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Mycobacterium tuberculosis antigen 85B and ESAT-6 expressed as a recombinant fusion protein in *Mycobacterium smegmatis* elicits cell-mediated immune response in a murine vaccination model

Anthony G. Tsolaki[a,*], Judit Nagy[b], Sergio Leiva[c], Uday Kishore[a], Ida Rosenkrands[d], Brian D. Robertson[b]

[a] *Centre for Infection, Immunity and Disease Mechanisms, Biosciences, School of Health Sciences and Social Care, Brunel University, London UB8 3PH, United Kingdom*
[b] *MRC Centre for Molecular Bacteriology & Infection, Department of Medicine, Flowers Building, Imperial College London, South Kensington, London SW7 2AZ, United Kingdom*
[c] *Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Casilla 567, Valdivia, Chile*
[d] *Statens Serum Institut, 5 Artillerivej, DK-2300 Copenhagen S, Denmark*

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ABSTRACT

In this study, we investigated the potential molecular and immunological differences of a recombinant fusion protein (Hybrid-1), comprising of the immunodominant antigens Ag85B and ESAT-6 from *Mycobacterium tuberculosis*, derived from two different expression systems, namely *Mycobacterium smegmatis* and *Escherichia coli*. The fusion protein was successfully expressed and purified from both bacterial hosts and analyzed for any host-dependent post-translational modifications that might affect the immunogenicity of the protein. We investigated the immunogenicity of Hybrid-1 expressed in the two host species in a murine vaccination model, together with a reference standard Hybrid-1 (expressed in *E. coli*) from the Statens Serum Institut. No evidence of any post-translation modification was found in the *M. smegmatis*-derived Hybrid-1 fusion protein, nor were there any significant differences in the T-cell responses obtained to the three antigens analyzed. In conclusion, the Hybrid-1 fusion protein was successfully expressed in a homologous expression system using *M. smegmatis* and this system is worth considering as a primary source for vaccination trials, as it provided protein of excellent yield, stability and free from lipopolysaccharide.

1. Introduction

Tuberculosis (TB) is still a major cause of morbidity and mortality in the world today. Currently, it is estimated that 2–3 million deaths occur worldwide from active TB and there are 8–10 million new cases per year, whilst a third of the world population are latently infected with the causative agent, *Mycobacterium tuberculosis* (Dye, 2006; Maher et al., 2007). During latency, *M. tuberculosis* is able to survive and persist as an intracellular pathogen for years, by being able to modulate phagosomal maturation, preventing phagosomal–lysosome fusion and reducing acidity within the phagosome (Gupta et al., 2012; Russell, 2001). *M. tuberculosis* also has significant interaction with components of the innate immune system e.g. toll-like receptors (TLRs), complement and lung surfactant proteins SP-A and SP-D, which play important roles in shaping adaptive immunity to TB infection in the host (Tsolaki, 2009). The only licensed vaccine being used currently against *M. tuberculosis* is *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), which has been used worldwide since the early 1900s. Whilst BCG has been shown to be protective against childhood forms of TB, its efficacy against adult active TB varies greatly, ranging from 0% to 85% (Andersen and Doherty, 2005; Fine, 1995). Improved vaccines are urgently needed, particularly to target the global epidemic of adult active TB, which is the most infectious form of the disease (Hanekom et al., 2008). During the last few years, a number of new candidate vaccines against TB have now been trialed, with several vaccines showing improved efficacy (Andersen, 2007). Among these candidates is the fusion protein Hybrid-1 or H1, which is based on the immunodominant antigens Antigen 85B (Ag85B) and the early secretory antigenic target (ESAT-6) from *M. tuberculosis*. The Hybrid-1 fusion protein is at the forefront of candidate vaccines against TB and has been extensively tested in a number of studies (Agger et al., 2008; Dietrich et al., 2007; Langermans et al., 2005; Olsen et al., 2004; van Dissel et al., 2010, 2011; Weinrich Olsen et al., 2001). A recent variant has also been fused to the latency associated protein Rv2660c (H56) that promises efficacy against pre- and post exposure (latent) TB (Aagaard et al., 2011). The majority of tested vaccine candidate antigens are heterologously expressed

Abbreviations: ESAT-6, early secretory antigenic target 6; Ag85, antigen 85; TB, tuberculosis; BCG, Bacillus Calmette-Guerin; LPS, lipopolysaccharide.

* Corresponding author. Tel.: +44 1895 266077.
E-mail address: anthony.tsolaki@brunel.ac.uk (A.G. Tsolaki).

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in various strains of the common laboratory organism *Escherichia coli* (*E. coli*), in spite of the fact that the protein folding and post-translational modification mechanism in the pathogen might be very different. Protein lacking the native mycobacterial milieu and modifications may be less immunologically active (Triccas et al., 1996). Post-translational modifications such as methylation have been shown to alter the immunogenicity of recombinant proteins when comparisons were made between native mycobacterial protein versus the recombinant protein expressed from *E. coli* (Menozzi et al., 1998; Pethe et al., 2002; Temmerman et al., 2004). The purification of native proteins from *M. tuberculosis* or *M. bovis* BCG for biochemical and immunological analysis is a complex and laborious process, often resulting in poor yields of protein (Delogu and Brennan, 1999; Menozzi et al., 1996). Recombinant expressions systems have been developed recently that can be used in fast-growing non-pathogenic mycobacteria. These systems have made use of modified mycobacterial plasmids engineered to over-express protein, via the hsp60 promoter (Curcic et al., 1994; Delogu et al., 2004; DeMaio et al., 1997; Dziadek et al., 2002; O'Donnell et al., 1994). Inducible systems for mycobacterial expression have also been developed, using tetracycline induction, which provide controlled amounts of protein expression (Blokpoel et al., 2005; Carroll et al., 2005; Ehrt et al., 2005; Triccas et al., 1998). Using such systems has potential in producing recombinant proteins with features that are native to those in *M. tuberculosis*, free from lipopolysaccharide (LPS) contamination, thus facilitating its use as an optimal vaccine candidate or new diagnostic marker (Masungi et al., 2002).

The aim of the present study was to develop an expression system in *Mycobacterium smegmatis* that would allow the production of antigens from *M. tuberculosis* and their efficient purification, with the resultant recombinant protein resembling the native antigen. We therefore describe the expression of the Hybrid-1 fusion protein in *E. coli* and *M. smegmatis*, using mycobacterial-shuttle plasmid vectors (Blokpoel et al., 2005; Wiles et al., 2005). The expression vector used here contains the *tetRO* region from the *Corynebacterium glutamicum* TetZ, making expression of genes cloned downstream of *tetRO* responsive to tetracycline We demonstrate the purification of the recombinant Hybrid-1 proteins from both bacterial hosts and analysis of their biochemical and immunological characteristics, in order to determine whether there are any differences in their immunogenicity.

2. Materials and methods

2.1. Bacterial strains and growth conditions

M. smegmatis mc^2-155 was grown at 37 °C, in Middlebrook 7H9 liquid broth or on Middlebrook 7H11 solid media, prepared according to the manufacturer's instructions, and supplemented with OADC (Difco), 0.08% glycerol and 0.05% Tween-80 (Sigma). All *E. coli* strains were grown on LB-agar plates or in LB broth. When needed, kanamycin was added at a final concentration of 50 μg/ml for *E. coli* and hygromycin was added at a concentration of 50 μg/ml for *M. smegmatis*.

2.2. Cloning of Hybrid-1 fusion into mycobacteria shuttle vectors

The pMCT6 plasmid containing the gene fusion Ag85B and ESAT-6 (Hybrid-1) was constructed as previously described (Weinrich Olsen et al., 2001). The Hybrid-1 fusion was amplified from pMCT6 using, Ag85B-HB-forward primer (5′-CGC GGA TCC ATG CAC CAC CAC CAC CAC CAC TTC TCC CGG CCG GGG CTG CC-3′) and ESAT-6-SpeI-reverse primer (5′-GGA CTA GTC TAT GCG AAC ATC CCA GTG ACG TTG CCT TC-3′). The forward primer contained a BamHI restriction site and the reverse primer contained a SpeI restriction site (underlined). The forward primer also contains a HIS-tag to facilitate purification. Amplification was carried out for 30 cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 90 s, using Biolase Taq polymerase (Bioline Reagents, UK). PCR products were purified (Qiagen, UK) and digested by BamHI and SpeI enzymes (New England Biolabs, UK) and positionally cloned into the tetracycline-inducible vector pMind, described previously (Blokpoel et al., 2005) and the pSHKLx1 shuttle vector (Wiles et al., 2005). Both plasmids pMind and pSHKLx1 are *E. coli*–mycobacteria shuttle plasmids containing kanamycin and hygromycin selection markers. The pSHKLx1 also contains the constitutively expressed promoter hsp60 from *M. tuberculosis* H37Rv (Wiles et al., 2005). The luxAB reporter genes in pSHKLx1 were replaced with the Hybrid-1 fusion, via the BamHI and SpeI sites. Both pMind (Hybrid-1) and pSHK (Hybrid-1) were transformed into *E. coli* strains DH5a and BL21 (Invitrogen, UK), using the $CaCl_2$ method (Sambrook and Russell, 2000) and pSHK (Hybrid-1) expressed in *E. coli* strain BL21 (Invitrogen, UK). Vectors pMind (Hybrid-1) and pSHK (Hybrid-1) were introduced into *M. smegmatis* by electroporation, using the protocol previously described (Parish and Stoker, 1998).

2.3. Expression conditions

For *M. smegmatis* starter cultures were set up consisting of pMind (Hybrid-1) transformants in triplicate 20 ml cultures of Middlebrook 7H9 liquid broth, supplemented with OADC, 0.08% glycerol and 0.05% Tween-80 at 37 °C, in 125 ml conical flasks and shaken at 200 rpm in an orbital shaker. Mycobacterial cultures were then sub-cultured at log phase, in 800 ml in 2 L flasks overnight. Induction of expression was achieved by adding 20 ng/ml of tetracycline. Cells were harvested after 4 h of induction. For *E. coli*, starter cultures were performed using triplicate 20 ml cultures in LB-broth, in 125 ml conical flasks, shaken at 200 rpm in an orbital shaker. *E. coli* cultures were then sub-cultured at log phase in 400 ml of 1 L LB and incubated overnight at 37 °C before harvesting. Samples were processed for protein purification as described below.

2.4. Protein purification

M. smegmatis cultures were harvested at 4400 × *g* and the pellet washed twice with ice cold PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). Cells were resuspended in PBS containing 0.05% Tween 80 (Sigma–Aldrich), protease inhibitor (P-8849, Sigma–Aldrich), lysozyme (Sigma–Aldrich, at a final concentration of 2.4 mg/ml) and incubated on ice for 30 min. Cells were disrupted twice with a cell disrupter French press machine (Constant Systems Ltd.) at a pressure of 1.1×10^8 Pa. *E. coli* cultures were harvested at 10,000 × *g* for 10 min and the pellet resuspended in BugBuster Reagent (5 ml/g wet pellet) (Novagen), Benzonase nuclease (Novagen) (25 U per 1 ml BugBuster), lysozyme (Sigma) (5 KU/g wet pellet) and protease inhibitor (P-8849, Sigma–Aldrich). The cell suspension was then incubated for 30 min at room temperature with gentle shaking. Insoluble debris was removed by centrifugation at 16,000 × *g* for 20 min at 4 °C.

Lysates from both *M. smegmatis* and *E. coli* were then size-fractionated using gel-filteration S-100 and S-10 spin columns (Amicon, Millipore) to exclude protein outside the range of 10–100 kDa. This fractionated lysate was then passed over a 1 ml HisTrap HP Ni-affinity sepharose column (GE Healthcare) and eluted with 500 mM imidazole. Eluted fractions were analyzed on SDS-PAGE and Coomassie staining. Positive fractions were then further purified by FPLC (AKTA, GE healthcare) by loading onto a 1 ml HisTrap HP column (GE Healthcare). For the purification a gradient