucleotide ($\text{FMN}(\text{H}_2)$), co-factor which is only found in living cells. Mice were infected intravenously in a lateral tail vein with 0.2 ml of bacteria and 8 weeks after challenge, mice were sacrificed and the number of bioluminescent bacteria was determined in spleen homogenates. For statistical analysis (one way ANOVA, Tukey's Multiple Comparison Test), results obtained in relative light units (RLUs)/spleen were converted to \log_{10} values [20].

2.16. Counting the number of CFUs

The CFU number in spleen was also determined by plating duplicate serial dilutions of organ homogenates in PBS on Middlebrook 7H11 (BD, Le pont de Claix, France)–OADC agar supplemented with mycobactin J (2 µg/ml, Allied Laboratories Inc. Synbiotics Europe). Petri dishes were sealed in plastic bags and incubated at 39 °C for 8 weeks, before counting the colonies visually.

2.17. BLAST analysis

BLAST analysis was performed using <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> protein database [33,34].

3. Results

3.1. Expression of MAP0586c and MAP4308c proteins in transiently transfected BHK-21 cells

Transfection of BHK-21 cells combined with Western blot analysis, using anti-histidine tag antibodies, confirmed that pV1J.ns-tPA-his-MAP0586c and pV1J.ns-tPA-his-MAP4308c effectively induced significant levels of expression of the corresponding antigens in eukaryotic cells (Fig. 1). Plasmids encoding MAP0586c and MAP4308c expressed a 34 and a 35 kDa protein, respectively. For MAP4308c protein, four protein bands were detected with the anti-histidine tag antibody, suggesting partial protein degradation of the antigen.

3.2. Th1 type cytokine secretion in response to MAP0586c and MAP4308c in plasmid DNA-vaccinated C57BL/6 and BALB/c mice

Vaccination of C57BL/6 and BALB/c mice with the MAP0586c DNA vaccine (including a vaccination regimen in which DNA vaccination was combined with a recombinant protein boost for the last immunization) induced elevated levels of antigen-specific IL-2 and IFN-γ (Table 2). IFN-γ levels were comparable (in the order of 7000 pg/ml) in C57BL/6 and BALB/c mice and with both immunization protocols. IFN-γ ELISPOT levels were two- to three-fold higher in mice that had received the combined DNA/protein immunization. IL-2 levels also tended to be higher in BALB/c (but not in C57BL/6) mice that had been vaccinated with the DNA/protein combination.

Table 2

MAP0586c-specific IL-2 and IFN-γ production in spleen cell cultures from C57BL/6 and BALB/c mice vaccinated with V1J.ns-tPA (plasmid control), only with V1J.ns-tPA-his-MAP0586c or boosted with MAP0586c protein

Vaccine used	IL-2 (CPM) ^a		IFN-γ (pg/ml) ^b		IFN-γ (SFC/10 ⁶ splenocytes) ^c	
	Unstimulated	Stimulated with recombinant protein	Unstimulated	Stimulated with recombinant protein	Unstimulated	Stimulated with recombinant protein
C57BL/6						
Control DNA 4X	744 ± 164	339 ± 118	47 ± 40	213 ± 24	1 ± 1	13 ± 12
DNA-MAP0586c 4X	905 ± 458	21,404 ± 5171	47 ± 80	7487 ± 842	9 ± 4	126 ± 16
DNA-MAP0586c 3X + rMAP0586c	843 ± 71	25,042 ± 7099	61 ± 13	8115 ± 1286	0	435 ± 87
BALB/c						
Control DNA 4X	1050 ± 163	1770 ± 200	35 ± 13	649 ± 107	31 ± 10	71 ± 24
DNA-MAP0586c 4X	1289 ± 373	8499 ± 5655	22 ± 4	5817 ± 625	18 ± 8	92 ± 36
DNA-MAP0586c 3X + rMAP0586c	1280 ± 106	22,915 ± 12,125	29 ± 15	7092 ± 3930	16 ± 17	185 ± 72

^a IL-2 levels (counts per minute [mean ± S.D.] for three to four mice tested individually) in spleen cell cultures stimulated or not for 24 h with purified protein MAP0586c (5 µg/ml). Mice were sacrificed 3 weeks after the last immunization.

^b IFN-γ levels (picograms per milliliter [mean ± S.D.] for three to four mice tested individually) in spleen cell cultures stimulated or not for 72 h with purified protein MAP0586c (5 µg/ml). Mice were sacrificed 3 weeks after the last immunization.

^c Numbers of IFN-γ producing T cells are expressed as spot-forming cells per million splenocytes (SFC/10⁶ splenocytes) for four mice tested in pool and in triplicate in spleen cell cultures stimulated or not for 48 h with protein purified protein MAP0586c (5 µg/ml). Mice were sacrificed 3 weeks after the last immunization.



Fig. 1. Expression of the proteins by the three plasmids: pV1J.ns-tPA (lane 2), pV1J.ns-tPA-his-MAP0586c (lane 3), pV1J.ns-tPA-his-MAP4308c (lane 4). Lysates of transfected BHK-21 cells were analysed by Western blot using mouse anti-histidine tag (MCA 1396) SEROTEC and peroxidase labeled rabbit anti-mouse IgG antibody (Dako). Positions of Rainbow molecular weight (Amersham) markers 30 kDa (carbonic anhydrase) and 45 kDa (ovalbumin) are given in lane 1.

MAP4308c-specific immune responses after vaccination of C57BL/6 and BALB/c mice are summarized in Table 3. As for MAP0586c, significant antigen-specific IL-2 and IFN-γ levels could be detected in both mouse strains. Overall, three immunizations with MAP4308c DNA induced higher IL-2 and IFN-γ levels than two DNA immunizations followed by a protein boost.

IL-2 and IFN-γ responses upon re-stimulation with crude culture filtrate of MAP (CF-P), of *M. bovis* (CF-B) or with purified protein derivative preparation from MAP (Johnin/PPD-P) and from *M. bovis* (PPD-B) were below detection level in all groups (data not show). It is possible, that the amounts of MAP0586c and MAP4308c pro-

Table 3
MAP4308c-specific IL-2 and IFN- γ production in spleen cell cultures from C57BL/6 and BALB/c mice vaccinated with V1J.ns-tPA (plasmid control), only V1J.ns-tPA-his-MAP4308c or boosted with MAP4308c protein

Vaccine used	IL-2 (CPM) ^a		IFN- γ (pg/ml) ^b		IFN- γ (SFC/10 ⁶ splenocytes) ^c	
	Unstimulated	Stimulated with recombinant protein	Unstimulated	Stimulated with recombinant protein	Unstimulated	Stimulated with recombinant protein
C57BL/6						
Control DNA 3X	3559 \pm 2394	1895 \pm 670	260 \pm 418	373 \pm 291	8 \pm 0	20 \pm 9
DNA-MAP4308c 3X	2751 \pm 326	32,425 \pm 3069	41 \pm 14	11,457 \pm 289	22 \pm 3	300 \pm 48
DNA-MAP4308c 2X + rMAP4308c	3270 \pm 739	6697 \pm 1456	138 \pm 127	3036 \pm 2440	10 \pm 14	75 \pm 23
BALB/c						
Control DNA 3X	2660 \pm 284	1753 \pm 239	72 \pm 40	1,933 \pm 1,214	10 \pm 2	57 \pm 14
DNA-MAP4308c 3X	2389 \pm 405	21,389 \pm 9215	51 \pm 28	7,108 \pm 505	14 \pm 9	291 \pm 8
DNA-MAP4308c 2X + rMAP4308c	4443 \pm 3195	46,028 \pm 15,952	148 \pm 137	6,924 \pm 1,842	13 \pm 3	161 \pm 24

^a IL-2 levels (counts per minute [mean \pm S.D.] for three to four mice tested individually) in spleen cell cultures stimulated or not for 24 h with purified protein MAP4308c (5 μ g/ml). Mice were sacrificed 3 weeks after the last immunization.

^b IFN- γ levels (picograms per milliliter [mean \pm S.D.] for three to four mice tested individually) in spleen cell cultures stimulated or not for 72 h with purified protein MAP4308c (5 μ g/ml). Mice were sacrificed 3 weeks after the last immunization.

^c The numbers of IFN- γ producing T cells are expressed as spot-forming cells per million splenocytes (SFC/10⁶ splenocytes) for four mice tested pool and in triplicate in spleen cell cultures stimulated or not for 48 h with protein purified protein MAP4308c (5 μ g/ml). Mice were sacrificed 3 weeks after the last immunization.

tein present in these crude preparations were insufficient to induce detectable levels of IL-2 and IFN- γ responses in mice vaccinated with these sub-unit type DNA vaccines, albeit that both proteins were identified using immunoproteomic analysis of MAP culture filtrate with sera from MAP infected cattle [19].

3.3. MAP0586c and MAP4308c-specific antibody production in plasmid DNA-vaccinated C57BL/6 and BALB/c mice

In contrast to the strong IL-2 and IFN- γ response, vaccination with plasmid DNA encoding MAP0586c induced only very weak levels of IgG1 antibodies in C57BL/6 and BALB/c mice (Fig. 2). Vaccination with MAP4308c DNA on the other hand effectively stimulated the production of IgG1 antibodies. Boosting with homologous recombinant protein resulted in significant increased antibody levels to both antigens (Fig. 2). Increased antibody responses following boosting were also observed for antibodies of IgG2a and of IgG2b isotype, reflecting the Th1 biased priming by both DNA vaccines (Fig. 3).

3.4. Identification of immunodominant H-2^b and H-2^d restricted epitopes of MAP0586c

In order to identify the immunodominant T cell epitopes on MAP0586c, spleen cells from DNA vaccinated C57BL/6 and BALB/c mice were stimulated with synthetic, overlapping 20-mer peptides spanning the entire MAP0586c sequence and supernatants were tested for IL-2 and IFN- γ content. C57BL/6 mice responded to peptide spanning aa 291–310 and the two flanking peptides (Fig. 4, upper panels) and cytokine levels were highest in mice vaccinated with the DNA/protein combination. Additionally, weaker T cell epitopes were located in the NH₂-terminal region of the protein, spanning aa 1–40 and in peptide spanning aa 161–180.

BALB/c mice vaccinated with MAP0586c recognized a larger number of peptides than C57BL/6 mice and overall IFN- γ responses tended to be higher (Fig. 4, lower panels). Strongest responses were directed against peptide spanning aa 171–190, but peptides spanning aa 1–30, 121–140, 241–270 and 291–313 also induced significant cytokine levels. Protein boosting did not increase IFN- γ responses of BALB/c mice to MAP0583c except to protein region spanning aa 241–270. All immunostimulatory MAP0586c peptides induced both IL-2 and IFN- γ , except for the carboxy-terminal peptide spanning aa 291–313 of MAP0586c against which a strong IFN- γ response was detected in the absence of an IL-2 response. It

remains to be determined whether this is indicative of the presence of an MHC class I restricted T cell epitope, as we have previously identified in mice vaccinated with DNA encoding the mycolyl-transferase Ag85A [35], the phosphate-binding PstS-3 lipoprotein [31] and the Dormancy Regulon encoded antigen Rv2626c [36].

3.5. Identification of immunodominant H-2^b and H-2^d restricted epitopes of MAP4308c

T cell epitope mapping in C57BL/6 mice vaccinated with MAP4308c DNA, using synthetic 15-mer overlapping peptides, revealed an immunodominant H-2^b restricted Th1 epitope spanning aa 187–201. Other peptides inducing IL-2 and IFN- γ spanned aa 13–27, 139–153 and 241–256 (Fig. 5, upper panels). Confirming the response observed in mice against the total MAP4308c protein, cytokine levels in response to peptide stimulation were higher in mice vaccinated three times with DNA than in mice that received the DNA prime/protein boost combination.

In BALB/c mice, an immunodominant H-2^d restricted epitope was identified on MAP4308c, spanning aa 241–261 (Fig. 5, lower panels). Weaker responses were detected in BALB/c mice against peptides spanning aa 1–15, 61–75 and 265–285.

3.6. Immune response against MAP0586c and MAP4308c in BALB/c and C57BL/6 mice infected with *M. avium* subsp. *paratuberculosis* S-23 or *M. bovis* AN5

As shown in Fig. 6, mice infected 5 weeks previously with MAP demonstrated robust IFN- γ responses against CF-P and these responses were partially species-specific and higher than against CF-B. Conversely, IFN- γ responses to CF-B were much stronger in mice infected with *M. bovis* than in mice infected with MAP. Although we have identified MAP0586c on the basis of its (serological) specificity for MAP as compared to *M. bovis*, the protein was recognized very strongly and with a similar magnitude by T cells from MAP and *M. bovis* infected C57BL/6 mice. *M. bovis* infected BALB/c mice also reacted to MAP0586c, but antigen-specific cytokine levels were about five-fold higher following MAP infection. Interestingly, even naïve uninfected mice, particularly of the BALB/c strain, also reacted to MAP0586c, which may be indicative of a cross-reactive response induced by previous sensitization to environmental (possibly *M. avium* subsp. *avium*) mycobacteria. IFN- γ responses were also examined in infected mice following stimulation with the peptides previously identified by plasmid DNA

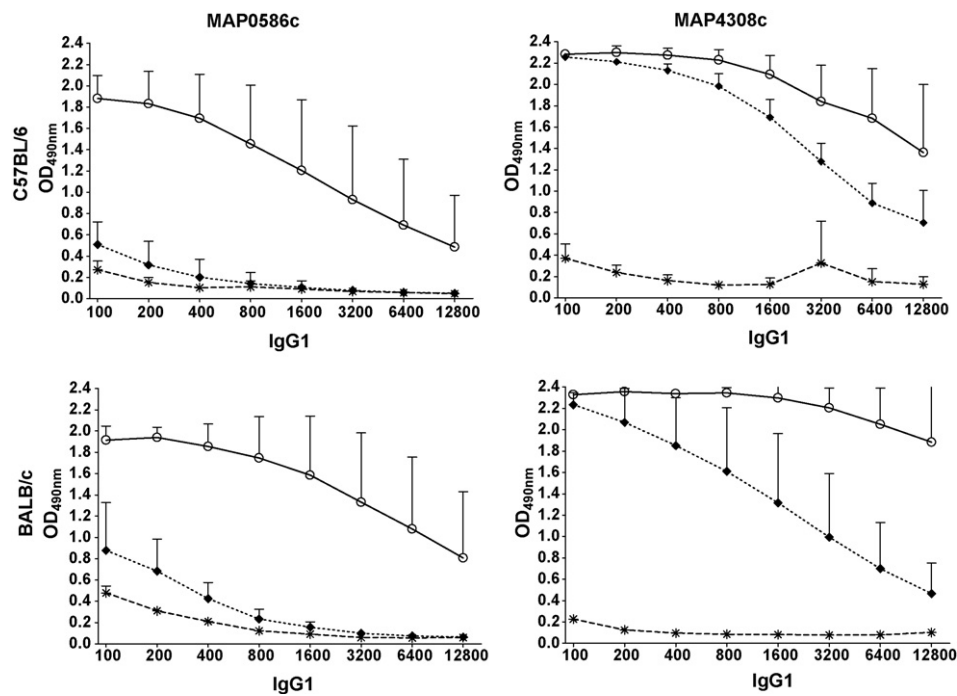


Fig. 2. MAP0586c- and MAP4308c-specific IgG1 antibodies in sera from C57BL/6 or BALB/c vaccinated with control DNA (stars), with MAP4308c-DNA or MAP0586c-DNA (diamond) or with MAP4308c-DNA or MAP0586c-DNA boosted with the respective recombinant proteins (circle). Sera were collected 3 weeks after the last immunization and results show mean and standard deviation of pooled OD₄₉₀ levels of ELISA performed as a serial dilution.

vaccination (aa 1–20, 161–180, 291–310 and 301–313 in C57BL/6 mice and aa 1–20, 121–140, 171–190, 241–280 and 291–310 in BALB/c mice). Responses were below detection level for all peptides (data not shown) except for peptide spanning aa 1–20, which was recognized in all four *M. bovis* (not MAP) infected C57BL/6 mice and for peptide spanning aa 121–140 which was strongly recognized in MAP infected mice and to a lesser extent in *M. bovis* infected BALB/c mice. Naïve uninfected mice also reacted to this peptide (Fig. 6).

In contrast, IFN- γ responses could not be detected in MAP or *M. bovis* infected mice against MAP4308c protein (Fig. 6) nor against any of the immunodominant peptides identified by DNA vaccination (aa 13–27, 139–153, 181–195 and 187–201 in C57BL/6 mice and aa 61–75, 241–255, 247–261 and 271–285 in BALB/c mice) (data not shown).

Ten weeks post-infection, IFN- γ responses to MAP0586c had further increased in mice infected with MAP and *M. bovis*, whereas

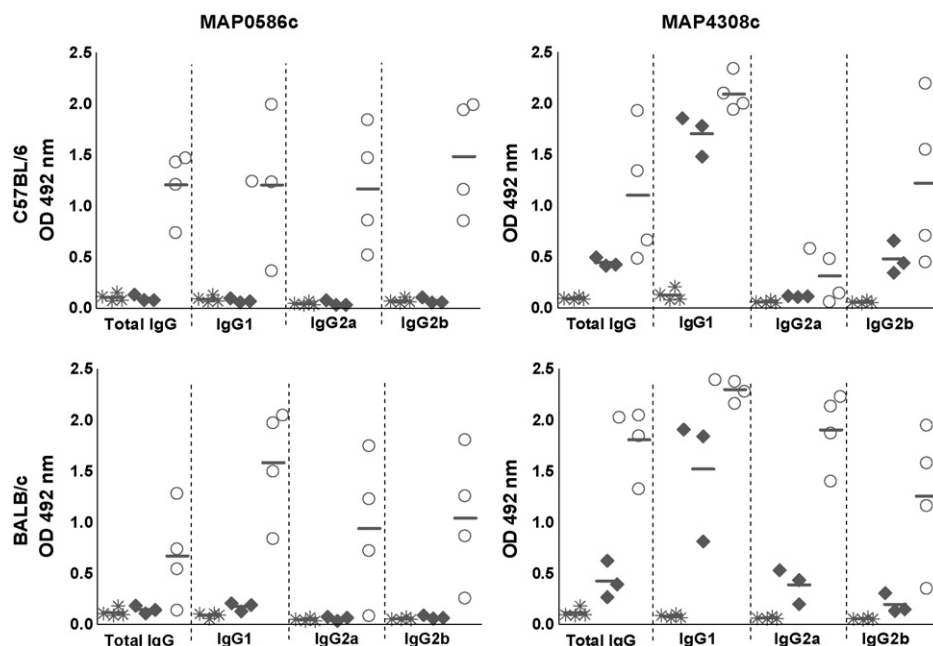


Fig. 3. MAP0586c- and MAP4308c-specific total IgG, IgG1, IgG2a and IgG2b antibodies in sera from C57BL/6 or BALB/c vaccinated with control DNA (stars), with MAP4308c-DNA or MAP0586c-DNA (diamond) or with MAP4308c-DNA or MAP0586c-DNA boosted with the respective recombinant proteins (circle). Sera were collected 3 weeks after the last immunization and results show individual OD₄₉₀ levels of ELISA performed at a 1:1600 dilution.

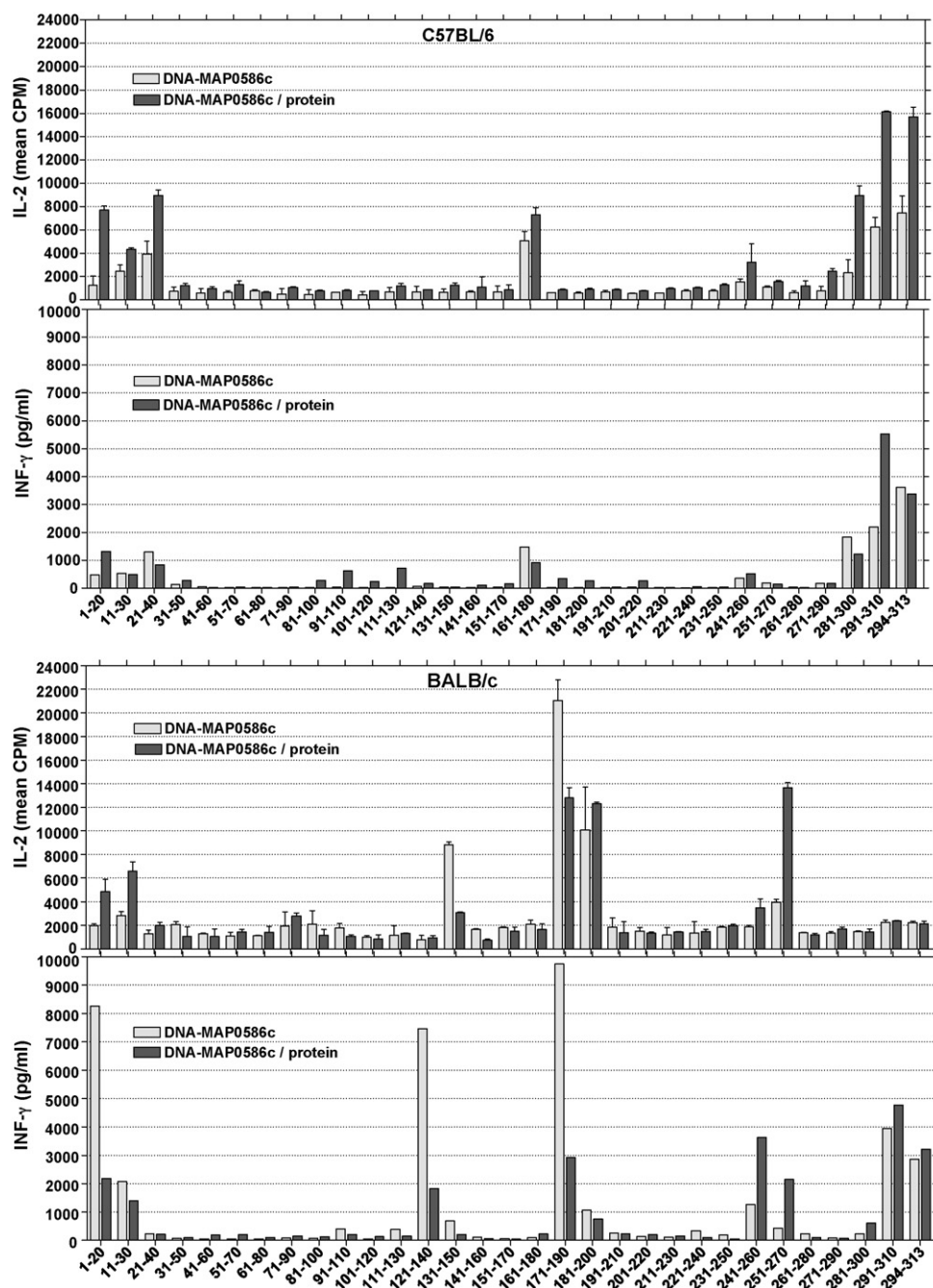


Fig. 4. IL-2 and IFN- γ levels as measured in 24- and 72-h spleen cell culture supernatants, respectively, from C57BL/6 or BALB/c mice stimulated with one of the 31 overlapping (10 aa) synthetic 20-mer peptides of MAP0586c (10 μ g/ml).

responses to MAP4308c protein were still undetectable (data not shown). MAP0586c- and MAP4308c-specific antibodies were below detection level in MAP infected C57BL/6 and BALB/c mice at both time points (data not shown).

3.7. Protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c

Immunization with MAP0586c DNA conferred partial protection to BALB/c mice from an experimental challenge infection with luminescent MAP (administered 6 weeks after the last immunization), as analyzed at 8 weeks post-infection and compared to mice vaccinated with empty vector (Table 4). Both the DNA and the

DNA/protein combination effectively reduced the number of bacteria in the spleen, as determined by luminometry and CFU plating. DNA/protein combination was as effective as *M. bovis* BCG vaccine ($p > 0.05$). Vaccination with MAP0586c DNA did not protect C57BL/6 mice against MAP challenge, possibly a reflection of the low MAP0586c-specific IFN- γ response induced in this mouse strain by infection, against the immunodominant peptide spanning aa 1–20, identified in vaccinated animals (Table 4). Bacterial replication was the same in mice vaccinated with empty vector and in mice vaccinated with empty vector followed by IFA adjuvant (data not shown). DNA vaccine encoding MAP4308c did not confer any protection against an experimental challenge infection with luminescent MAP 8 weeks post-infection (data not shown).

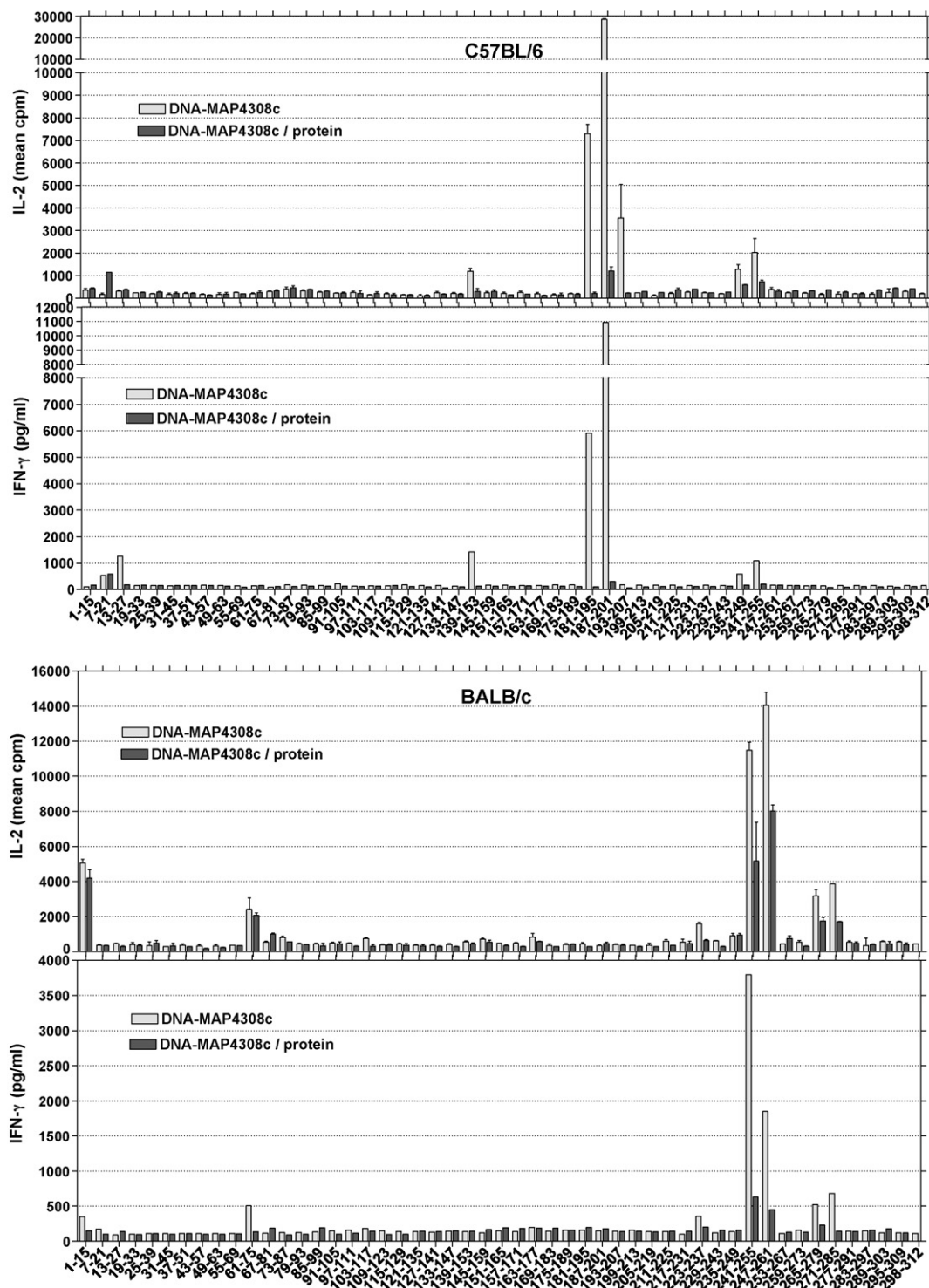


Fig. 5. IL-2 and IFN- γ activities as measured in 24- and 72-h spleen cell culture supernatants, respectively, from C57BL/6 or BALB/c mice stimulated with one of the 51 overlapping (9 aa) synthetic 15-mer peptides of MAP4308c (10 μ g/ml).

4. Discussion

Several countries have initiated control programs to stop the spreading of Johne's disease. However, increasing national and international trade, the slow multiplication rate, the long survival time of MAP bacteria in the environment and the large proportion of subclinically infected animals – difficult to identify with present diagnostic tools – hamper seriously the control of this disease [4].

Vaccines against MAP are currently available in some countries. They are composed of whole bacteria (killed or live attenuated) formulated in mineral oil adjuvant but they confer only partial protection against MAP, do not prevent infection and interfere with skin testing for bovine tuberculosis and with indirect, immune diagnosis of MAP [10]. The development of a marked subunit vaccine composed of one or more MAP specific and protective antigens, which would not interfere with current immunodiagnosis of bovine

Table 4
Protective efficacy of MAP0586c vaccine in C57BL/6 and BALB/c mice

	C57BL/6			BALB/c		
	log ₁₀ RLU ^a	log ₁₀ CFU ^b	Δ ^c	log ₁₀ RLU ^a	log ₁₀ CFU ^b	Δ ^c
Control DNA	5.05 ± 0.25 (5)	5.66 ± 0.15 (5)		5.30 ± 0.19 (5)	5.94 ± 0.04 (5)	
DNA-MAP0586c	4.97 ± 0.15 (5) ^{ns}	5.85 ± 0.15 (5) ^{ns}	−0.08	4.80 ± 0.27 (4) [*]	5.56 ± 0.23 (4) [*]	−0.50
DNA-MAP0586c + rMAP0586c	5.05 ± 0.14 (5) ^{ns}	5.90 ± 0.14 (5) ^{ns}	0.0	4.72 ± 0.19 (5) ^{**}	5.38 ± 0.21 (5) ^{***}	−0.58
<i>M. bovis</i> BCG	3.90 ± 0.21 (5) ^{***}	nd	−1.15	4.61 ± 0.22 (3) ^{**}	nd	−0.69

Bacterial replication in spleen from C57BL/6 and BALB/c mice vaccinated with control DNA, MAP0586c-DNA, MAP0586c-DNA boosted with recombinant MAP0586c protein in IFA adjuvant, and vaccinated with *M. bovis* BCG and infected with bioluminescent *M. avium* subsp. *paratuberculosis* ATCC 19698 8 weeks before. Spleens from individual infected mice were homogenized, and the number of bacteria/organ was enumerated by luminometry (results in RLU converted to log₁₀)^a or by plating on Middlebrook 7H11 agar (results in CFU converted to log₁₀)^b. Data represent mean ± S.D. values of 3–5 mice (numbers given in parentheses). Statistical analysis was performed using GraphPad Prism 4: One-way-ANOVA Tukey: *** ($p < 0.001$), ** ($0.001 < p < 0.01$), * ($p < 0.05$) and ns = not significantly different. nd: not done. Superscript letter (c): as compared to log₁₀ RLU values in control DNA vaccinated mice.

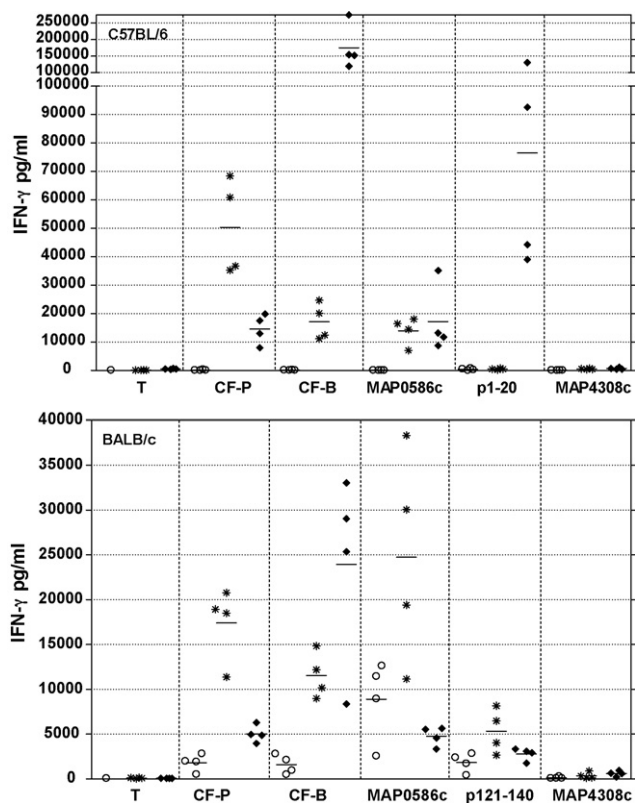


Fig. 6. IFN- γ levels as measured in 72-h spleen cell culture supernatants, from C57BL/6 or BALB/c mice either non-infected (open circles), infected with MAP-S23 (stars) or *M. bovis* AN5 (black diamonds) and non-stimulated (T) or stimulated with culture filtrate from MAP (CF-P) or *M. bovis* (CF-B), with purified MAP0586c protein, with MAP0586c_{1–20} (C57BL/6), MAP0586c_{121–140} (BALB/c) or with purified MAP4308c protein.

tuberculosis and paratuberculosis, would be an invaluable tool for the control of Johne's disease.

Several research groups have identified MAP antigens for use in serodiagnosis [19,37–40] and have identified antigens for T cell based diagnosis with a strong IFN- γ inducing activity [22,41–46]. However, only few of these antigens have been analyzed for their actual vaccine potential, particularly in target species. Koets et al. reported on vaccination experiments with recombinant MAP Hsp70 protein mixed with dimethyl dioctadecyl ammonium bromide (DDA) adjuvant in 40 female calves. Results demonstrated that rHsp70 could be successfully used as a subunit vaccine against bovine paratuberculosis, significantly reducing shedding of bacteria in feces during the first 2 years following experimental infection with an – admittedly – low dose of about 2×10^4 CFU. This vaccine

had little direct and long-term side effects and enabled the serological differentiation between vaccinated and infected animals, the latter presenting weak hsp70 specific antibodies. However, the (probable) interference of this hsp70 based vaccine with tuberculin skin-testing still needs to be evaluated [47].

Subunit protein vaccines based on other MAP antigens, such as the 65 kDa heat-shock protein (GroEL homolog) [48], a 16 kDa protein [10] and the 30 kDa mycolyl-transferase Ag85B [10] are currently under investigation. Recombinant viral vectors have also been used to study the vaccine potential of MAP antigens. Bull et al reported on recombinant Adenovirus 5 and Modified Vaccinia Ankara virus, expressing a 95 kDa fusion protein, consisting of fragments of 2 secreted (MAP1589c/AphC and MAP1234/Gsd) and two cell surface (MAP2444c and MAP1235/Mpa) proteins. Significant IFN- γ ELISPOT responses were observed in vaccinated C57BL/6 mice and an Ad5 prime/MVA-boost protocol conferred some protection against subsequent challenge, as measured by qPCR in spleen and liver [49].

Using an immunoproteomic analysis of MAP culture filtrate with sera from MAP and *M. bovis* infected cattle, we have recently identified MAP0586c (NP_959520.1) and MAP4308c (NP_963242.1) as two putatively MAP-specific antigenic proteins, the latter protein clearly having a serodiagnostic potential [19]. Interestingly, Cho et al. have also identified MAP4308c in MAP culture filtrate and evaluated its use in paratuberculosis diagnosis [38,50]. In order to analyze the vaccine potential of MAP0586c and MAP4308c we used the technique of DNA vaccination. It is well established that intramuscular DNA vaccination of mice is an easy and effective method for generating strong humoral and Th1 biased cellular immune responses, enabling high throughput screening of possible vaccine candidates [17]. We have previously reported on the immunogenicity of DNA vaccines encoding the mycolyl-transferases Ag85A and Ag85B from MAP, two abundantly secreted proteins that are highly conserved among all mycobacterial species [51]. DNA vaccines encoding Ag85A from *M. bovis* BCG and from *M. avium* and Hsp65 from MAP have also been evaluated in sheep for protection against an oral infection with 20×10^8 MAP and showed considerable protection as assessed by histopathology of post mortem tissue sections 1 year later [48]. Using expression library immunization, Huntley et al. have reported on the protective potential of a clone encoding 26 MAP antigens, that conferred significant protection of BALB/c against intraperitoneal challenge with 10^8 CFU of MAP. Genes in the protective clone were identified as transport/binding, membrane and virulence proteins and as mycobactin/polyketide synthases [52]. To the best of our knowledge, the analysis of the 26 individual proteins has not been reported so far.

Here we have shown that MAP0586c and MAP4308c DNA vaccination and homologous DNA-prime/protein boost vaccination induced substantial production of antigen-specific IFN- γ and IL-2 in two MAP susceptible mouse strains. Importantly, IFN- γ and

IL-2 responses against tuberculin PPD-B and Johnin PPD-P were below detection level in these DNA vaccinated mice, suggesting that vaccines composed of these antigens might not interfere with the existing skin test or *ex vivo* IFN- γ test of bovine tuberculosis. Despite the induction of strong Th1 responses, mice vaccinated with MAP0586c DNA vaccine produced only very low antibody levels prior to protein boosting. However, MAP0586c DNA vaccine conferred significant protection against MAP challenge in BALB/c mice, showing (albeit indirectly) that MAP0586c-specific antibodies were not responsible for this protection. In contrast, MAP0586c DNA vaccination did not protect C57BL/6 mice against MAP challenge. Although BALB/c and C57BL/6 mice both show the susceptible phenotype during the first month following intravenous MAP infection, control of bacterial replication is different in these two mouse strains during the two subsequent months [21]. Thus, in MAP infected C57BL/6 mice, CFU counts in spleen and liver decrease dramatically between week 4 and 12 (in the absence of any vaccination), whereas in BALB/c mice CFU counts remain more or less stable during the same period. As a consequence of this inherent more 'resistant' phenotype of C57BL/6 mice, probably linked to its higher Th1 "proneness", measuring protective efficacy (by comparison of replication in non-vaccinated or control vaccinated animals) is more difficult and this could explain why the MAP0586c DNA vaccine was found to be effective in BALB/c but not in C57BL/6 mice.

MAP0586c but not MAP4308c protein induced robust IFN- γ responses in mice upon experimental infection with MAP and (somewhat unexpectedly in the light of its previous identification as a serologically specific antigen), also in mice infected with virulent *M. bovis*. It is interesting to note that even naïve, uninfected BALB/c mice showed a considerable T cell reactivity to MAP0586c, possibly reflecting a sensitization to *M. avium* subsp. *avium* via the drinking water [53]. Indeed, sequences of MAP0586c and MAV0681 protein are highly conserved (E -value = 3×10^{-174}), and these proteins differ only in two amino-acids, namely in position 48 and 120 where a valine substitutes a leucine and an alanine substitutes a proline in *M. avium* subsp. *avium* protein. Although overall similarity with *M. bovis* sequences was weak, NCBI Blast:Protein analysis revealed an certain homology with a possible membrane protein encoded by Mb1262c from *M. bovis* AF2122/97 in MAP sequence spanning aa 161–264 (E -value = 5×10^{-06}). However, looking at the NH₂ terminal MAP0586c sequence, strongly recognized in *M. bovis* infected C57BL/6 mice, revealed a very strong homology (10/12 residues identical or conserved) with a class A beta-lactamase encoded by 1090361 blaC from *M. bovis*. Likewise, MAP0586c sequence spanning aa 121–140 (recognized in naïve and in *M. bovis* infected BALB/c mice) revealed a significant homology with hypothetical Mb2201c from *M. bovis* (52% identical or conserved residues) for region spanning aa 110–145 of MAP0586c. These homologies may explain the cross-reactive IFN- γ responses observed in the *M. bovis* infected mice.

MAP0586c protein has a putative transglycosylase function, as it contains a domain spanning aa 175–257, which has strong homology with a soluble lytic transglycosylase (SLT) domain from *Ralstonia eutropha* H16 (cd00254 E -value = 1×10^{-10}), a Gram-negative soil bacterium, known to produce and sequester polyhydroxyalkanoate plastics when exposed to excess amounts of sugar substrate. Lytic transglycosylases (LT) cleave the β -glycosidic bond between *N*-acetyl-muramic acid and *N*-acetylglucosamine residues within peptidoglycan by a transglycosylase reaction [54]. LTs are thought to play an important role in the synthesis and recycling of peptidoglycan and as such are important for the integrity of the bacterial cell wall [55]. Furthermore, transglycosylases may play a role in reactivation phenomena as dormant bacilli require peptidoglycan hydrolysis which alters the mechanical properties of the cell wall to

facilitate cell division, or the release of lytic products that function as anti-dormancy signals [56].

Sequences of MAP4308c and MAV5271, a fructose-bisphosphate aldolase Class I of *M. avium* subsp. *avium* 104 are also highly conserved, MAV5271 lacking the first 15 amino acids and substituting a valine by an alanine in position 167 (expect = 2×10^{-170}). In contrast, sequence comparison with *M. bovis* AF2122/97 genome does not reveal any homology with MAP4308c sequence. This glycolytic enzyme catalyses the reversible reaction between fructose-1,6-bisphosphate, and dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate. Initially, it was assumed that Class II (metallo) aldolases were more primitive than the Class I (Schiff-base) analogs, since the latter had only been found in eukaryotes. However, with the identification of Class I aldolases in Archaeobacteria and Eubacteria, an alternative scheme of evolution has been suggested, proposing that both aldolase classes are evolutionary ancient. Thus Class I aldolases have been described amongst others in *Halobacterium* ssp., *Staphylococci*, *Peptococci* and the mycobacterial species *M. smegmatis* [57].

In conclusion, our results show that MAP0586c (NP.959520.1) but not MAP4308c (NP.963242.1) is an immunodominant T cell antigen in MAP infected mice. Moreover, vaccination with DNA encoding this putative transglycosylase induces strong Th1 type cytokine production and can protect BALB/c mice against experimental challenge with MAP ATCC 19698 to the same extent as *M. bovis* BCG vaccination, while inducing low antibody levels and no reaction to bovine PPD-B or Johnin PPD-P. It is obvious that more work is needed to confirm these promising findings using more physiological routes of challenge such as oral or eventually peritoneal infection and using target species of Johnie's disease, such as sheep, goats and cattle.

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