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Novel human recombinant antibodies against *Mycobacterium tuberculosis* antigen 85B

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Abstract

Background: Tuberculosis is the leading cause of death due to bacterial infections worldwide, mainly caused by *Mycobacterium tuberculosis*. The antigen 85 complex comprises a set of major secreted proteins of *M. tuberculosis*, which are potential biomarkers for diagnostic.

Results: In this work, the first human single chain fragment variable (scFv) antibodies specific for the tuberculosis biomarker 85 B were selected by phage display from naïve antibody gene libraries (HAL7/8). Produced as scFv-Fc in mammalian cells, these antibodies were further characterized and analysed for specificity and applicability in different tuberculosis antigen detection assays. Sandwich detection of recombinant 85 B was successful in enzyme linked immunosorbent assay (ELISA), lateral flow immunoassay and immunoblot. Whereas detection of *M. tuberculosis* cell extracts and culture filtrates was only possible in direct ELISA and immunoblot assays. It was found that the conformation of 85 B, depending on sample treatment, influenced antigen detection.

Conclusions: Recombinant antibodies, selected by phage display, may be applicable for 85 B detection in various assays. These antibodies are candidates for the development of future point of care tuberculosis diagnostic kits. Using 85 B as a biomarker, the antigen conformation influenced by sample treatment is important.

Background

Approximately 8.7 million people worldwide fell ill with tuberculosis (TB) in 2011 and 1.4 million deaths were reported [1]. Worldwide TB ranks as the second major cause of death from an infectious disease. One third of the world population is estimated to be infected with *M. tuberculosis* (Mtb), yet they remain asymptomatic. This is defined as latent TB infection (LTBI) [2]. Only 66% of the TB-cases worldwide are correctly diagnosed [1]. The gold standard in TB diagnosis remains the preparation of liquid cultures in selective media from sputum or tissue/body fluid specimens [3]. This is followed by further Mtb specific tests or drug susceptibility testing (i.e. nucleic acid amplification tests such as the Gene Xpert MTB/RIF [4]).

Diagnosis of TB in most low- and middle-income countries continues to rely on sputum smear microscopy for acid-fast bacilli (Ziel-Neelsen stain) [1]. This technique detects only 40 – 60% of pulmonary TB cases and is not able

to differentiate between Mtb and other ubiquitous mycobacteria [5,6]. This is less sensitive in children, HIV co-infected patients and in patients with extrapulmonary TB [7,8]. The diagnosis of TB in developing countries is limited by equipment and infrastructure [1]. Therefore, in these countries, a simple diagnostic tool without the need for sophisticated instruments is needed. Accuracy, simplicity, affordability and technical robustness are important factors for a point of care (POC) TB-test. The main advantage of this is a rapid diagnosis, which allows initiation of treatment while the patient is still accessible [9]. Several anti-tuberculosis antibody detection systems are available. However the WHO recommended against the use of these assays because of their lack of sensitivity and specificity [10]. Direct detection of Mtb antigens in human specimens would allow specific diagnosis of active TB to be made, independent of the host's immune response. Furthermore, the use of specific antibodies to Mtb antigens in a lateral flow immuno assay (LFIA) would potentially provide a rapid POC test in a cost effective, easy-to-use format. Potential target antigens for POC TB-detection in human samples should be selected by the following

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criteria: substantial expression by bacteria *in vivo*, presence in the extracellular environment or on the mycobacterial cell wall, and resistance to degradation by host enzymes [11]. The 85 complex is a major secretion product of Mtb [12,13] which comprises three variant 85 proteins (A, B and C) that are encoded by three different genes (*fbpA*, *fbpB* and *fbpC2*, [14]). Antigens 85 A, 85 B and 85 C possess mycoyltransferase activity and are involved in the formation of trehalose monomycolate (TMM) and trehalose dimycolate (TDM), which are components of the mycobacterial cell wall [15,16]. Besides the 85 complex there is another 85 protein, known as 85 D or *FbpC1*. 85 D shows structural similarity to the 85 complex proteins, yet does not possess mycoyltransferase activity [15] and its function remains unknown. Disruption of *fbpA* results in the inability of Mtb to replicate within macrophages, indicating a key role in virulence [17]. Furthermore, it was shown that 85 complex proteins interact with gelatine-binding sites of human fibronectin, enhancing complement-mediated phagocytosis by macrophages [18-20]. Antibodies specific for only one of the 85 proteins would allow for further investigation of their individual roles in the pathogenesis of TB. However only antibodies cross reacting with all other 85 complex proteins have been reported so far [21-23].

The presence of the 85 complex was demonstrated in human serum [24], urine [20], cerebrospinal fluid [25] and sputum [26] of TB infected individuals. This makes it a prominent marker for TB. Although other mycobacteria express similar 85 complex proteins, these antigens could be used as TB markers in combination with other antigens to enhance specificity.

For the future development of diagnostic assays, recombinant antibodies generated by phage display are an alternative to polyclonal and monoclonal antibodies [27-32]. Naïve antibody libraries allow generation of antibodies to epitopes which are not recognized by any immune system. In addition, recombinant human antibodies could be used in therapeutic applications. In this work, single chain Fv (scFv) were isolated from a human naïve antibody gene library using phage display [33] and produced as scFv-Fc (yumab) [34]. These antibodies were further characterized

and analysed for their suitability in an antigen detection assay targeting Mtb 85 B.

Results

Selection of human antibodies against 85 B

For antibody selection, panning was performed on immobilized 85 B followed by a screening ELISA using soluble scFv (see Additional file 1: Figure S1). Finally, five unique scFv were confirmed by DNA sequencing. The corresponding human germline sequences according to VBASE2 [35] are displayed in Table 1. All α -85 B antibodies contain a variable gene segment of the heavy chain (HV) of subfamily V_H3. Four binders have a lambda light chain (V_L λ) and one has a kappa light chain (V_L κ). All V_L λ clones have a joining segment of the heavy chain (HJ) of subfamily J4, a joining segment of the light chain (LJ) of subfamily J3, and a variable gene segment of the heavy chain (HV) of subfamily V_H3 gene 30.

Cloning and production of scFv-Fc (Yumabs)

The scFv were subcloned into pCSE2.5-hIgG1-Fc-XP [34], produced in 50 mL scale and purified from the culture supernatant via Protein A. The purified scFv-Fc (Yumabs) were analysed by SDS-PAGE, silver staining, α -human IgG(Fc) immunoblot and reducing gel analysis via Tape Station. No degradation was detected (data not shown). The purity of the obtained antibody preparations was determined to 93.4 – 96.9%.

Validation of antigen binding

The antigen binding of the Yumabs was analysed by titration ELISA (Figure 1A). The antigen binding was confirmed for all Yumabs. Antigen detection limits of the α -85 B scFv-Fc were determined by antigen titration ELISA (Figure 1B). About 5 ng/mL were detected by MFU50-C10, ~ 10 ng/mL by MFU50-A10 and MFU50-E2, and ~ 30 ng/mL by MFU50-D4 and MFU50-D7.

Epitope mapping

To determine whether the antibodies bind to linear or conformational epitopes, the α -85 B scFv-Fc were analysed by immunoblot (data not shown). All antibodies

Table 1 Comparison of heavy and light chain gene segments of α -85 B antibodies

Clone	Heavy chain			Light chain	
	HV	D	HJ	LV	LJ
MFU50-A10	IGHV3-30*04	IGHD6-19*01	IGHJ4*02	IGLV8-61*01	IGLJ3*02
MFU50-C10	IGHV3	IGHD2-15*01	IGHJ6*02	IGKV1-12*01	IGKJ2*01
MFU50-D4	IGHV3-30*18	IGHD3-3*02	IGHJ4*02	IGLV2-14*01	IGLJ3*01
MFU50-D7	IGHV3-30*04	IGHD4-17*01	IGHJ4*02	IGLV3-21*02	IGLJ3*02
MFU50-E2	IGHV3-30*03	IGHD6-13*01	IGHJ4*02	IGLV7-43*01	IGLJ3*02

Abbreviations: HV variable (V) gene segment of the heavy chain, D diversity gene segment, HJ joining (J) gene segment of the heavy chain, LV variable gene segment of the light chain, LJ joining gene segment of the light chain.

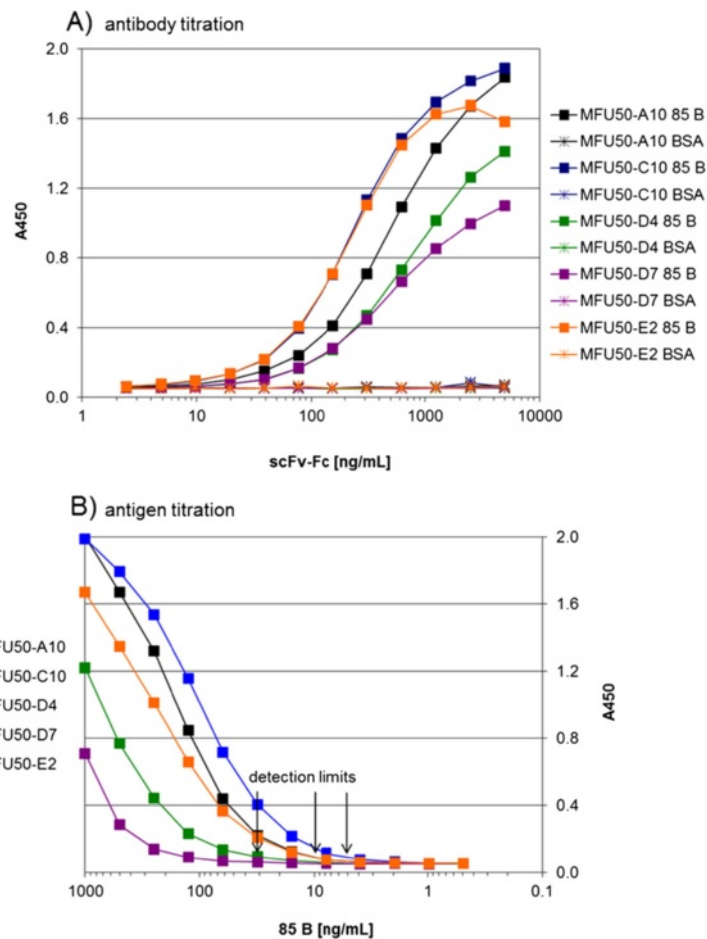


Figure 1 Analysis of antigen binding. **A)** Antibody titration and **B)** antigen titration in ELISA with α -85 B scFv-Fc. **A)** Dilution series of scFv-Fc were used for detection of directly coated antigen (85 B) or BSA (negative control). Detection of bound scFv-Fc with goat α -human IgG(Fc)-HRP and development with TMB. **B)** Different dilutions of antigen (85 B) were directly coated to the wells. Antigen detection with a scFv-Fc concentration of half maximal saturation. Detection of bound scFv-Fc with goat α -human IgG(Fc)-HRP, development with TMB. Negative control BSA A450 = 0.05.

recognized linear epitopes (data not shown). Epitope mapping of the α -85 B scFv-Fc was carried out on PepSpot membranes (Figure 2A). Through the overlap of the peptide sequences the epitopes were determined (Figure 2B). The crystal structure of antigen 85 B was determined by Anderson et al. [36] and the epitopes were visualized on the 3D structure of the antigen (Figure 2C). The distance between epitope regions “AFSRPGPLV(EYL)” and “SPAVYL” was computed to ~ 2.5 nm, between “SPAVYL” and “SSDPAWERN(DPT)” to ~ 4 nm and between “SSDPAWERN(DPT)” and “AFSRPGPLV(EYL)” to ~ 5 nm. Considering the width of an antibody (~ 4 nm, [37]), sandwich detection was likely targeting epitopes on different sites of the antigen.

Cross reactions

Sequence comparison of the epitopes of the α -85 B antibodies with corresponding sequences of the other 85 complex antigens provided information about possible cross

reactions (Table 2). The complete epitope “AFSRPGPLV(EYL)” is present in antigen 85 A and 85 C, but not in 85 D. Homologous regions of the epitopes “SPAVYL” and “SSDPAWERN(DPT)” can be found in 85 A, C, and D. Experimental study of 85 complex cross reactivity was carried out by indirect ELISA (Figure 3). MFU50-A10 reacted with 85 A and 85 D. MFU50-D4 displayed a weak cross reactivity with 85 A as predicted. MFU50-E2 bound antigen 85 B weakly and showed no cross reactions. MFU50-D7 reacted weakly with 85 B. No reaction with the other 85 complex antigens was detected. MFU50-C10 was also not cross reactive. A summary of the expected cross reactions in comparison to determined cross reactions is outlined in Table 2.

Detection of 85 B in Mtb cell extracts and culture filtrates

The antibody binding to Mtb/BCG cell extracts and culture filtrates was analysed by indirect ELISA (Figure 4). In this assay only MFU50-A10 reacted with Mtb culture

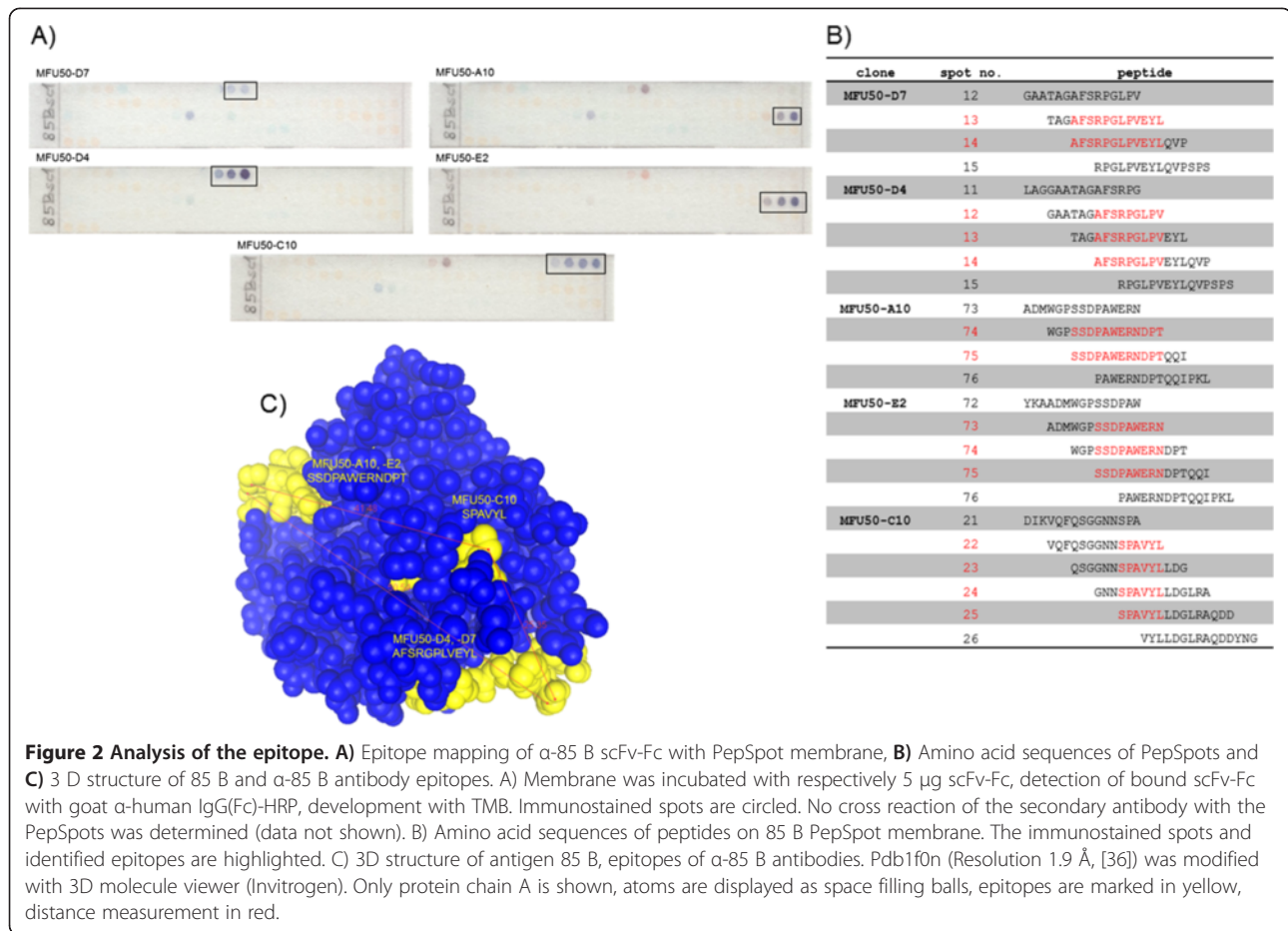


Figure 2 Analysis of the epitope. **A)** Epitope mapping of α -85 B scFv-Fc with PepSpot membrane, **B)** Amino acid sequences of peptides on 85 B PepSpot membrane and **C)** 3 D structure of 85 B and α -85 B antibody epitopes. A) Membrane was incubated with respectively 5 μ g scFv-Fc, detection of bound scFv-Fc with goat α -human IgG(Fc)-HRP, development with TMB. Immunostained spots are circled. No cross reaction of the secondary antibody with the PepSpots was determined (data not shown). B) Amino acid sequences of peptides on 85 B PepSpot membrane. The immunostained spots and identified epitopes are highlighted. C) 3D structure of antigen 85 B, epitopes of α -85 B antibodies. Pdb1f0n (Resolution 1.9 Å, [36]) was modified with 3D molecule viewer (Invitrogen). Only protein chain A is shown, atoms are displayed as space filling balls, epitopes are marked in yellow, distance measurement in red.

Table 2 Cross reactions of α -85 B antibodies with other 85 complex antigens

sequence comparison (corresponding sequences to α -85 B epitopes)					
Clone	85 A	85 B	85 C*	85 D	Expected cross reaction
MFU50-A10	KEDPAWQRNDPL	SSDPAWERNPT	SSDPAWKRNDPM	SDPA	85 A, 85 C, 85 D
MFU50-C10	SPALYL	SPAVYL	PHAVYL	PHAVYL	85 A, 85 C, 85 D
MFU50-D4	AFSRPGLPV	AFSRPGLPV	AFSRPGLPV	-	85 A, 85 C
MFU50-D7	AFSRPGLPVEYL	AFSRPGLPVEYL	AFSRPGLPVEYL	-	85 A, 85 C
MFU50-E2	KEDPAWQRN	SSDPAWERN	SSDPAWKRN	SDPA	85 A, 85 C, 85 D
Reaction with 85 complex antigens in ELISA					
Clone	85 A	85 B	85 C*	85 D	Determined cross reaction
MFU50-A10	Strong	Strong	-	Strong	85 A, 85 D
MFU50-C10	None	Strong	-	None	None
MFU50-D4	Weak	Strong	-	None	Weak 85 A
MFU50-D7	None	Weak	-	None	None
MFU50-E2	None	Weak	-	None	None

*Purified antigen 85 C was not available for immunological assays, nevertheless it is listed for completeness of the illustration.

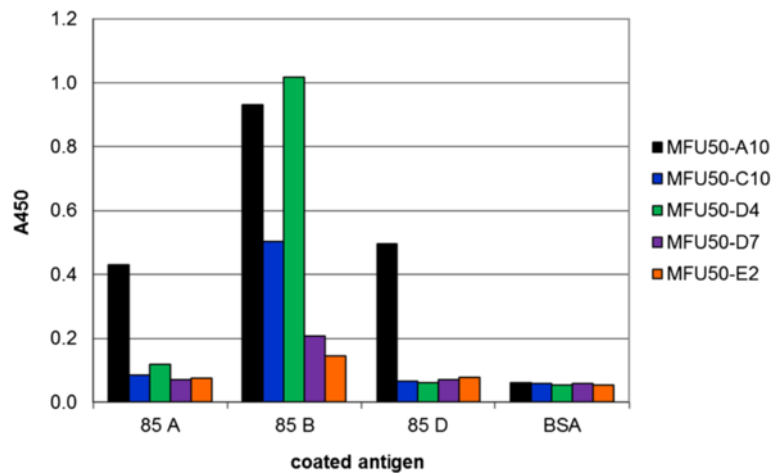


Figure 3 Cross reactions of α -85 B scFv-Fc with other 85 complex antigens in ELISA. 100 ng of the antigens were directly coated to the wells. 85 B detection with α -85 B scFv-Fc (using concentrations at half maximal saturation), detection of bound scFv-Fc with goat α -human IgG (Fc)-HRP, development with TMB.

filtrate and Mtb/BCG cell extracts. The other antibodies bound only to recombinant 85 B. A weak cross reaction of MFU50-A10 with 7H9 medium was detected.

Development of a sandwich ELISA

Due to the availability of five different antibodies with three different epitopes on the target antigen a sandwich α -85 B assay was performed. Therefore MFU50-A10 (epitope “SSDPAWERNDPT”), MFU50-C10 (epitope “SPAVYL”) and MFU50-D4 (epitope “AFSRPGLPV”) scFv-Fc were conjugated to HRP. Sandwich ELISA detection of the antigen was carried out with MFU50-A10-HRP, MFU50-C10-HRP or MFU50-D4-HRP as detection antibodies. Capturing was conducted with corresponding antibodies recognizing different epitopes (than the detection antibody)

on the target (Figure 5A, B, C). Blocking reagent only was used as a negative control ($A_{450} = 0.02 - 0.05$). Sandwich detection of the recombinant antigen was successful with all combinations. The antigen detection limit for capturing with MFU50-A10, MFU50-C10, MFU50-D4 or MFU50-D7 was determined to ~ 10 ng/mL, independent from the detection antibody. Capturing with MFU50-E2 resulted in a detection limit of ~ 25 ng/mL. The most suitable combination was obtained with MFU50-A10 as the capture antibody and MFU50-C10-HRP as detection antibody-conjugate. Mtb culture filtrate (Mtb cultivated in 7H9 + ADC + Tween for 3 months at 37°C) was analysed by α -85 B sandwich titration ELISA (Figure 6A, red curve). This resulted in weak binding barely distinguishable from the medium control (Figure 6A, pink curve). Mtb culture

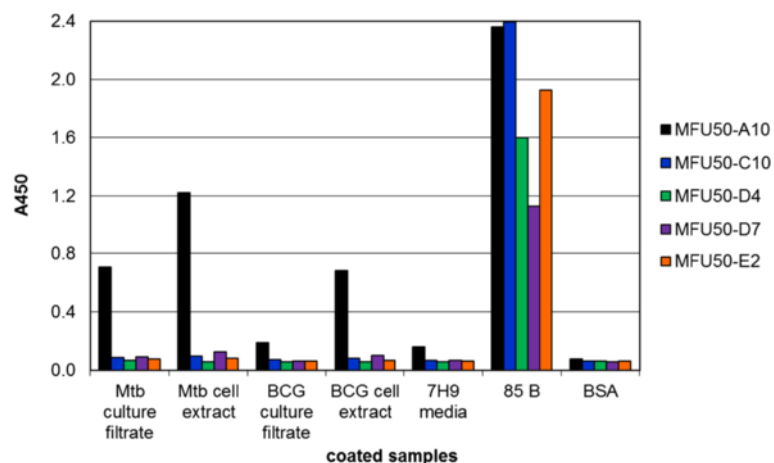
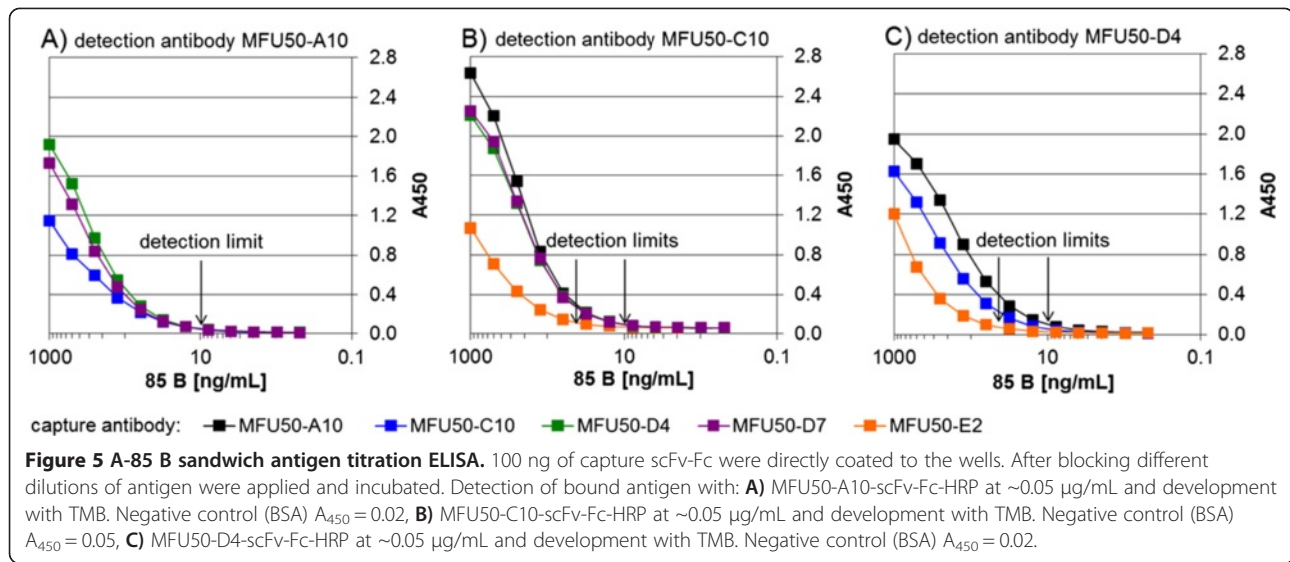


Figure 4 Reaction of α -85 B scFv-Fc with Mtb/BCG cell extracts and culture filtrates determined by indirect ELISA. 100 ng/100 μ L of the different samples were directly coated to the well, reaction was determined with a scFv-Fc concentration of 2.5 μ g/mL. Detection of bound scFv-Fc with goat α -human IgG(Fc)-HRP. Development with TMB.

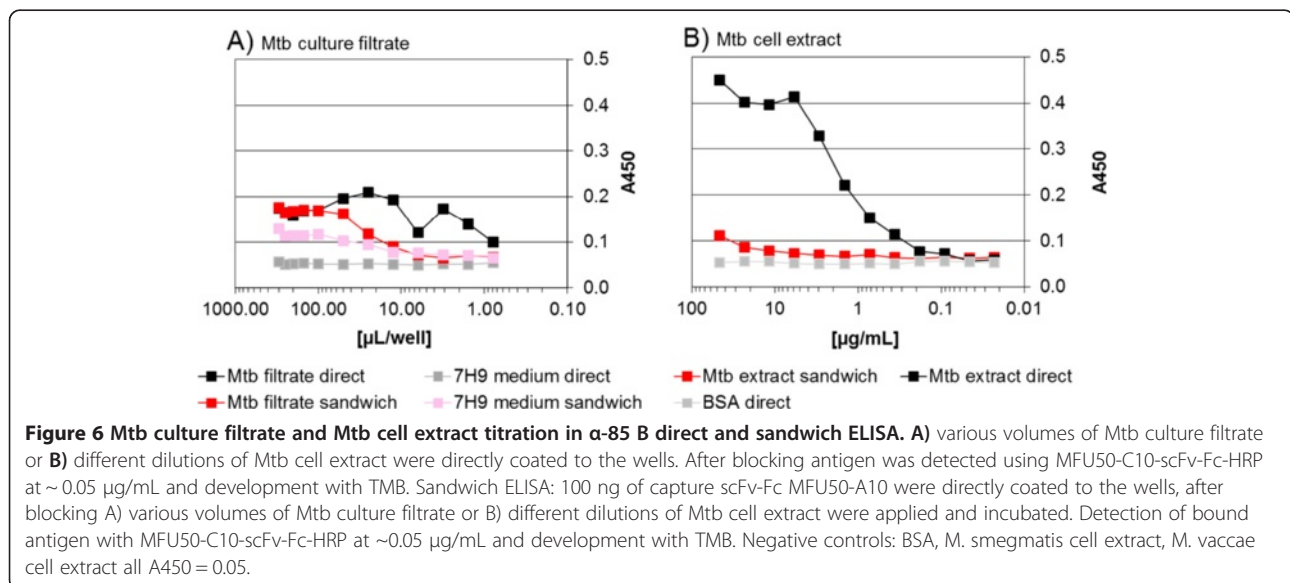


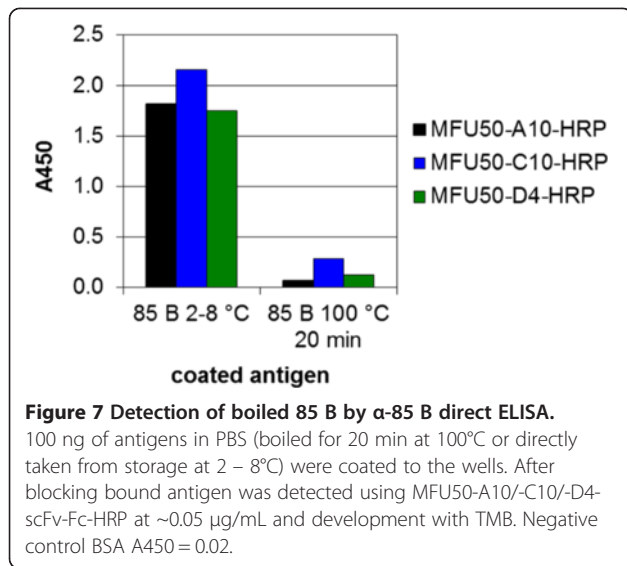
filtrate was further analysed by direct α -85 B ELISA (Figure 6A, black curve). In this assay there was no increase of the signal by increasing of sample volume. Additionally, Mtb cell extract was analysed by α -85 B sandwich titration ELISA (Figure 6B, red curve). This showed no binding distinguishable from the negative control except for the highest concentration (Figure 6B, grey curve). However, in a direct α -85 B ELISA specific binding to Mtb cell extract was observed (Figure 6B, black curve).

Effect of sample treatment on 85 B detection

The failed detection of 85 B in Mtb cell extract in sandwich ELISA compared to the successful detection in direct ELISA was further analysed. It was hypothesized

that the sample treatment, especially autoclaving, was the cause for differing antigen detection. Cell extracts were prepared by autoclaving at 121°C for 20 min. The influence of sample pretreatment by heat on 85 B detection was investigated by direct ELISA (Figure 7). When 85 B was boiled, antigen binding was reduced. Further examination of the susceptibility of 85 B to heat was carried out by analytical SEC. The theoretical molecular mass of an 85 B monomer was computed with the ExPASy prot param tool [38] to 34.6 kDa. The untreated sample of 85 B displayed a small monomer peak at 33.8 kDa and a dominant multimer peak with a molecular mass out of the measurement range but definitely greater than 669 kDa (Figure 8A). In comparison the boiled 85 B



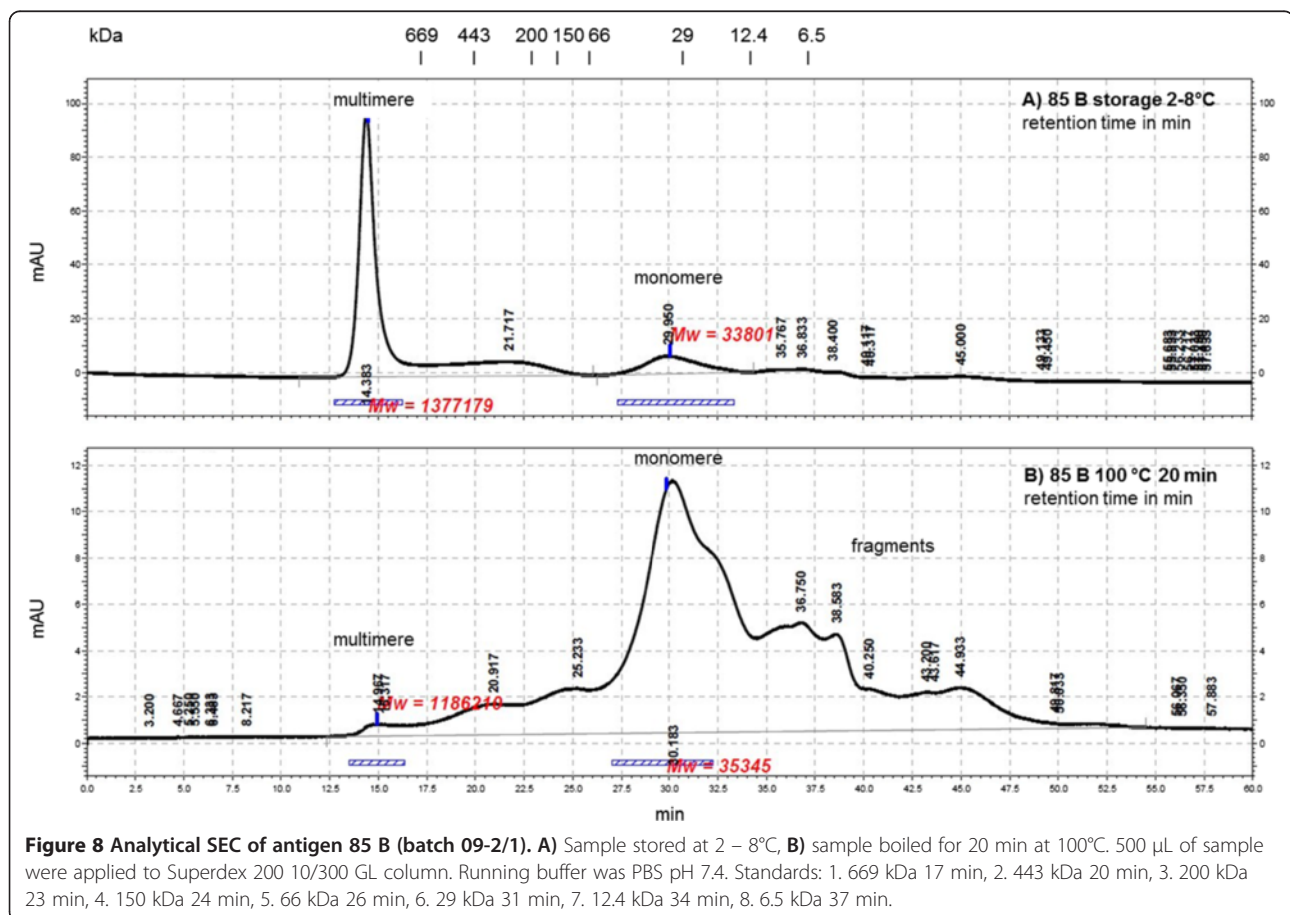


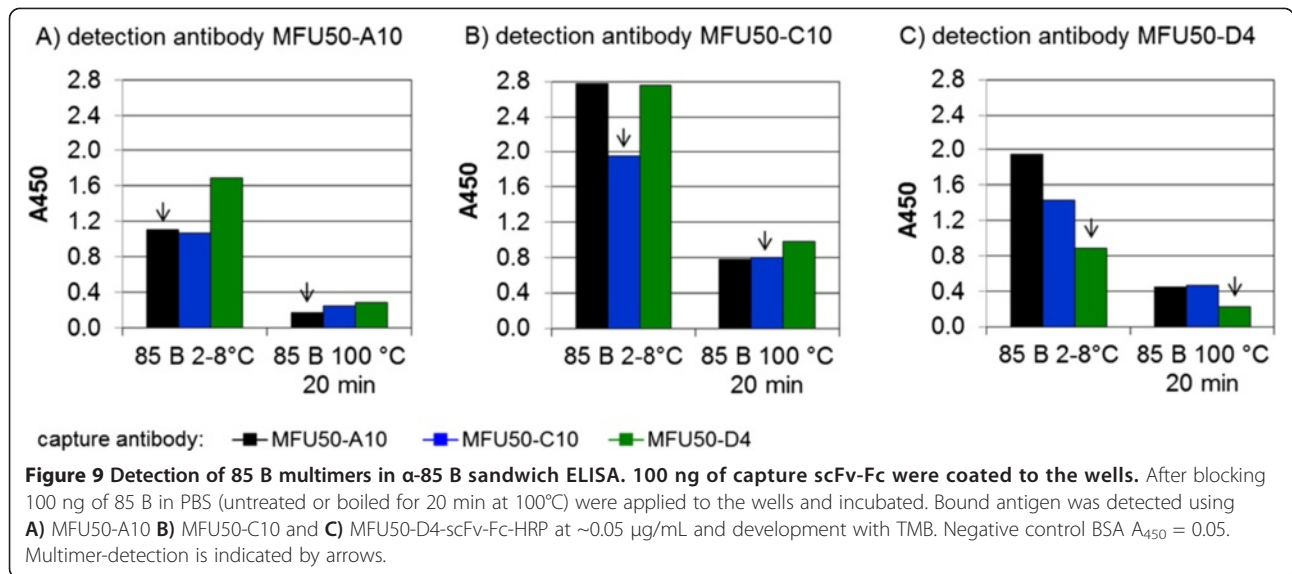
sample offered a dominant peak at the size of a monomer (34.6 kDa), multiple peaks with lower molecular mass and a few non-dominant multimer peaks (Figure 8B). Interestingly, aggregation was greater in the untreated sample than in the boiled sample. It was concluded that the

aggregation improved sandwich detection, and if multimers were detected by sandwich ELISA, the use of the same antibody for capturing and detection should be possible. Experimental study by sandwich ELISA (Figure 9) verified this assumption. In the untreated as well as in the boiled sample, sandwich detection with only one antibody was possible (Figure 9, arrows).

Development of a lateral flow immuno assay

To develop a Lateral Flow Immuno Assay (LFIA) all α-85 B scFv-Fc were conjugated to 40 nm colloidal gold. Sandwich 85 B detection was performed with all available antibodies for capturing and all antibody-gold conjugates for detection. The most suitable combination, MFU50-A10 as capture antibody and MFU50-D4-gold as detection antibody, was further analysed. A procedure that allowed sensitive antigen detection and low background combined with good feasibility was developed (data not shown). The 85 B detection limit was determined by sandwich LFIA to ≤ 5 ng/mL (Figure 10). Mtb cell extract or culture filtrate (concentrated and unconcentrated) were analysed by α-85 B sandwich LFIA. Unfortunately, 85 B was not detectable in any sample in this assay.





Development of an immunoblot assay for the detection of 85B

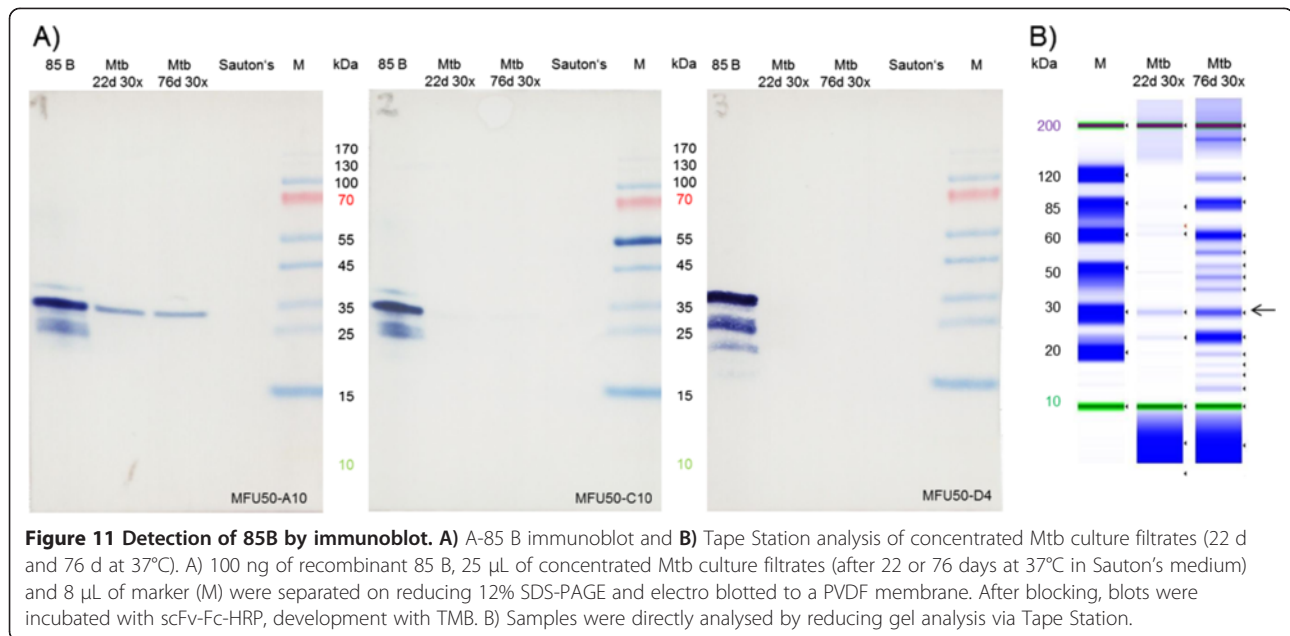
Sandwich detection of antigen 85 B in Mtb culture filtrates and cell extracts was complex in previously described sandwich ELISA and LFIA. As an alternative a direct α -85 B immunoblot assay was developed to avoid the challenge of sample pretreatment in sandwich detection. Furthermore, Mtb was cultured in Sauton's minimal medium and culture filtrates were concentrated to increase the antigen quantity. Weekly samples were taken, concentrated 20 to 30 fold and analysed by reducing gel analysis via Tape Station and α -85 B immunoblot. No 85 B

was detected in 7 and 16 days old cultures by either means (data not shown). After 22 days a protein band at \sim 30 kDa (according to Tape Station analysis, Figure 11B) was recognized by MFU50-A10 in immunoblot (Figure 11A). Additionally, 85 B expression was found in 61 and 70 days old cultures (data not shown). The Tape Station and α -85 B immunoblot results for 22 and 76 days old cultures are given in Figure 11.

Discussion

Tuberculosis is the leading cause of death due to bacterial infections worldwide [1]. State of the art TB detection





is mostly time consuming or needs expensive infrastructure [39]. In developing countries, a rapid, cheap and easy to use POC TB test is needed [9]. The WHO does not recommend the detection of antibodies against Mtb [10]. The only available assay for direct Mtb antigen detection is that against mycobacterial Lipoarabinomannan (LAM). However this test displays poor sensitivity apart from in patients with advanced HIV/AIDS [40].

In this work, we isolated and characterized specific antibodies against Mtb antigen 85 B. These antibodies were proved suitable for 85 B sandwich detection in ELISA and LFIA. In addition, an Mtb immunoblot assay was developed.

Five unique antibodies were selected from the human naïve libraries HAL7/8 [33]. The examined antibodies displayed homogeneity in the subfamily of the variable gene segment of the heavy chain (all V_{H3}). This correlates with the overrepresentation of V_{H1} and V_{H3} in the HAL7/8 libraries [41] and *in vivo* [42]. Four antibodies have a lambda light chain, one a kappa light chain. According to Løset et al. [43] lambda scFv are expressed in higher yields in *E. coli* compared to kappa scFv, which may lead to an advantage in phage display [33,41]. The antibody MFU50-C10 has a germline combination of V_{H3} and $V_{\kappa 1}$, which is common *in vivo* and in naïve libraries [33,42,44]. The lambda germline sequences present in the other antibodies are from the subfamilies 2, 3, 7 and 8. Subfamilies $V_{\lambda 1,2}$ and 3 are dominant *in vivo*, whereupon $V_{\lambda 7}$ and 8 are rare *in vivo* [42,44]. In the HAL7 library $V_{\lambda 7}$ and 8 are more frequent than *in vivo* [33].

Ferrara et al. [22] selected 48 antibodies directed against the Mtb 85 complex in a combination of yeast and phage display. None of these antibodies were specific for an

individual 85 antigen but, cross reacted with all other 85 complex proteins. Landowski et al. [45] isolated an α -85 B chicken IgY which cross reacted with 85 complex proteins. Drowart et al. [21] generated seven monoclonal α -85 complex antibodies, which all cross reacted with other mycobacterial species however none were specific for antigen 85 B. This study shows the isolation of the first human recombinant antibodies (MFU50-C10, MFU50-D7 and MFU50-E2) specific for antigen 85 B. Furthermore four α -85 A antibodies (MFU12-D8, MFU53-A3, MFU53-F3 and MFU53-G2) and three α -85 D antibodies (MFU51-A6, MFU51-B10 and MFU53-C2) were generated (unpublished data). MFU12 -D8 was found to be specific for antigen 85 A and MFU51-B10 was specific for 85 D. These antibodies allow for the discrimination between individual components of the 85 complex. Antibody phage display using human naïve antibody gene libraries allows for the selection against non-immunogenic proteins and epitopes. We propose these epitopes, which would allow discrimination between the different 85 proteins, are not immunogenic. We propose this because of the fact that no antibodies, specific for one component of the 85 complex, were isolated by hybridoma technology [21,23].

Easy applicability to *in vitro* assays was showed in this study by conjugation to colloidal gold or HRP. Due to the recombinant nature of the explored antibodies, they can easily be altered to different formats, fused to different Fc-parts [34] or fused to markers such as green fluorescent protein (GFP, [46]) for *in vivo* experiments. Thereby the role of particular 85 proteins in Mtb cell wall biosynthesis and evasion of the host's immune response may be investigated.

All antibodies generated in this study recognized continuous sequences of the antigen, and the corresponding epitopes were determined by screening overlapping peptides immobilized on a cellulose membrane. MFU50-D4 recognized the epitope "AFSRPGLPVEYL" and MFU50-D7 recognized the epitope "AFSRPGLPV". This epitope region seems to be a potent T cell antigen since synthetic peptides including this sequence were found to induce cytokine release or immune cell proliferation in peripheral blood of individuals with varying TB status [47-50]. However only weak human B cell responses against these peptides were reported [49,51]. Interestingly, no antibodies against this epitope were generated by immunization. In this work, human antibodies were successfully generated by screening phage display libraries, fortifying the advantage of phage display technology over conventional immunization methods. Protein sequence comparison of the 85 complex proteins revealed the presence of the epitope in antigen 85 A and 85 C, suggesting cross reactivity. In ELISA and immunoblot analysis MFU50-D7 showed no 85 A binding, however MFU50-D4 showed slight cross reactivity with 85 A. During this study 85 C was not available for examination. Furthermore, a protein blast search (BLASTP, [52]) disclosed the existence of the entire epitope in several different mycobacterial strains (i.e. *M. bovis*, *M. ulcerans*, *M. marinum*, *M. smegmatis*, *M. vaccae*, etc.), suggesting cross reactivity.

The α -85 B antibodies MFU50-A10 and MFU50-E2 recognize the epitopes "SSDPAWERNNDPT" and "SSDPAWERN" respectively. Landowski et al. [45] generated an oligoclonal chicken IgY antibody against the peptide "SSDPAWERNNDPT" (epitope ID 60953, IEDB) and demonstrated 55% sensitivity and 85% specificity for detection of circulating 85 B in human blood by an immunoblot approach. Shen et al. [51] reported the synthetic peptide "GPSSDPAWERNNDPTQ QIPKL" (epitope ID 21797, IEDB) was recognized by sera (IgG) of TB + individuals. Similar peptides containing the epitope "SSDPAWERNNDPT" were found to induce cytokine release or T cell proliferation in various assays with samples of TB + individuals [49,50,53-55]. The entire amino acid sequence "SSDPAWERNNDPT" is present only in 85 B of *M. tuberculosis* and *M. bovis* BCG. Homologous sequences in Mtb 85 A or 85 C and in 85 complex proteins from other mycobacterial species (identified by BLASTP) differed primarily by substitution of one amino acid. Cross reactivity of MFU50-A10 with Mtb 85 A and *M. bovis* BCG cell extract and culture filtrate was detected by indirect ELISA and immunoblot. Landowski et al. [45] found a "SSDPAWERNNDPT" specific chicken IgY antibody to be cross reactive with Mtb 85 A and 85 C as well as 85 complex proteins of *M. avium*. The reaction of MFU50-A10 with 85 A, B and D indicates a possible

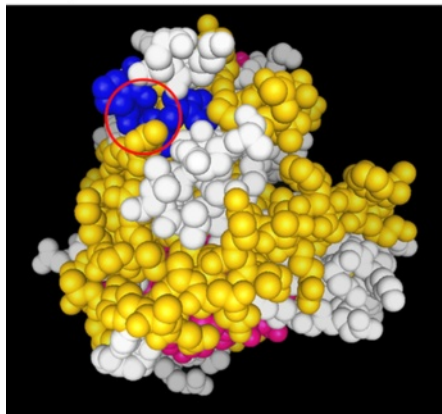
smaller epitope than determined by epitope mapping. The amino acid sequence "DPA" is present in antigen 85 A, B and C, but the adjacent amino acids differ. In 85 B the "DPA" is flanked by serine on the N-terminal site and tryptophan on the C-terminal site ("SDPAWE"). In 85 A glutamic acid is N-terminal and tryptophan C-terminal ("EDPAWQ"). In 85 D serine is N-terminal and alanine C-terminal ("SDPAAM"). It seems possible that the epitope of MFU50-A10 is "SDPAW" or "DPAWE", which would mean that two amino acids are different in 85 A or 85 D respectively. This would explain the weaker recognition of 85 A and 85 D. Furthermore, the comparison of the 3D structures of antigen 85 A and 85 B (Figure 12) revealed that the glutamic acid in 85 B forms a pin structure in front of the "DPAW" region. This formation is missing in 85 A, instead of glutamic acid there is glutamine. Glutamic acid can mediate strong electrostatic attractions and hydrogen bridges through the loaded carboxylgroup, whereupon glutamine is uncharged and can only mediate hydrogen bridges through the amino- and the ketogroup [56]. Thus, it is reasoned that the glutamic acid pin structure is needed structurally for correct docking, and physico-chemically for full strength binding to the antigen.

MFU50-C10 recognizes the epitope "SPAVYL", which is close to the suggested active site of antigen 85 B [36], offering a possible inhibitory effect by steric hindrance [57]. Synthetic peptides including this epitope were found to induce cytokine release and T cell proliferation in peripheral blood mononuclear cells of TB + humans [49,50,54,58] and antibodies against these peptides were detected in sera of TB + individuals [51,59]. Sequence comparison of Mtb 85 complex proteins revealed no homology in this area. According to BLASTP equivalent sequences are present in several other mycobacterial species (i.e. *M. bovis*, *M. marinum*, *M. leprae*, *M. vaccae*, *M. ulcerans*, *M. avium*, etc.). No cross reactivity with 85 A and 85 D was detected by indirect ELISA and immunoblot, suggesting specificity for antigen 85 B. Surprisingly, no cross reactions with *M. bovis* BCG cell extract and culture filtrate were detected in an indirect ELISA.

Sandwich ELISA detection of recombinant 85 B with the α -85 B Yumabs was successful in all performed assays. The most suitable combination was capturing with MFU50-A10 and detection with MFU50-C10-HRP, reaching a detection limit of ~10 ng/mL. Sandwich LFIA detection of recombinant 85 B was successful capturing with MFU50-A10 and detecting with MFU50-D4-gold. A detection limit of 5 ng/strip (0.03 ng/mL) was evaluated. There are no published reports on single antigen 85 B sandwich detection, only whole 85 complex sandwich assays [20,22-24,39,45,60].

Sandwich detection of recombinant 85 B was enhanced by multimeric antigen conformation, and reduced by

85 A) DPAWQ



85 B) DPAWE

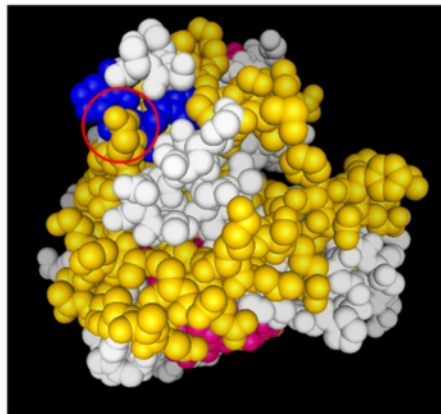


Figure 12 3 D structures of antigens A) 85 A and B) 85 B around the amino acid sequence "DPAW". A) Pdb1sfr (Resolution 2.7 Å, [75]) and B) Pdb1f0n (Resolution 1.9 Å, [36]) were modified with 3D molecule viewer (Invitrogen). Respectively only protein chain A is shown, atoms are displayed as space filling balls, "DPAW" is marked in blue, glutamic acid in 85 B is encircled in comparison to glutamine in 85 A.

monomeric antigen conformation. The small size of the antigen (~5 nm diameter, [36]) may be responsible for this finding. Considering the length of a human scFv of ~4.5 nm [37] and the Fc-mediated homodimerisation of scFv-Fc fusions [34,61], sterical inhibition by the capture antibody is possible. The commercially available immunochromatographic assays for MPT64 antigen detection in Mtb cultures are sandwich assays with monoclonal antibodies [62]. The Capilia TB Test Kit [63] uses only one monoclonal antibody for a sandwich assay, indicating multimer detection. Recombinant antigen MPT64 showed multimerization by disulphide bonds in two unrelated studies [64,65] and no difference in immunogenicity compared to native antigen [64,66]. These findings imply multimerization of native MPT64 and that these aggregates are detected in sandwich assays. Detection of 85 B in culture filtrates was only possible with MFU50-A10 in direct ELISA and immunoblot. MFU50-A10 was cross reactive with 85 A and 85 D in ELISA and immunoblotting. It has been reported to cross react with 85 C by Landowski et al. [45].

Conclusions

This work identifies the first recombinant human scFv antibodies specific for antigen 85 B selected by phage display from naïve antibody gene libraries (HAL7/8). These antibodies allow the discrimination between 85 complex proteins, and showed suitability for the establishment of different assays for Mtb 85 B detection. Antigen conformation influenced by sample treatment was shown to be important. The presented antibodies are candidates for the future development of a POC TB diagnostic kit. However, for this purpose an affinity maturation of the antibodies would be necessary to improve the sensitivity.

Material and methods

All chemicals used were p.a. purity grade and purchased from Sigma Aldrich, Merck or Carl Roth (all located in Germany) or as indicated otherwise.

Antigen purification

Genes *fbpA*, B, D coding for proteins of antigen 85 complex of *M. tuberculosis* were amplified by PCR and inserted into pET expression vectors (Novagen, Germany) by site directed cloning (Table 3).

Exchanges L78Q and S196T within the derived amino acid sequence of 85B, and exchange F54L within 85C were detected by DNA sequencing.

Recombinant 85A was produced in *E. coli* strain BL21 (DE3) [pLysS] (Novagen, Germany) by growth in LB [67] containing 400 µg/mL ampicillin and 34 µg/mL chloramphenicol. Expression was induced by addition of 1 mM IPTG at mid-log-phase. Cells were harvested 4 hours after induction. 85 B and 85 C were produced using BL21(DE3) (Novagen, Germany) as host strains grown in LB containing 50 µg/mL kanamycin. After induction with 1 mM IPTG at mid-log-phase, cells were incubated overnight and then harvested.

Table 3 Cloned genes of *M. tuberculosis* antigen 85 complex and properties of the recombinant gene products

Gene	Vector	Gene product		
		Antigen	Signal sequence	His-tag
<i>fbpA</i> (Rv3804c)	pET26b	85 A	No	N-terminal
<i>fbpB</i> (Rv1886c)	pET22b	85 B	Yes	no
<i>fbpD</i> (Rv3803c)	pET28c	85 D	Yes	N-terminal

85 B purification

Cells were suspended in 10 mM tris/HCl, pH 8.0 containing 1% Triton X-100 and 10 mM EDTA (10 mL per g wet weight). After cell disruption by sonification and centrifugation the pellet was washed once using the same buffer. The pellet was denatured in 20 mM tris/HCl, pH 8.0, 1% Triton X-100 buffer containing 8 M urea and 2 mM DTT.

After centrifugation the supernatant containing the denatured 85 B antigen was bound on a Q-Sepharose High Performance column (GE Healthcare) and eluted by a linear gradient from 0 to 500 mM NaCl. Fractions containing 85 B antigen were pooled and underwent a buffer exchange step on a Sephadex G25 fine column (GE Healthcare) into the buffer described above without Triton X-100. The protein containing pool was subjected to another chromatography step on a Q-Sepharose High Performance column (GE Healthcare). Again protein elution was performed by a linear gradient from 0 – 500 mM NaCl. Pure antigen 85 B containing fractions were pooled and underwent a final refolding and buffer exchange step on a Sephadex G25 fine column (GE Healthcare) into 10 mM NH₄HCO₃, 100 μM DTT, pH 7.9.

The final protein preparation is a mixture of protein containing the signal sequence and lacking the signal sequence. Approximately 90% of the protein shows the signal sequence and approximately 10% lacks the signal sequence.

85 A purification

Cells were suspended in 20 mM tris/HCl, 100 mM NaCl, pH 8.0 (10 mL per gram wet weight). After cell disruption by sonification and centrifugation, imidazole was added to the supernatant to a final concentration of 5 mM. This solution was applied on a Ni-NTA Superflow column (GE Healthcare) and eluted by a linear gradient from 0 to 500 mM imidazole. Fractions containing 85 A antigen were pooled and underwent a buffer exchange step on a Sephadex G25 fine column (GE Healthcare) into 20 mM tris/HCl, pH 8.0. Chromatography using a Q-Sepharose High Performance column (GE Healthcare) was performed on the pooled protein sample. Protein elution was performed using a linear gradient from 0 – 500 mM NaCl. Pure antigen 85 A containing fractions were pooled and underwent a final buffer exchange step on a Sephadex G25 fine column (GE Healthcare) into 10 mM NH₄HCO₃, pH 7.9.

85 D purification

Cells were suspended in 20 mM tris/HCl, 100 mM NaCl, pH 8.0 containing 1% Triton X-100 (10 mL per gram wet weight). After cell disruption by sonification and centrifugation the pellet was washed once using 20 mM tris/HCl, 100 mM NaCl, pH 8.0 containing 1% Triton X-100. Afterwards the pellet was denatured in 20 mM tris/

HCl, 100 mM NaCl, pH 8.0 containing 8 M urea. After centrifugation the supernatant containing the denatured 85 D antigen underwent a refolding step on a Sephadex G25 fine column (GE Healthcare) into 20 mM tris/HCl, 100 mM NaCl, pH 8.0. The protein peak of this refolding step was collected and then bound on a Q-Sepharose High Performance column (GE Healthcare). Protein elution was performed by a linear gradient from 0 to 300 mM NaCl. Fractions containing pure 85 D antigen were pooled and underwent a buffer exchange step on a Sephadex G25 fine column (GE Healthcare) into 10 mM NH₄HCO₃, pH 7.9. For final endotoxin removal the protein solution was passed four times over a 1 mL Profos endotrap red column (Hyglos GmbH).

Selection of human antibodies using phage display

ScFv were isolated in vitro from the human naïve phage libraries HAL7/8 by panning on decreasing amounts (10, 3 and 1 μg) of immobilized recombinant antigen 85 B according to [68].

Production of soluble scFv antibodies for screening ELISA

The identification of monoclonal binders was performed as described in [69] with the following modifications: 96-well polypropylene (PP) micro titer plates (Greiner, Germany) containing 150 μL 2xYT-G/A (2xYT [67] supplemented with 100 mM glucose and 100 μg/mL ampicillin) were inoculated with colonies from the titration plate of the third panning round. Micro titer plates (MTP) were incubated overnight at 37°C and 850 rpm in a MTP shaker (PST-60HL-4, Lab4you, Austria). A volume of 180 μL 2xYT-G/A per well in PP-MTP was inoculated with 10 μL of the overnight culture and grown at 37°C and 850 rpm for two hours. Bacteria were harvested by centrifugation for 10 min at 3,220 × g and 180 μL supernatant were removed. The pellets were resuspended in 180 μL 2xYT supplemented with 50 mM sucrose and 100 μg/mL ampicillin + 50 μM IPTG and incubated at 30°C and 850 rpm overnight. Bacteria were pelleted by centrifugation for 15 min at 3,220 × g and 4°C. The scFv-containing supernatant was transferred to a new PP-MTP and stored at 4°C prior to analysis.

Screening ELISA

96 wells of Microolon MTP (Greiner, Germany) were coated with 100 ng of recombinant 85 B in 100 μL PBS buffer pH 7.4 or BSA in 100 μL PBS buffer pH 7.4 (Carl Roth, Germany) as a negative control [67] overnight at 4°C. After coating, the wells were blocked with 300 μL PBST-B (PBS supplemented with 0.1% (w/v) Tween-20 and 1% (w/v) BSA) for 2 h at RT. This was followed by three washing steps with PBST0.05 (PBS supplemented with 0.05% (w/v) Tween-20). For identification of binders, supernatants containing monoclonal scFv were incubated

in the antigen coated plates for 1.5 h at RT followed by three PBST washing cycles. Bound scFv were detected using mouse α -c-Myc-tag 9E10 (culture supernatant, 100 μ L, 1:1,000 in PBST-B; 1.5 h at RT) followed by goat α -mouse IgG (Fc)-HRP (A0168, Sigma-Aldrich, Germany) (100 μ L, 1:30,000 in PBST-B; 45 min at RT). After three washing steps with PBST0.05 the reactions were visualized with 100 μ L 3,3',5,5'-tetramethylbenzidine (TMB, Seramun, Germany) as a substrate. The staining reaction was terminated by addition of 100 μ L 0.2 M H₂SO₄. Absorbance at 450 nm (620 nm reference) was measured using MRX ELISA reader (Dynatec, Germany).

Antibody titration ELISA

For each antibody, 24 wells of Greiner Microlon MTP were coated with 100 ng antigen in PBS at 4°C overnight. BSA was coated as a negative control. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST0.05. Twelve dilutions of antibody (differing, depending on the antibody) in PBST-B were applied in duplicates on the antigen and BSA controls and incubated for 1.5 h at RT. Bound scFv-Fc were detected using goat α -human IgG (Fc)-HRP (A0170, Sigma-Aldrich, Germany) (100 μ L, 1:130,000 in PBST-B; 45 min at RT). The assay was further processed as described above.

Antigen titration ELISA

Antigen in PBS at twelve dilutions (1 μ g/mL to 0.5 ng/mL) were coated in duplicates to wells of Greiner Microlon MTP at 4°C overnight. BSA coated wells were used as a negative control. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST. Antigen detection was carried out with a concentration of antibody at half maximal saturation (determined by antibody titration ELISA) in PBST-B for 1.5 h at RT followed by three PBST washing cycles. Bound scFv-Fc were detected using goat α -human IgG (Fc)-HRP (100 μ L, 1:130,000 in PBST-B; 45 min at RT). The assay was further processed as described above.

Direct ELISA

Analyte (antigen or antibody) was coated to the surface of Greiner Microlon 96 Well MTP at various concentrations in PBS buffer overnight at 4°C. Detection of antigen was carried out with an scFv-Fc antibody conjugated to HRP (by EZ-Link Plus Activated Peroxidase Kit, Pierce, Germany, according to the manufacturer's instructions) diluted in PBST-B for 1 h at RT. Detection of antibody was carried out with Goat α -human IgG (Fc)-HRP diluted (100 μ L, 1:130,000 in PBST-B) 45 min at RT. The assay was further processed as described above.

Sandwich antigen titration

100 ng of capture antibodies were coated to the surface of 96 wells of Greiner Microlon MTP in PBS buffer overnight at 4°C. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST. Twelve dilutions of antigen in PBST-B were applied in duplicates onto the antibody coated wells and incubated for 1.5 h at RT, followed by three washing steps with PBST. Detection of bound antigen was performed with HRP conjugated scFv-Fc (exact dilutions described in results) in PBST-B for 1.5 h at RT, followed by three washing steps with PBST. The assay was further processed as described above.

Epitope Mapping

The protein sequence of 85 B (Rv1886c, UniProt ID P0C5B9, sequence source: <http://genolist.pasteur.fr/TubercuList/>) was divided into overlapping peptide fragments, each consisting of 15 amino acids, with an offset of three amino acids. This array of peptides was synthesized by the SPOT technique [70,71] on an amino-pegylated cellulose membrane (AIMS Scientific Products GmbH, Wolfenbüttel, Germany) as described previously [72]. Peptides are N-terminally acetylated and remain covalently attached to the membrane via their carboxy-terminus. The membrane bound peptide array was probed with the α -85 B antibodies for binding according to established procedures [30,72].

Immunoblot

Proteins were separated on 12% SDS-PAGE [73,74] and semi-dry blotted onto polyvinylidene fluoride (PVDF) membranes (Carl Roth, Germany) according to the manufacturer's instructions. Blocking was performed with MPBST (PBST0.1 supplemented with 2% (w/v) dry milk) for minimum 30 min at RT. Subsequent incubation with protein-specific antibody was carried out in MPBST for a minimum of 60 min at RT. Three washing steps with PBST0.1 for 5 min were performed. The PVDF membrane was incubated with a secondary antibody coupled to HRP in MPBST for at least 45 min at RT. The blot was washed another three times with PBST0.1 and then developed with TMB peroxidase membrane substrate (Seramun, Germany) until an adequate signal was obtained. Development was stopped by three short washing steps with deionized water.

LFIA assembly + execution

5 – 40 μ L cm⁻¹ of colloidal gold-antibody conjugates were dispensed onto 8 mm glass fibre pads (Millipore, Germany) using the xyz-dispenser (Biodot, USA) and dried at 37°C. 1 μ L cm⁻¹ of different concentrations of capture antibody solution (sandwich assay) or antigen (direct assay) in PBS were dispensed in a line onto Unisart CN 95 nitrocellulose

membranes (20 mm, Sartorius, Germany) that were already assembled onto 300 mm backing cards (Jieyi Biotechnology, China), and dried at 37°C. The glass fibre conjugate pads were laminated onto the backing cards, overlapping the nitrocellulose membrane at the connection point for ~0.5 – 1 mm. Then cellulose fibre pads (Millipore, Germany) were laminated onto the backing cards as sample and wicking pad, overlapping the nitrocellulose membrane at the connection point for ~0.5 – 1 mm. The assembled cards were cut into strips of 0.4 cm width with the CM4000 guillotine-cutter (Biodot) and the test strips were placed into Lateral Flow Strip Test (LFST) cassettes (Jieyi Biotechnology, China).

Several experiments were carried out to determine a procedure that would allow sensitive antigen detection and a low background combined with good feasibility (data not shown). The best option for a manageable LFIA was to add a diluent to the sample before applying it to the sample well. Therefore 1/3 sample volume (50 µL) of conjugation buffer (2 mM borax pH 8.8) enriched with 8% (w/v) BSA were mixed with 100 µL sample and applied to the LFIA. After 15 – 20 min results were optically evaluated.

Yumab production + purification

All scFv isolated from antibody-phage display were subcloned into pCSE2.5-hIgG1-Fc-XP and produced as scFv-Fc (yumab) in HEK293-6E cells (National Research Council (NRC), Biotechnological Research Institute (BRI), Montreal, Canada). HEK293-6E cells were cultured in chemical defined F17 medium (Invitrogen, Life Technologies, Darmstadt, Germany) supplemented with 1 g/L pluronic F68 (Appligene, Darmstadt, Germany), 4 mM L-glutamine (PAA) and 25 mg/L G418 (PAA) as previously described [34]. DNA was transiently transfected into 25 mL HEK293-6E cells in 125 mL Erlenmeyer shake flasks. After 48 hours of cultivation at 110 rounds per minute (rpm) in a Minitron orbital shaker (Infors, Bottmingen, Switzerland) at 37°C and 5% CO₂ atmosphere, one volume culture medium and a final concentration of 0.5% (w/v) of tryptone N1 (TN1, Organotechnie S.A.S., La Courneuve, France) were added. ScFv-Fc were purified via UNOsphere SUPRA column (Biorad, Munich, Germany) using a Profinia automate (Biorad, Hercules, California, USA), according to the manufacturer's instructions.

Analytical SEC

For analytical size exclusion chromatography (SEC) a Knauer PLATINblue HPLC Plus system (Knauer, Germany) with a Superdex 200 10/300 GL column (GE Healthcare, USA) was used according to the manufacturer's instructions. The column was equilibrated with the Gel Filtration HMW/LMW Calibration Kits (GE Healthcare, USA) according to the manufacturer's instructions.

Tape Station

Protein solutions were analysed with the Screen Tape P200 Protein Standard Kit (Agilent, USA) under reducing conditions on a 2200 Tape Station system (Agilent, USA) according to the manufacturer's instructions. The "P200 molecular weight standard" (Agilent) was used as a molecular marker.

Additional file

Additional file 1: Figure S1. Screening ELISA for 85 B binding scFv in HAL7/8. Culture supernatants containing soluble scFv of 92 single clones (3. panning round) were screened for their ability to bind antigen (85 B) and BSA (negative control). On positions H9 and H12 an anti-lysozyme antibody was used on lysozyme as control for scFv production and ELISA detection system. Detection of bound scFv with mouse α-c-Myc-tag 9E10 IgG followed by goat α-mouse IgG(Fc)-HRP, development with TMB.

Competing interests

The authors have no competing financial or non-financial interests. This study was financially supported by the German Federal Ministry of Commerce, Grant No. KF 2584301. All aspects of cooperation and the generated results by the partner organisations have been mentioned in the cooperation agreement of the grant as required by the German Federal Ministry of Commerce. We did not receive any other financial or personal contribution by any other third party. Currently, there are no plans to file a patent. Following the rules of the German Federal Ministry of Commerce, LIONEX reserves its rights to develop any product based on the results or materials of this manuscript but agrees to provide the materials to research organisations only, exclusively for research use.

Authors' contributions

MF performed most of the experiments, participated in the design of the study, analysed data and drafted the manuscript. SK, SH, WP, RS, WO and RF performed some of the experiments, analysed data and provided material. SD participated in the design of the study and analysed data. MS and MH conceived the project and wrote the grant application, participated in the design of the study, analysed data and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We kindly acknowledge the technical support of Susanne Daenicke and Franziska Resch. Further, we acknowledge the financial support of ZIM BMBF (contract No. KF 2584301). We also thank Elizabeth O'Brien for the careful correction of this manuscript.

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Received: 22 April 2014 Accepted: 1 July 2014

Published: 17 July 2014

References

1. WHO: *Global Tuberculosis Report 2012*. Geneva, Switzerland: 2012.
2. Dye C, Scheele S, Dolin P, Pathania V, Ravignone MC: **Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project.** *JAMA* 1999, **282**:677–686.
3. Kumar V, Robbins SL: *Robbins basic pathology*. 8th edition. Philadelphia, PA: Saunders/Elsevier; 2007.
4. Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, Kop J, Owens MR, Rodgers R, Banada P, Safi H, Blakemore R, Lan NTN, Jones-Lopez EC, Levi M,

- Burday M, Ayakaka I, Mugerwa RD, McMillan B, Winn-Deen E, Christel L, Dailey P, Perkins MD, Persing DH, Alland D: **Rapid Detection of Mycobacterium tuberculosis and Rifampin Resistance by Use of On-Demand, Near-Patient Technology.** *J Clin Microbiol* 2010, **48**:229–237.
5. Rai DR, Kshetry NT, Bhargava D, Pokhrel BM: **Comparison of Ziehl-Neelsen staining microscopy and immunochromatographic tuberculosis test for diagnosis of pulmonary tuberculosis.** *J Inst Med* 2006, **28**:15–18.
6. van Deun A, Portaels F: **Limitations and requirements for quality control of sputum smear microscopy for acid-fast bacilli.** *Int J Tuberc Lung Dis* 1998, **2**:756–765.
7. Steingart KR, Ng V, Henry M, Hopewell PC, Ramsay A, Cunningham J, Urbanczik R, Perkins MD, Aziz MA, Pai M: **Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review.** *Lancet Infect Dis* 2006, **6**:664–674.
8. Perkins MD, Cunningham J: **Facing the crisis: improving the diagnosis of tuberculosis in the HIV era.** *J Infect Dis* 2007, **196**(Suppl 1):S15–S27.
9. Dheda K, Ruhwald M, Theron G, Peter J, Yam WC: **Point-of-care diagnosis of tuberculosis: Past, present and future.** *Respirology* 2013, **18**:217–232.
10. WHO: *An International Roadmap for Tuberculosis Research.* Geneva, Switzerland: WHO; 2011.
11. Flores LL, Steingart KR, Dendukuri N, Schiller I, Minion J, Pai M, Ramsay A, Henry M, Laal S: **Systematic Review and Meta-Analysis of Antigen Detection Tests for the Diagnosis of Tuberculosis.** *Clin Vaccine Immunol* 2011, **18**:1616–1627.
12. Wiker HG, Harboe M: **The Antigen 85 Complex: a Major Secretion Product of Mycobacterium tuberculosis.** *Microbiol Rev* 1992, **56**:648–661.
13. Sonnenberg MG, Belisle JT: **Definition of Mycobacterium tuberculosis culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry.** *Infect Immun* 1997, **65**:4515–4524.
14. Wiker HG, Sletten K, Nagai S, Harboe M: **Evidence for three separate genes encoding the proteins of the mycobacterial antigen 85 complex.** *Infect Immun* 1990, **58**:272–274.
15. Kremer L, Maughan W, Wilson R, Dover L, Besra GS: **The M. tuberculosis antigen 85 complex and mycolyltransferase activity.** *Lett Appl Microbiol* 2002, **34**:233–237.
16. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS: **Role of the major antigen of Mycobacterium tuberculosis in cell wall biogenesis.** *Science* 1997, **276**:1420–1422.
17. Armitige LY, Jagannath C, Wanger AR, Norris SJ: **Disruption of the genes encoding antigen 85A and antigen 85B of Mycobacterium tuberculosis H37Rv: effect on growth in culture and in macrophages.** *Infect Immun* 2000, **68**:767–778.
18. Abou-Zeid C, Ratliff T, Wiker H, Harboe M: **Characterization of Fibronectin-Binding Antigens Released by Mycobacterium tuberculosis and Mycobacterium bovis BCG.** *Infect Immun* 1988, **56**:3046–3051.
19. Abou-Zeid C, Garbe T, Lathiga R, Wiker HG, Harboe M, Rook G, Young B: **Genetic and Immunological Analysis of Mycobacterium tuberculosis Fibronectin-Binding Proteins.** *Infect Immun* 1991, **59**:2712–2718.
20. Bentley-Hibbert SJ, Quan X, Newman T, Huygen K, Godfrey HP: **Pathophysiology of antigen 85 in patients with active tuberculosis: antigen 85 circulates as complexes with fibronectin and immunoglobulin G.** *Infect Immun* 1999, **67**:581–588.
21. Drowart A, de Bruyn J, Huygen K, Damiani G, Godfrey HP, Stelandre M, Yernault JC, Van Vooren JP: **Isoelectrophoretic characterization of protein antigens present in mycobacterial culture filtrates and recognized by monoclonal antibodies directed against the Mycobacterium bovis BCG antigen 85 complex.** *Scand J Immunol* 1992, **36**:697–702.
22. Ferrara F, Naranjo LA, Kumar S, Gaiotto T, Mukundan H, Swanson B, Bradbury AR, Andrew RM: **Using phage and yeast display to select hundreds of monoclonal antibodies: application to antigen 85, a tuberculosis biomarker.** *PLoS One* 2012, **7**:e49535.
23. Mukundan H, Kumar S, Price DN, Ray SM, Lee Y, Min S, Eum S, Kubicek-Sutherland J, Resnick JM, Grace WK, Anderson AS, Hwang SH, Cho SN, Via LE, Barry C3, Sakamuri R, Swanson BI: **Rapid detection of Mycobacterium tuberculosis biomarkers in a sandwich immunoassay format using a waveguide-based optical biosensor.** *Tuberculosis (Edinb)* 2012, **92**:407–416.
24. Kashyap RS, Rajan AN, Ramteke SS, Agrawal VS, Kelkar SS, Purohit HJ, Taori GM, Dagainawala HF: **Diagnosis of tuberculosis in an Indian population by an indirect ELISA protocol based on detection of Antigen 85 complex: a prospective cohort study.** *BMC Infect Dis* 2007, **7**:74.
25. Kashyap RS, Dobos KM, Belisle JT, Purohit HJ, Chandak NH, Taori GM, Dagainawala HF: **Demonstration of components of antigen 85 complex in cerebrospinal fluid of tuberculous meningitis patients.** *Clin Diagn Lab Immunol* 2005, **12**:752–758.
26. Wallis RS, Perkins M, Phillips M, Joloba M, Demchuk B, Namale A, Johnson JL, Williams D, Wolski K, Teixeira L, Dietze R, Mugerwa RD, Eisenach K, Ellner JJ: **Induction of the antigen 85 complex of Mycobacterium tuberculosis in sputum: a determinant of outcome in pulmonary tuberculosis treatment.** *J Infect Dis* 1998, **178**:1115–1121.
27. Kirsch MI, Hülseweh B, Nacke C, Rülker T, Schirrmann T, Marschall HJ, Hust M, Dübel S: **Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV).** *BMC Biotechnol* 2008, **8**:66.
28. Meyer T, Stratmann-Selke J, Meens J, Schirrmann T, Gerlach GF, Frank R, Dübel S, Strutzberg-Minder K, Hust M: **Isolation of scFv fragments specific to OmpD of Salmonella Typhimurium.** *Vet Microbiol* 2011, **147**:162–169.
29. Meyer T, Schirrmann T, Frenzel A, Miethel S, Stratmann-Selke J, Gerlach GF, Strutzberg-Minder K, Dübel S, Hust M: **Identification of immunogenic proteins and generation of antibodies against Salmonella Typhimurium using phage display.** *BMC Biotechnol* 2012, **12**:29.
30. Schütte M, Thullier P, Pelat T, Wezler X, Rosenstock P, Hinz D, Kirsch MI, Hasenberg M, Frank R, Schirrmann T, Gunzer M, Hust M, Dübel S: **Identification of a Putative Crf Splice Variant and Generation of Recombinant Antibodies for the Specific Detection of Aspergillus fumigatus.** *PLoS One* 2009, **4**:e6625.
31. Chan C, Zhao B, Cazenave-Gassiot A, Pang S, Bendt A, Wenk M, Macary P, Hanson B: **Novel phage display-derived mycolic acid-specific antibodies with potential for tuberculosis diagnosis.** *J Lipid Res* 2013, **54**:2924–2932.
32. Lillo AM, Ayris JE, Shou Y, Graves SW, Bradbury AR: **Development of phage-based single chain Fv antibody reagents for detection of Yersinia pestis.** *PLoS One* 2011, **6**:e27756.
33. Hust M, Meyer T, Voedisch B, Rülker T, Thie H, El-Ghezal A, Kirsch MI, Schütte M, Helmsing S, Meier D, Schirrmann T, Dübel S: **A human scFv antibody generation pipeline for proteome research.** *J Biotechnol* 2011, **152**:159–170.
34. Jäger V, Büsow K, Wagner A, Weber S, Hust M, Frenzel A, Schirrmann T: **High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells.** *BMC Biotechnol* 2013, **13**:52.
35. Retter I, Althaus H, Münch R, Müller W: **VBASE2, an integrative V gene database.** *Nucleic Acids Res* 2004, **33**:D671.
36. Anderson DH, Harth G, Horwitz MA, Eisenberg D: **An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the Mycobacterium tuberculosis 30 kda major secretory protein (antigen 85B), a mycolyl transferase.** *J Mol Biol* 2001, **307**:671–681.
37. Boehm MK, Woof JM, Kerr MA, Perkins SJ: **The Fab and Fc fragments of IgA1 exhibit a different arrangement from that in IgG: a study by X-ray and neutron solution scattering and homology modelling.** *J Mol Biol* 1999, **286**:1421–1447.
38. Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, Duvaud S, Flegel V, Fortier A, Gasteiger E, Grosdidier A, Hernandez C, Loannidis V, Kuznetsov D, Liechti R, Moretti S, Mostaguir K, Redaschi N, Rossier G, Xenarios I, Stockinger H: **ExpASY: SIB bioinformatics resource portal.** *Nucleic Acids Res* 2012, **40**:W597.
39. Bekmurzayeva A, Sypabekova M, Kanayeva D: **Tuberculosis diagnosis using immunodominant, secreted antigens of Mycobacterium tuberculosis.** *Tuberculosis* 2013, **93**:381–388.
40. Lawn SD: **Point-of-care detection of lipoarabinomannan (LAM) in urine for diagnosis of HIV-associated tuberculosis: a state of the art review.** *BMC Infect Dis* 2012, **12**:103.
41. Frenzel A, Fröde D, Meyer T, Schirrmann T, Hust M: **Generating Recombinant Antibodies for Research, Diagnostics and Therapy Using Phage Display.** *Curr Biotechnol* 2012, **1**:33–41.
42. Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellenhofer G, Hoess A, Wolle J, Plückthun A, Virnekas B: **Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides.** *J Mol Biol* 2000, **296**:57–86.
43. Loset GA, Lobersli I, Kavlie A, Stacy JE, Borgen T, Kausmally L, Hvattum E, Simonsen B, Hovda MB, Brekke OH: **Construction, evaluation and refinement of a large human antibody phage library based on the IgD and IgM variable gene repertoire.** *J Immunol Methods* 2005, **299**:47–62.
44. Schofield DJ, Pope AR, Clementel V, Buckell J, Chapple SD, Clarke KF, Conquer JS, Crofts AM, Crowther SR, Dyson MR, Flack G, Griffin GJ, Hooks Y,

- Howat WJ, Kolb-Kokocinski A, Kunze S, Martin CD, Maslen GL, Mitchell JN, O'Sullivan M, Perera RL, Roake W, Shadbolt SP, Vincent KJ, Warford A, Wilson WE, Xie J, Young JL, McCafferty J: **Application of phage display to high throughput antibody generation and characterization.** *Genome Biol* 2007, **8**:R254.
45. Landowski CP, Godfrey HP, Bentley-Hibbert SI, Liu X, Huang Z, Sepulveda R, Huygen K, Gennaro ML, Moy FH, Lesley SA, Haak-Frendscho M: **Combinatorial Use of Antibodies to Secreted Mycobacterial Proteins in a Host Immune System-Independent Test for Tuberculosis.** *J Clin Microbiol* 2001, **39**:2418–2424.
46. Kremer L, Baulard A, Estaquier J, Poulain-Godefroy O, Lochet C: **Green fluorescent protein as a new expression marker in mycobacteria.** *Mol Microbiol* 1995, **17**:913–922.
47. Huygen K, Lozes E, Gilles B, Drowart A, Palfiet K, Jurion F, Roland I, Art M, Dufaux M, Nyabenda J: **Mapping of TH1 helper T-cell epitopes on major secreted mycobacterial antigen 85A in mice infected with live *Mycobacterium bovis* BCG.** *Infect Immun* 1994, **62**:363–370.
48. Launois P, DeLays R, Niang MN, Drowart A, Andrien M, Dierckx P, Cartel JL, Sarthou JL, Van Vooren JP, Huygen K: **T-cell-epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy.** *Infect Immun* 1994, **62**:3679–3687.
49. Roche PW, Peake PW, Billman-Jacobe H, Doran T, Britton WJ: **T-cell determinants and antibody binding sites on the major mycobacterial secretory protein MPB59 of *Mycobacterium bovis*.** *Infect Immun* 1994, **62**:5319–5326.
50. Commandeur S, Lin MY, van Meijgaarden KE, Friggen AH, Franken KL, Drijfhout JW, Korsvold GE, Oftung F, Geluk A, Ottenhoff TH: **Double- and monofunctional CD4(+) and CD8(+) T-cell responses to *Mycobacterium tuberculosis* DosR antigens and peptides in long-term latently infected individuals.** *Eur J Immunol* 2011, **41**:2925–2936.
51. Shen G, Behera D, Bhalla M, Nadas A, Laal S: **Peptide-based antibody detection for tuberculosis diagnosis.** *Clin Vaccine Immunol* 2009, **16**:49–54.
52. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**:403–410.
53. Lightbody KA, Girvin RM, Pollock DA, Mackie DP, Neill SD, Pollock JM: **Recognition of a common mycobacterial T-cell epitope in MPB59 of *Mycobacterium bovis*.** *Immunology* 1998, **93**:314–322.
54. Valle MT, Megiovanni AM, Merlo A, Li Pira G, Bottone L, Angelini G, Bracci L, Lozzi L, Huygen K, Manca F: **Epitope focus, clonal composition and Th1 phenotype of the human CD4 response to the secretory mycobacterial antigen Ag85.** *Clin Exp Immunol* 2001, **123**:226–232.
55. D'Souza S, Rosseels V, Romano M, Tanghe A, Denis O, Jurion F, Castiglione N, Vanonckelen A, Palfiet K, Huygen K: **Mapping of murine Th1 helper T-Cell epitopes of mycolyl transferases Ag85A, Ag85B, and Ag85C from *Mycobacterium tuberculosis*.** *Infect Immun* 2003, **71**:483–493.
56. Murphy K, Travers P, Walport M, Janeway C: *Janeway's immunobiology*. 7th edition. New York: Garland Science; 2008.
57. Aron R: **Enzyme inhibition by antibodies.** *Acta Endocrinol Suppl (Copenh)* 1975, **194**:133–153.
58. Mustafa AS, Abal AT, Shaban F, El-Shamy AM, Amoudy HA: **HLA-DR binding prediction and experimental evaluation of T-cell epitopes of mycolyl transferase 85B (Ag85B), a major secreted antigen of *Mycobacterium tuberculosis*.** *Med Princ Pract* 2005, **14**:140–146.
59. Gaseitsiwe S, Valentini D, Mahdaviifar S, Magalhaes I, Hoft DF, Zerweck J, Schutkowski M, Andersson J, Reilly M, Maeurer MJ: **Pattern recognition in pulmonary tuberculosis defined by high content peptide microarray chip analysis representing 61 proteins from *M. tuberculosis*.** *PLoS One* 2008, **3**:e3840.
60. Nguyen YH, Ma X, Qin L: **Rapid identification and drug susceptibility screening of ESAT-6 secreting *Mycobacteria* by a NanoELIwell assay.** *Sci Rep* 2012, **2**:635.
61. Powers DB, Amersdorfer P, Poul M, Nielsen UB, Shalaby MR, Adams GP, Weiner LM, Marks JD: **Expression of single-chain Fv-Fc fusions in *Pichia pastoris*.** *J Immunol Methods* 2001, **251**:123–135.
62. Yin X, Zheng L, Lin L, Hu Y, Zheng F, Hu Y, Wang Q: **Commercial MPT64-based tests for rapid identification of *Mycobacterium tuberculosis* complex: A meta-analysis.** *J Infect* 2013, **67**:369–377.
63. Ngamlert K, Sinthuwattanawibool C, McCarthy KD, Sohn H, Starks A, Kanjanamongkolsiri P, Anek-vorapong R, Tasaneeyapan T, Monkongdee P, Diem L, Varma JK: **Diagnostic performance and costs of Capilia TB for *Mycobacterium tuberculosis* complex identification from broth-based culture in Bangkok, Thailand.** *Trop Med Int Health* 2009, **14**:748–753.
64. Geisbrecht BV, Nikonenko B, Samala R, Nakamura R, Nacy CA, Sacksteder KA: **Design and optimization of a recombinant system for large-scale production of the MPT64 antigen from *Mycobacterium tuberculosis*.** *Protein Expr Purif* 2006, **46**:64–72.
65. Chu TJ, Yuann JP: **Expression, purification, and characterization of protective MPT64 antigen protein and identification of its multimers isolated from nontoxic *Mycobacterium tuberculosis* H37Ra.** *Biotechnol Appl Biochem* 2011, **58**:185–189.
66. Oettinger T, Holm A, Haslov K: **Characterization of the delayed type hypersensitivity-inducing epitope of MPT64 from *Mycobacterium tuberculosis*.** *Scand J Immunol* 1997, **45**:499–503.
67. Sambrook J, Russell D: *Molecular Cloning: A Laboratory Manual*. 3rd edition. New York: Cold Spring Harbor Laboratory Press; 2001.
68. Schirrmann T, Hust M: **Construction of human antibody gene libraries and selection of antibodies by phage display.** *Methods Mol Biol* 2010, **651**:177–209.
69. Hust M, Steinwand M, Al-Halabi L, Helmsing S, Schirrmann T, Dübel S: **Improved microtitre plate production of single chain Fv fragments in *Escherichia coli*.** *N Biotechnol* 2009, **25**:424–428.
70. Frank R: **Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support.** *Tetrahedron* 1992, **48**:9217–9232.
71. Frank R: **The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports—principles and applications.** *J Immunol Methods* 2002, **267**:13–26.
72. Frank R, Dübel S: **Analysis of protein interactions with immobilized peptide arrays synthesized on membrane supports.** In *Protein-Protein Interactions, A Molecular Cloning Manual*. 2nd edition. Edited by Golemis E, Adams PD. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2005.
73. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227**:680–685.
74. Schägger H, von Jagow G: **Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa.** *Anal Biochem* 1987, **166**:368–379.
75. Ronning DR, Vissa V, Besra GS, Belisle J, Sacchettini JC: ***Mycobacterium tuberculosis* Antigen 85A and 85C Structures Confirm Binding Orientation and Conserved Substrate Specificity.** *J Biol Chem* 2004, **279**:36771–36777.

doi:10.1186/1472-6750-14-68

Cite this article as: Fuchs et al.: Novel human recombinant antibodies against *Mycobacterium tuberculosis* antigen 85B. *BMC Biotechnology* 2014 14:68.

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