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Original article

Potential of *Mycobacterium tuberculosis* resuscitation-promoting factors as antigens in novel tuberculosis sub-unit vaccines

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Abstract

Novel vaccines are needed to control tuberculosis (TB), the bacterial infectious disease that together with malaria and HIV is worldwide responsible for high levels of morbidity and mortality. TB can result from the reactivation of an initially controlled latent infection by *Mycobacterium tuberculosis* (*Mtb*). *Mtb* proteins for which a possible role in this reactivation process has been hypothesized are the five homologs of the resuscitation-promoting factor of *Micrococcus luteus*, namely *Mtb* Rv0867c (*rpfA*), Rv1009 (*rpfB*), Rv1884c (*rpfC*), Rv2389c (*rpfD*) and Rv2450c (*rpfE*). Analysis of the immune recognition of these 5 proteins following *Mtb* infection or *Mycobacterium bovis* BCG vaccination of mice showed that Rv1009 (rpfB) and Rv2389c (rpfD) are the most antigenic in the tested models. We therefore selected rpfB and rpfD for testing their vaccine potential as plasmid DNA vaccines. Elevated cellular immune responses and modest but significant protection against intra-tracheal *Mtb* challenge were induced by immunization with the *rpfB* encoding DNA vaccine. The results indicate that rpfB is the most promising candidate of the five rpf-like proteins of *Mtb* in terms of its immunogenicity and protective efficacy and warrants further analysis for inclusion as an antigen in novel TB vaccines.

Keywords: *Mycobacterium tuberculosis*; Vaccine development; Resuscitation-promoting factor

1. Introduction

Mycobacterium tuberculosis (*Mtb*) and the other members of the *Mtb* complex are the etiological agents of human tuberculosis (TB), which globally is the most prevalent disease caused by a single bacterial pathogen. In 2009, worldwide there were an estimated 14 million prevalent cases of TB (among these 9.4 million were new cases) and 1.3 million HIV-negative persons died of the disease. Classified as HIV-fatalities, another 380,000 HIV-infected individuals died of TB [1]. Furthermore, it is estimated that one third of the world population is latently infected with TB bacilli, thus at risk to develop the active pulmonary form of TB and transmit the infection to others [2].

The only vaccine currently available to prevent TB is the live, attenuated *Mycobacterium bovis* Bacille Calmette Guérin (BCG) vaccine. Neonatal BCG vaccination protects children against TB meningitis and against disseminated, miliary disease, but confers a variable protection (ranging from 0% to 80%) against pulmonary TB in adults and has been found to be of variable efficacy in a number of clinical trials [3–5]. A clear explanation for the failure of BCG to protect against pulmonary TB is still not available. Waning of immune memory,

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interference of environmental mycobacteria and genetic variations, both in host populations and BCG-vaccine strains used, explain the variable efficacy of BCG to some extent, although other factors may also be involved [5]. Such as the fact that upon BCG vaccination no – or only very weak – T cell immune responses against latency-associated antigens are detected, responses that have been associated with control of latent *Mtb* infection in endemic and non-endemic countries [6–8]. Therefore, incorporating these latency antigens could complement and improve the current BCG vaccine.

With the same purpose to develop an improved TB vaccine, it is also important to characterize the vaccine potential of antigens possibly associated with reactivation of a latent *Mtb* infection. Several studies have analyzed the potential role in this process of five *Mtb* proteins homologous to the resuscitation-promoting factor (rpf) of *Micrococcus luteus* (reviewed by Kana and Mizrahi [9]). The rpf of *M. luteus* is an essential secreted 17 kDa protein restoring active growth of *M. luteus* cultures rendered dormant by prolonged incubation in stationary phase [10,11]. The *Mtb* homologs of *M. luteus* rpf are *rpfA* (Rv0867c), *rpfB* (Rv1009), *rpfC* (Rv1884c), *rpfD* (Rv2389c) and *rpfE* (Rv2450c), all share the *M. luteus* rpf conserved ~70 amino acid fragment (the rpf-like domain) and all are putatively secreted or membrane anchored proteins. Moreover, they can mediate the resuscitation activity of *M. luteus* rpf [12,13]. *In vitro* and *in vivo* transcriptional profiling studies have shown that the five *rpf* genes of *Mtb* are expressed at varying levels in a growth-phase-dependent manner [12,14,15]. Moreover, in a rabbit model of latent TB it was shown that *rpfB* is expressed *in vivo* and preferentially expressed in dexamethasone treated rabbits versus latently infected animals [16]. *In vitro*, it was demonstrated that actively growing BCG bacilli express the five *rpf* genes, but no transcript was detected for any of these genes in BCG grown to the stationary phase or following prolonged starvation [12]. To our knowledge no data are available regarding the *in vivo* expression of the *rpf* genes of BCG.

Besides being involved *in vitro* in resuscitation from dormancy, indications of the fact that *Mtb* rpfs are involved in reactivation processes *in vivo* come from studies in which the phenotypes of *Mtb* strains lacking single or multiple *rpf* genes were analyzed in mouse models. Indeed, delayed aminoguanidine-induced reactivation from chronic TB was observed in mice infected with a strain lacking *rpfB* [17], while no attenuation was observed in terms of its ability to proliferate in organs of infected animals or in terms of the induced immunopathology (granulomatous inflammation and tissue damage) compared to the wild-type strain [14,17]. A more severe defect in aminoguanidine-induced reactivation was observed with *ΔrpfAB* and *ΔrpfBD* double knockout strains [18] and with these strains also an attenuation of growth in the infected organs was observed. Similarly, inability to transiently reactivate the infection and a phenotype of attenuation for growth *in vivo* was also observed for double, triple and quadruple knockout strains [19–21]. Collectively, these studies indicate an *in vivo* role of the rpf proteins in immune-suppression induced reactivation but also in virulence.

Concerning the immune responses induced by the *Mtb* rpf homologs, a note published by Yeremeev et al., reported that administration to mice of recombinant *Mtb* rpf proteins in adjuvant led to specific humoral and cellular immune responses, which were protective against an intravenous challenge infection [22]. More recently, rpfB, rpfC or rpfE specific IFN-γ expression was observed in $CD4^+CD45RO^+$ memory T cells of latently *Mtb* infected individuals (LTBI). Recombinant rpfA failed to induce significant IFN-γ expression while recombinant rpfD was not tested in this study. This study also showed significantly higher T cell responses against recombinant rpfB and rpfE in LTBI than in patients suffering from active TB [23]. *In vivo* studies on *Mtb* rpfs thus indicate that they are immunogenic in a mouse model, and that some are preferentially recognized by humans latently infected with *Mtb*. Against this background we were interested to analyze in more detail in the mouse model the potential of the five *Mtb* rpfs as antigens for novel TB sub-unit vaccines. Here we report on the immune responses specific for these proteins after *Mtb* infection, BCG vaccination and immunization with plasmid DNA vaccines coding for single *Mtb* rpfs. We also demonstrate that vaccination with *rpfB* encoding DNA vaccine results in a modest but significant protective efficacy against an experimental *Mtb* challenge infection.

2. Materials and methods

2.1. Mice

Female BALB/c and C57BL/6 mice were bred in the Animal Facilities of the Scientific Institute of Public Health-Site Ukkel, from breeding couples originally obtained from Bantin & Kingman (United Kingdom). All animals were 6–8 weeks old at the start of the experiments.

2.2. Plasmid vaccination

The genes encoding two homologs of the resuscitation-promoting factor of *M. luteus* from *M. tuberculosis* H37Rv (Rv1009 (*rpfB*) and Rv2389c (*rpfD*)) were recombined into a Gateway® (Invitrogen) adapted pV1J.ns-tPA vector. In pV1J.ns-tPA vector (pDNA) the genes of interest are expressed under the control of the promotor of IE1 antigen from cytomegalovirus, including intron A preceded by the signal sequence of human tissue plasminogen activator [24]. Mice were anesthetized with ketamine/xylazine and injected three times intramuscularly in both quadriceps muscles with 2 × 50 μg of pDNA at three week intervals.

2.3. BCG vaccination and M. tuberculosis infection of mice

M. bovis BCG (strain GL2) and luminescent *M. tuberculosis* H37Rv [25] were grown as a surface pellicle on synthetic Sauton medium as described before [26]. Bacteria were harvested after 2 weeks and aliquots were stored frozen at −70° until use.

Mycobacterial loads in lungs and spleen of *Mtb* infected mice were quantified using a bioluminescence assay (determination of relative light units – RLU) [27].

To assess the rpf-specific immune responses in BCG vaccinated and *Mtb* infected mice, BALB/c and C57BL/6 mice were vaccinated intravenously with 10^6 CFU of BCG or infected intravenously with 10^5 CFU of *M. tuberculosis*. Mice were sacrificed at specified time-points.

For the protective efficacy studies, mice were challenged 6 weeks after the third pDNA immunization by the intratracheal route with 10.000 CFU of luminescent *M. tuberculosis* H37Rv. The BCG control group had received 10^6 CFU of BCG by the intravenous route at the time of the first pDNA vaccination.

2.4. Purification of recombinant rpfs of M. tuberculosis antigens

Recombinant proteins were produced as previously described [7,28]. Briefly, nucleotide sequences of selected *M. tuberculosis* H37Rv genes were obtained from http://genolist.pasteur.fr/TubercuList. Genes were amplified by PCR from genomic DNA of *M. tuberculosis* H37Rv and cloned by Gateway Technology (Invitrogen, San Diego, CA) in pDEST™17, a bacterial expression vector containing an N-terminal hexa-histidine tag for rapid purification with nickel-chelating resin. The proteins were over expressed in *Escherichia coli* BL21 (DE3) and purified as previously described. Sequencing was performed to confirm the identity of the cloned DNA fragments. Size and purity were checked by gel electrophoresis and Western blotting with anti-His antibodies (Invitrogen). Residual endotoxin levels were determined with a Limulus Amoebocyte Lysate assay (Cambrex) and were found to be below 50 IU/mg recombinant protein.

2.5. Synthetic peptides

Synthetic 20-mer peptides, overlapping by ten amino-acid residues, were synthesized as previously described [29]. Peptides were initially dissolved in DMSO and stock solutions were subsequently prepared in RPMI-1640 culture medium at 1 mg/ml, and aliquots were stored frozen at −20° until use.

2.6. Antibody assay

Sera from plasmid vaccinated mice were collected 3 weeks after the third vaccination by retro-orbital bleeding. Levels of total-IgG antibodies specific for the *Mtb* rpfB and rpfD were determined by an enzyme-linked immunosorbent assay (ELISA) on individual sera, using recombinant antigens for coating (400 ng/well), a secondary peroxidase-labeled rat anti-mouse κ light chain monoclonal antibody (LO-MK-1, Experimental Immunology Unit, U. C. L., Brussels) and tetramethyl-benzidine (TMB) Substrate Solution (e-Bioscience) for revelation. Data represent the average and standard deviation of the optical densities at 450 nm obtained for specified dilutions of the sera (three individual animals tested/group) against recombinant rpfB or rpfD.

2.7. Cytokine production

Plasmid DNA vaccinated mice were sacrificed 3 weeks after the third immunization and infected mice were sacrificed at specified time-points.

Spleens were removed aseptically and gently homogenized. Spleen cells (4×10^6 WBC/ml in RPMI-1640 medium supplemented with 10%FCS, 5×10^{-5} M 2-mercapto-ethanol and antibiotics) from three to five mice per group were tested individually for cytokine response to the five recombinant rpfs and as a pool for responses to rpf-peptides.

Cells were incubated at 37 °C in a humidified CO_2 incubator, in round-bottom 96 well microtiter plates and stimulated with purified recombinant antigens (5 μg/ml) or synthetic peptides (10 μg/ml). Culture supernatants were harvested after 24 h for 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. Experiments were performed at least three times and data from one representative experiment are reported.

2.8. IL-2 and IFN-γ assays

IL-2 activity was measured by sandwich ELISA using coating antibody JES6-1A12 and biotinylated detection antibody JES6-5H4 (both eBioscience™). Detection limit of IL-2 ELISA was 2 pg/ml. IFN-γ activity was quantified by sandwich ELISA using coating antibody R4-6A2 and biotinylated detection antibody XMG1.2 (both BD Pharmingen™). Detection limit of IFN-γ ELISA was 5 pg/ml.

3. Results

3.1. Th1-type cytokine secretion to the five rpfs of Mtb in C57BL/6 and BALB/c infected with Mtb or vaccinated with BCG

First we analyzed whether and which of the five *Mtb* rpf homologs induce Th1 type immune responses in *Mtb*-infected or BCG-vaccinated mice. For this purpose, C57BL/6 and BALB/c mice were inoculated with *Mtb* or BCG. At week 4, 8, 12 and 16 after *Mtb* infection or BCG vaccination, mice were sacrificed and leukocytes isolated from spleen and lungs − the latter only for *Mtb* infected animals − were stimulated with each of the five recombinant *Mtb* rpfs (rpfA to rpfE). IL-2 and IFN-γ concentrations were evaluated by sandwich ELISA in day 1 and day 3 culture supernatants respectively.

Elevated levels of IFN-γ production were detected as early as 4 weeks after *Mtb* infection or BCG vaccination in spleen cell cultures from C57BL/6 and BALB/c mice in response to recombinant rpfB and rpfD (Fig. 1). Interestingly, these IFN-γ levels decreased at the later time-points tested, indicating that rpfB- and rpfD-specific responses peak at the moment of peak replication. Indeed, in *Mtb* infected C57BL/6 mice, rpfB and rpfD specific responses respectively decreased by a factor of 4 and 5 between week 4 and week 8, 12 and 16 (Fig. 1A). This reduction in the rpfB and rpfD specific IFN-γ responses was

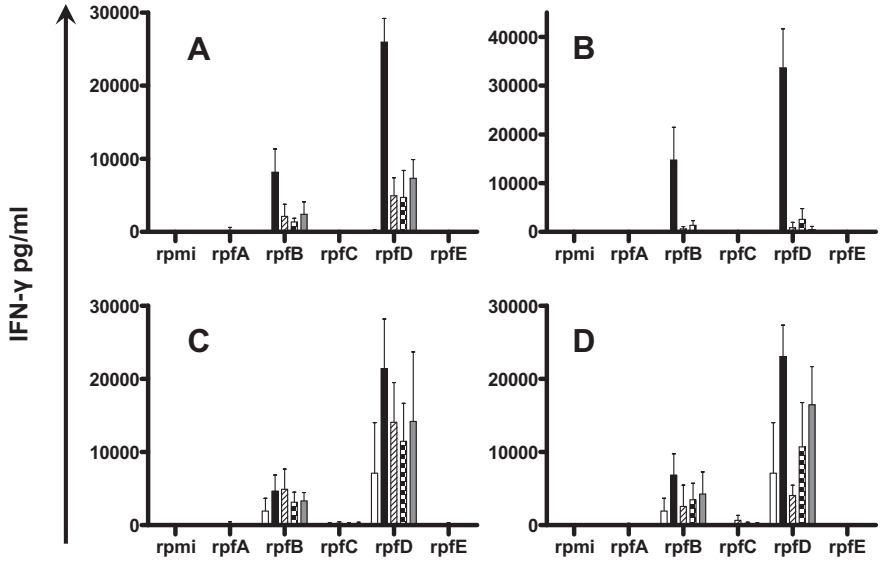

Fig. 1. Rpf-specific IFN-γ responses in *M. tuberculosis* infected and in *M. bovis* BCG vaccinated C57BL/6 and BALB/c mice. IFN-γ levels in 72 h spleen cell culture supernatant from C57BL/6 (A and B) and BALB/c (C and D) mice 4, 8, 12 and 16 weeks after infection with *M. tuberculosis* (A and C) or vaccination with BCG (B and D). Cells were left unstimulated (rpmi) or were stimulated with 5 μg/ml of recombinant rpfA, rpfB, rpfC, rpfD or rpfE proteins. Uninfected naïve control mice (white bars), 4 weeks (black bars), 8 weeks (hashed bars), 12 weeks (bars with squares) and 16 weeks (gray bars). Results represent mean ± SD values of three mice tested individually in each group and are representative of three independent experiments.

even more pronounced in BCG vaccinated C57BL/6 mice, in which a drop of respectively 25 and 40 times in specific IFN-γ response was seen between week 4 and week 8 (Fig. 1B). At week 16, rpfB and rpfD specific IFN-γ responses were close to negative background levels in BCG vaccinated C57BL/6 mice (Fig. 1B). This reduction in rpfB and rpfD specific responses was also observed in BALB/c mice, but to a lesser extent (Fig. 1C and D). Stimulation with antigenic peptides well known to induce significant cellular responses in *Mtb* or BCG treated mice, such as Esat-6_{1-20} for *Mtb* infected C57BL/6 [30], $Ag85B_{241-260}$ for C57BL/6 administered *Mtb* or BCG [31], or $Ag85A_{101-120}$ for BALB/c [32] resulted in significant IL-2 and IFN-γ responses (Fig. 2A and B), while none of the recombinant *Mtb* rpfs induced a significant production of IL-2 (Fig. 2A). Finally, while elevated IFN-γ levels could be induced in response to antigenic peptides Esat-6_{1-20} and $Ag85A_{101-120}$ in lung cell cultures of intravenously *Mtb* infected animals (Fig. 2B), rpf-specific IFN-γ levels in lung cell cultures were only slightly increased in response to rpfB and rpfD, indicative of a weak recruitment of rpf-specific effector T cells to the principal (pulmonary) site of infection (data not shown).

3.2. Immune responses induced in C57BL/6 and BALB/c mice vaccinated with plasmid DNA encoding Mtb *rpfB and rpfD*

To further analyze the antigenic potential of rpfB and rpfD – the two members of the *Mtb* rpfs which were recognized

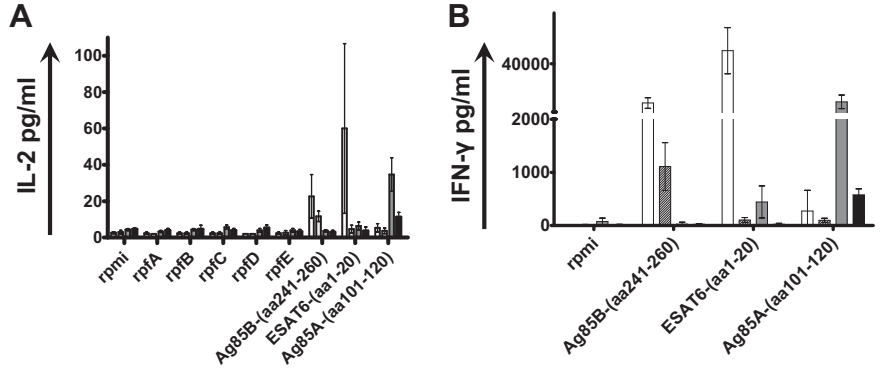

Fig. 2. IL-2 and IFN-γ responses in *M. tuberculosis* infected and in *M. bovis* BCG vaccinated C57BL/6 and BALB/c mice. IL-2 (A) and IFN-γ (B) levels in respectively 24 h and 72 h spleen cell culture supernatant from C57BL/6 and BALB/c mice 4 weeks after infection with *M. tuberculosis* or vaccination with BCG. Cells were left unstimulated (rpmi) or were stimulated with 5 μg/ml of recombinant rpfA, rpfB, rpfC, rpfD or rpfE or were stimulated with 10 μg/ml of $Ag85B_{(aa241-260)}$, Esat-$6_{(aa1-20)}$ or $Ag85A_{(aa101-120)}$. C57BL/6 *Mtb* infected (white bars), C57BL/6 BCG vaccinated (hashed bars), BALB/c *Mtb* infected (gray bars) and BALB/c BCG vaccinated (black bars). Results represent mean ± SD values of three mice tested individually in each group and are representative of three independent experiments.

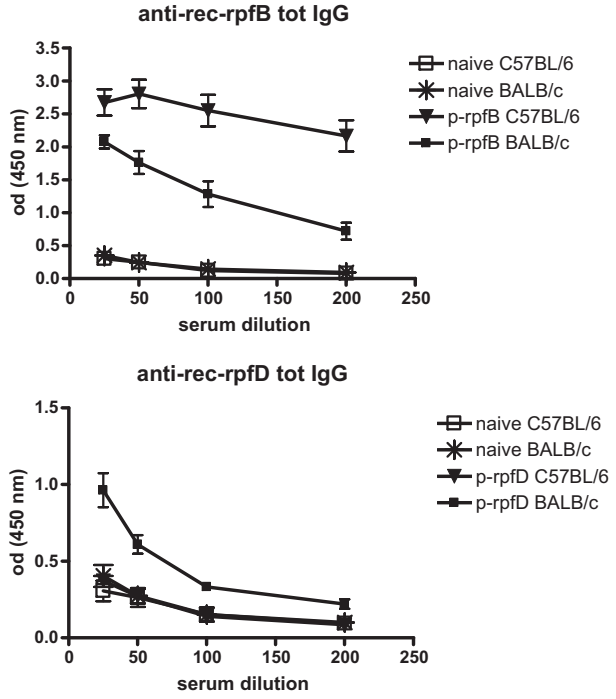

Fig. 3. RpfB and rpfD specific total IgG in sera from C57BL/6 and BALB/c mice vaccinated with DNA vaccines encoding specified rpfs. Three weeks after the third vaccination, sera were collected from vaccinated animals and naïve controls. Data represent the average and standard deviation of the optical densities at 450 nm obtained for 1:25 to 1:200 diluted sera tested against the specified recombinant rpfs. Data are representative of three independent experiments.

following *Mtb* infection and BCG vaccination – we evaluated the immunogenicity of plasmid DNA vaccines encoding these two rpf homologs in C57BL/6 and BALB/c mice. For this purpose, C57BL/6 and BALB/c mice were vaccinated intramuscularly 3 times at 3 weeks interval with 100 μg of pV1J.ns-tPA plasmid DNA encoding rpfB or rpfD. Three weeks after the third vaccination, sera were collected for analysis of the humoral response and mice were sacrificed for spleen restimulation experiments with recombinant rpfB and rpfD proteins. IL-2 and IFN-γ concentrations in respectively day 1 and day 3 culture supernatants were evaluated by sandwich ELISA.

As shown in Fig. 3, vaccination of C57BL/6 and BALB/c mice with p-*rpfB* resulted in elevated levels of specific total-IgG antibodies, while vaccination with p-*rpfD*, resulted in only weak specific IgG antibody responses in BALB/c mice. Interestingly, vaccination of BALB/c with p-*rpfD* also resulted in the generation of antibodies recognizing recombinant rpfB (data not shown), indicating some levels of cross-reactivity.

Regarding the cellular immune responses, immunization of C57BL/6 mice with p-*rpfB* induced elevated levels of IL-2 and IFN-γ when stimulated with rec-rpfB (Fig. 4), while vaccination with p-*rpfD* induced no antigen-specific IL-2 and only a weak production of IFN-γ (1775 ± 926 pg/ml) in C57BL/6 mice. In BALB/c mice, p-*rpfB* vaccination also induced IL-2 and IFN-γ responses, while vaccination with p-*rpfD* induced basically no specific IL-2 responses but some production of IFN-γ. In addition, some cross-reactive responses were observed, with p-*rpfB* vaccinated BALB/c also producing IFN-γ in response to recombinant rpfD and p-*rpfD* vaccinated BALB/c also producing IFN-γ in response to recombinant rpfB, again indicating some levels of cross-reactivity between these two rpf-like proteins.

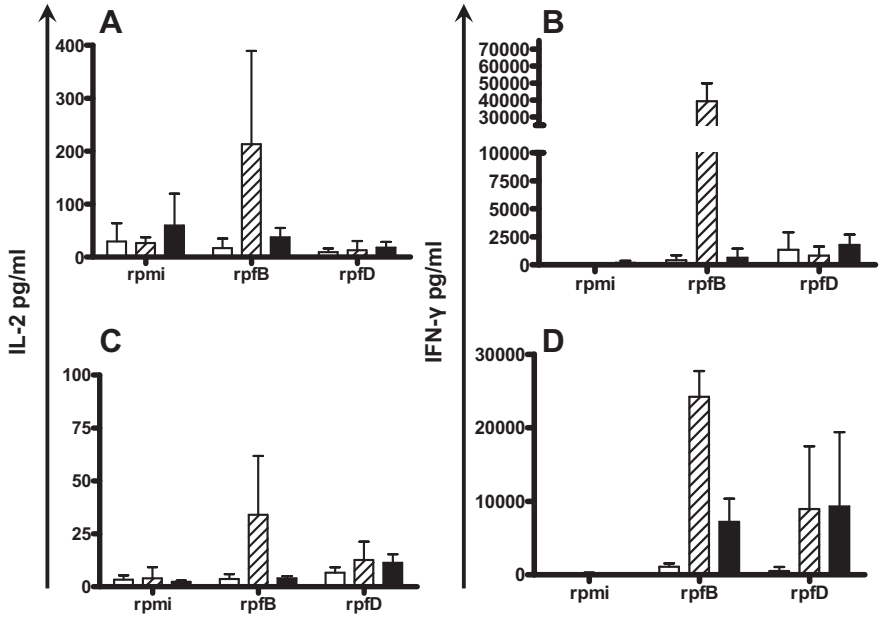

Fig. 4. Rpf-specific IL-2 and IFN-γ responses in C57BL/6 and BALB/c mice vaccinated with pDNAs encoding *rpfB* and *rpfD*. IL-2 levels in 24 h (A, C) and IFN-γ levels in 72 h (B, D) spleen cell culture supernatant from C57BL/6 (A and B) and BALB/c (C and D) naïve (rpmi) mice or mice vaccinated with plasmid DNA encoding *rpfB* and *rpfD*. Cells were left unstimulated (white bars) or were stimulated with 5 μg/ml of recombinant rpfB (hashed bars) or rpfD (black bars). Results represent mean ± SD values of three mice tested individually in each group and are representative of three independent experiments.

3.3. Identification of immunodominant H-2^d – and H-2^b – restricted epitopes of rpfB and rpfD

As vaccination with plasmid DNA coding for *rpfB* induced significant IL-2 and IFN-γ responses in C57BL/6 and BALB/c mice, we identified the immunodominant H-2^d – and H-2^b – restricted epitopes of this member of the rpf family of *Mtb* following plasmid DNA vaccination. We have previously shown that plasmid vaccination is a powerful tool for the mapping of CD4$^+$ as well as of CD8$^+$ T cell epitopes in mice [31,33,34]. C57BL/6 and BALB/c mice vaccinated with pV1J.ns-tPA-*rpfB* vector were sacrificed and spleen cells were stimulated with 20-mer synthetic peptides overlapping by 10 amino acids and spanning the entire rpfB sequence.

Results for the mapping in C57BL/6 and BALB/c vaccinated with p-*rpfB* and tested for IL-2 and IFN-γ production are shown in Table 1. Overall a higher number of T cell epitopes were identified in C57BL/6 than in BALB/c mice. Moreover, some regions were recognized both in C57BL/6 and BALB/c, indicating possible presence of promiscuously recognized epitopes.

Vaccination with p-*rpfB* induced strong IL-2 and IFN-γ responses and long epitope stretches were identified. Indeed, p-*rpfB* vaccinated C57BL/6 reacted to two peptides spanning rpfB$_{51-80}$, to five peptides spanning rpfB$_{121-180}$ and to three peptides spanning rpfB$_{231-270}$. In addition, also rpfB$_{91-110}$ induced elevated IL-2 and IFN-γ responses in C57BL/6. One peptide i.e. rpfB $_{241-270}$ was recognized by both C57BL/6 and BALB/c plasmid vaccinated mice and in addition, BALB/c mice also reacted against rpfB$_{21-40}$. No specific responses to the rpf-like domain – spanning rpfB$_{290-360}$ – were observed in p-*rpfB* vaccinated animals.

In addition, we also tested splenocytes from p-*rpfD* vaccinated mice with rpfD synthetic overlapping peptides. As expected from the weak T cell response to rpfD protein in p-*rpfD* vaccinated C57BL/6 mice, no H-2^b restricted IL-2 or IFN-γ inducing peptides could be identified. BALB/c mice showed modest levels of IFN-γ (600 pg/ml compared to 27 pg/ml for unstimulated controls) after restimulation with rpfD$_{11-30}$ and some IFN-γ production (610 pg/ml) was observed following restimulation with rpfD$_{135-154}$.

Table 1
T cell epitope mapping in C57BL/6 and BALB/c mice vaccinated with p-*rpfB*.

	C57BL/6		BALB/c	
	IL-2 (pg/ml)	IFN-γ (pg/ml)	IL-2 (pg/ml)	IFN-γ (pg/ml)
rpmi	22	215	4	92
rpfB aa:				
1–20	22	bdl	5	226
11–30	105	103	8	864
21–40	10	bdl	**15**	**1026**
31–50	16	116	**113**	**6798**
41–60	25	1100	8	369
51–70	**52**	**6298**	7	41
61–80	**59**	**7271**	11	30
71–90	58	42	5	76
81–100	42	bdl	8	52
91–110	**78**	**4450**	9	211
101–120	bdl	96	8	69
111–130	7	bdl	7	67
121–140	**112**	**5348**	8	71
131–150	**61**	**3970**	10	90
141–160	**85**	**12,816**	8	324
151–170	**118**	**7552**	7	48
161–180	**67**	**12,177**	7	26
171–190	162	13	5	119
181–200	6	bdl	16	424
191–210	12	157	8	41
201–220	42	21	6	46
211–230	50	1569	7	26
221–240	52	bdl	6	37
231–250	**210**	**9635**	6	115
241–260	***285***	***68,979***	***56***	***2945***
251–270	***398***	***107,882***	***80***	***6133***
261–280	20	bdl	10	132
271–290	27	23	4	80
281–300	24	18	8	102
291–310	17	bdl	5	59
301–320	53	bdl	6	28
311–330	35	1065	4	50
321–340	15	bdl	18	298
331–350	19	211	16	137
341–360	7	21	16	140
351–362	15	26	15	236

Three weeks after the third p-*rpfB* vaccination spleen cells cultures were prepared from sacrificed vaccinated animals and naïve controls and restimulated with negative control culture medium (rpmi) or with 20-mer synthetic peptides (10 μg/ml), spanning the entire rpfB sequence and over-lapping by 10 aa. Respectively, day 1 and day 3 supernatants were tested for IL-2 or IFN-γ concentration with sandwich ELISA tests. Restimulation with peptides in naïve control mice did not induce any significant IL-2 or IFN-γ production. "bdl" below detection limit. Values in bold are positive for one of the tested mouse strains and values in bold italic are positive for both strains. Data are representative of three independent experiments.

3.4. A DNA vaccine encoding rpfB protects C57BL/6 against intra-tracheal Mtb infection

As immunization with pDNA encoding rpfB induced specific antibodies as well as elevated Th1-type immune responses, we assessed the protective potential of p-*rpfB* in an experimental model of pulmonary TB. For that purpose, C57BL/6 mice were vaccinated with plasmid DNA coding for rpfB or with empty plasmid control or with BCG and subsequently infected intratracheally with 10^4 CFU of luminescent *M. tuberculosis* H37Rv. Five weeks after TB challenge, mice were sacrificed and the number of bacilli was evaluated in spleen and lungs by luminometry. Characteristic for this pulmonary TB experimental model is a minimal bacterial dissemination to other peripheral organs [35]. Thus, as expected, bacterial numbers in spleen were low in all groups albeit higher in unvaccinated and plasmid vaccinated mice than in BCG immunized animals (Fig. 5A). At the pulmonary level, immunization with p-*rpfB* resulted in a moderate but significant reduction of the bacterial burden compared to the control group vaccinated with empty vector (Fig. 5B). Indeed, in the control DNA group 4.68 ± 0.33 $\log_{10}$(mRLU) were detected, while lower bacterial numbers were found in the p-*rpfB* vaccinated group (4.12 ± 0.23 $\log_{10}$(mRLU), $\Delta\log_{10}$: 0.56, $0.010 < p < 0.025$). Levels of BCG induced protection were more substantial (3.64 ± 0.25 $\log_{10}$(mRLU) $\Delta\log_{10}$: 1.04 and $p < 0.005$ as compared to the control DNA group).

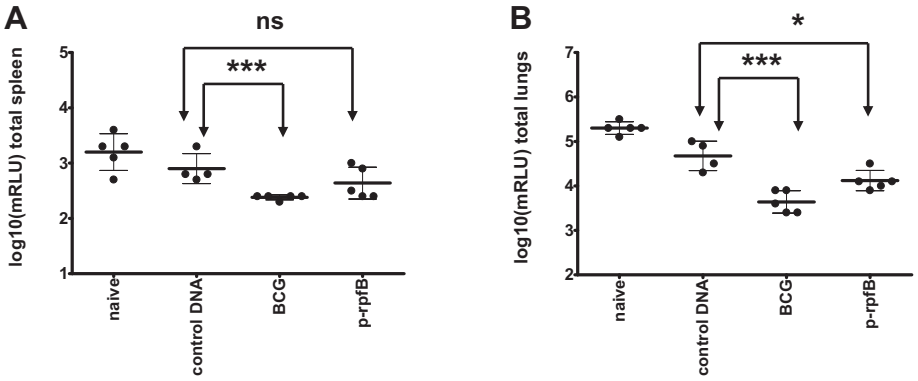

Fig. 5. Protective efficacy of pDNA encoding *rpfB* in C57BL/6 mice. Replication of luminescent *M. tuberculosis* H37Rv 5 weeks after an intra-tracheal challenge as measured by luminometry in spleen (A) and lungs (B) of C57BL/6 mice that were unvaccinated (naïve) or previously vaccinated as specified on the *x* axis with control DNA, BCG or pDNA-*rpfB*. Dots represent the mean $\log_{10}$(mRLU) values obtained for individually tested animals. Thick horizontal bars represent the mean value for each group and the thin horizontal bars represent 1 SD for each group. Results are representative of three independent experiments. Statistical significance between groups is represented in the figure by * ($0.010 < p < 0.025$) and *** ($p < 0.005$) and was evaluated by Student's *t*-test.

4. Discussion

Improved TB vaccines are needed to achieve a better control of this most prevalent bacterial disease caused by infection with *M. tuberculosis*. Indeed, the only currently available vaccine BCG is poorly efficient in protecting against the contagious pulmonary TB form. Half of the pulmonary TB cases arise from the reactivation of a latent *Mtb* infection and it is estimated that one third of the world population is latently infected with *Mtb*. In this study we have analyzed the antigenic potential of proteins to which a role in this reactivation process has been attributed [9]. Namely the five *Mtb* homologs of the resuscitation-promoting factor (rpf) of *M. luteus*: Rv0867c (*rpfA*), Rv1009 (*rpfB*), Rv1884c (*rpfC*), Rv2389c (*rpfD*) and Rv2450c (*rpfE*).

In the first part of the work, we analyzed the immune responses specific to the five *Mtb* rpf homologs in *Mtb* infected and BCG vaccinated mice. Rpf specific antibody levels in these mice were comparable to levels in control mice, indicating that no specific humoral response to these proteins is induced after *Mtb* or BCG administration (data not shown). Regarding cellular immune responses, strong rpfB and rpfD specific IFN-γ responses were detected in spleen of *Mtb* infected and BCG vaccinated C57BL/6 and BALB/c mice, whereas only weak rpfB and rpfD specific responses were detected in lungs of *Mtb* infected mice. As these weak rpfB- and rpfD-specific pulmonary IFN-γ responses were observed following intravenous infection, rpf-specific responses were also tested in BALB.B10 mice infected with *Mtb* by the intra-tracheal route. Here again strong rpfB- and rpfD-specific IFN-γ responses were observed in spleen but not in lungs (data not shown). Interestingly, the rpfB and rpfD specific IFN-γ responses observed in spleen of *Mtb* infected and BCG vaccinated C57BL/6 and BALB/c mice were strongest at the moment of peak replication (week 4) and diminished at later time-points. Such an early induction of specific responses against two of the five rpf homologs is indicative of an immediate expression of *rpfB* and *rpfD* during establishment of infection by *Mtb* and BCG, and this is somewhat counterintuitive with regard to their so-called resuscitation-promoting function. At the later time-points post-infection or vaccination, rpfB- and rpfD-specific IFN-γ levels decreased as compared to the levels achieved at week 4. This decrease was more pronounced in C57BL/6 than in BALB/c mice, and rpfB and rpfD responses were close to background levels at week 16 in BCG vaccinated C57BL/6. A more effective immune-mediated control of *Mtb* and BCG in C57BL/6 as compared to BALB/c mice, accompanied by lower levels of bacterial replication, can possibly explain these observations [36]. It is indeed tempting to hypothesize that the elevated rpfB and rpfD specific responses observed at week 4 correlate with an elevated expression of these proteins in the phase during which *Mtb* and BCG replicates in the host. This is further supported by the data of Rubin and collaborators demonstrating a role of rpfB in cell division. Indeed, rpfB interacts and co-localize to the septa of actively growing mycobacteria with ripA (rpf interacting protein A), which is a peptidoglycan hydrolyzing endopeptidase essential for cell division [37,38]. Hence, the early detection of rpfB-specific IFN-γ responses after *Mtb* and BCG administration might be linked to the fact that rpfB plays a role in *Mtb* cell division and is therefore expressed and presented to the immune system during the establishment of infection in the mouse. As less is known about the specific functions of rpfD, we can only speculate that rpfD would play a similar role in the initial bacterial replication phase.

Detection of rpfB- and rpfD-specific cellular immune responses following BCG vaccination indicated that at least these two members of the rpf protein family are also expressed *in vivo* by BCG at an early stage. It was previously demonstrated *in vitro* that actively growing BCG bacilli express the five *rpf* genes, but to our knowledge the results reported here are the first indication of the *in vivo* expression of rpfs by BCG in mice [12]. Of note is the fact that also in a study analyzing rpfA- and rpfD-specific immune responses in BCG-vaccinated humans, rpfD resulted in significant IFN-γ responses compared to non-BCG-vaccinated, TST-negative, healthy controls [39]. The fact that rpfA, rpfC and rpfE were not

recognized in terms of antibody nor of Th1 type cellular responses after *Mtb* infection and BCG vaccination in our mouse model, does not exclude that these proteins might be presented and recognized by the immune system during later infection stages. Indeed, although not recognized by acutely *Mtb* infected or BCG vaccinated mice, this defect was not caused by an intrinsic lack of antigenicity, as significant humoral and cellular immune responses could be induced with pDNA vaccines encoding these three rpf-like proteins (data not shown). Moreover, studies in which *in vitro* rpf gene expression in *Mtb* cells grown under different conditions was analyzed, do indicate that different rpfs may play different physiological roles during hypoxia, nutrient starvation, acidic conditions, stationary-, nonculturable- and resuscitation-phase-like conditions [15].

As our analysis of the immune response in *Mtb* infected and BCG vaccinated mice enabled us to identify rpfB and rpfD as the two most antigenic rpf homologs of *Mtb*, we examined in the second part of this work their immunogenicity as encoded in pDNA sub-unit vaccines. DNA vaccination is a powerful and easy method for screening the vaccine and immune potential of mycobacterial antigens and identifying their immunodominant MHC class I and MHC class II restricted T cell epitopes [40]. In addition, though first generation DNA vaccines were poorly immunogenic in animals larger than mice, progress in the field of DNA vaccines has improved the potential of this sub-unit vaccine delivery platform. More sustained cellular responses and more consistent antibody responses are being reported in human trials and a number of DNA vaccine products have been approved for veterinary use in large animals [41]. Analysis of the immune responses to rpfB and rpfD in plasmid DNA vaccinated C57BL/6 and BALB/c mice demonstrated that both members of the *Mtb* rpf family are immunogenic, with rpfB being more immunogenic than rpfD. Strong rpfB specific total IgG responses were detected in C57BL/6 and BALB/c mice, while vaccination with p-*rpfD* induced a specific IgG response only in BALB/c mice. The role of antigen-specific antibodies induced by immunization with mycobacterial proteins in protection against TB is still unclear [42]. However, a protective role of rpfB specific antibodies can not be totally excluded at this point and more work is needed to examine whether they could possibly neutralize the enzymatic activity of this antigenic enzyme. In parallel to the humoral responses, we analyzed Th1 cellular responses following vaccination with rpfB and rpfD DNA vaccines. Vaccination with p-*rpfB* induced significant antigen-specific Th1 responses in C57BL/6 and BALB/c mice, while vaccination with p-*rpfD* induced only weak antigen-specific IFN-γ responses. In parallel, we used overlapping synthetic peptides to identify the T cell epitopes of rpfB and rpfD. This enabled us to identify a number of IL-2 and IFN-γ inducing regions on the rpfB molecule. Overall, our results show that *Mtb* rpfB encompasses numerous MHC class II H-2^d − and H-2^b − restricted epitopes, whereas MHC class I restricted epitopes could not be identified in this study. Finally, although vaccination with p-*rpfB* of C57BL/6 and BALB/c induces responses specific to large stretches of the rpfB protein (Table 1), none of these regions encompasses the conserved ~70 amino acid long rpf-like domain ($rpfB_{290-360}$), which is shared between the five *Mtb* rpf homologs and *M. luteus* rpf. Somewhat surprisingly, given the strong rpfD-specific IFN-γ responses observed in *Mtb* infected and BCG vaccinated mice, C57BL/6 mice vaccinated with DNA encoding rpfD showed only weak humoral and cellular responses to rpfD protein and to the corresponding peptides. BALB/c mice vaccinated with p-*rpfD* reacted somewhat better than C57BL/6 mice.

In conclusion, our results indicate that rpfB is a member of the *Mtb* rpf family, which should be further analyzed as possible component of multi-antigenic TB sub-unit vaccines. Indeed, rpfB-specific responses were observed following *Mtb* and BCG administration and vaccination with p-*rpfB* resulted in a significant protection when pulmonary bacterial burden in this group was compared to that obtained in control vaccinated animals. However, the level of protection was moderate compared to the one observed in BCG vaccinated animals. Protection was also modest if compared to the one observed with other mycobacterial antigens such as the PPE44 antigen that in a similar experimental set-up − when administered as a pDNA vaccine or as a recombinant protein in adjuvant − is able to induce protective efficacies comparable to the one achieved by BCG immunization [43]. In addition, no protection of p-*rpfB* DNA was observed at a later time point tested (week 10 post-infection, data not shown). It is possible that rpfB expression decreases in the course of *Mtb* infection (as suggested by the decreased rpfB-specific immune response that we have observed). Decreased expression could hamper the ability of an rpfB-specific T cell population to exert its protective effector function, similarly to what has been observed for other antigens such as Ag85B [44].

In conclusion, our data seem to point to an *in vivo* role of rpfB in mycobacterial replication rather than to a direct role in reactivation. In a rabbit model of *Mtb* infection, Kesavan et al. showed that rpfB is preferentially expressed in dexamethasone treated rabbits versus latently infected animals [16]. In the light of our results, it could be argued that this increased expression in dexamethasone treated rabbits was reflecting the role of rpfB in the cell division process of *Mtb* resuming its growth, rather than a direct role of rpfB in resuscitation of the infection. Nevertheless, to further dissect its potential as possible component of multi-antigenic TB sub-unit vaccines, it would be interesting to analyze the protective efficacy of rpfB-specific responses induced by vaccination in models mimicking reactivation of latent *Mtb* infection. A major limitation for that purpose is the lack of satisfactory reactivation models. Indeed for example in the recently described rat model of latent TB infection, reactivation is induced by a harsh immunosuppressive treatment with dexamethasone that will affect vaccine generated memory immunity [45]. A possible model to test protective efficacy of vaccine induced rpfB-specific responses against reactivation of latent *Mtb* infection is the Cornell mouse model in which reactivation is induced by aminoguanidine (AG) [46]. AG is indeed an inhibitor of iNOS (inducible nitric oxide synthase) and will not affect vaccine generated effector T cell responses, but operate downstream of these.

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