Functional Analysis of Early Secreted Antigenic Target-6, the Dominant T-cell Antigen of *Mycobacterium tuberculosis*, Reveals Key Residues Involved in Secretion, Complex Formation, Virulence, and Immunogenicity *

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Proteins of the 6-kDa early secreted antigenic target (ESAT-6) secretion system-1 of *Mycobacterium tuberculosis* are not only strongly involved in the anti-mycobacterial Th1-host immune response but are also key players for virulence. In this study, protein engineering together with bioinformatic, immunological, and virulence analyses allowed us to pinpoint regions of the ESAT-6 molecule that are critical for its biological activity in *M. tuberculosis*. Mutation of the Trp-Xaa-Gly motif, conserved in a wide variety of ESAT-6-like proteins, abolished complex formation with the partner protein CFP-10, induction of specific T-cell responses, and virulence. Replacement of conserved Leu residues interfered with secretion, coiled-coil formation, and virulence, whereas certain mutations at the extreme C terminus did not affect secretion but caused attenuation, possibly because of altered ESAT-6 targeting or trafficking. In contrast, the mutation of several residues on the outer surface of the four-helical bundle structure of the ESAT-6-CFP-10 complex showed much less effect. Construction of recombinant BCG expressing ESAT-6 with a C-terminal hexahistidine tag allowed us to co-purify ESAT-6 and CFP-10, experimentally confirming their strong interaction both in and outside of the mycobacterial cell. The strain induced potent, antigen-specific T-cell responses and intermediate *in vivo* growth in mice, suggesting that it remained immunogenic and biologically active despite the tag. Together with previous NMR data, the results of this study have allowed a biologically relevant model of the ESAT-6-CFP-10 complex to be constructed that is critical for understanding the structure-function relationship in tuberculosis pathogenesis.

Several attenuated or avirulent members of the *Mycobacterium tuberculosis* complex, like *Mycobacterium bovis* BCG (BCG), the vole bacillus *Mycobacterium microti*, or the dassie bacillus, are deleted for an overlapping portion of the genome, known as region of difference 1 (RD1) 1–3. This segment is localized close to the origin of replication (4) in all fully virulent members of the complex (5) and harbors genes *esxA*, coding for the 6-kDa early secreted antigenic target (ESAT-6), and *esxB*, encoding the 10-kDa culture filtrate protein (CFP-10). The region was recently shown to be required for full virulence of *M. tuberculosis* (6–8) and, when integrated into BCG, improved the ability of the recombinant BCG strain to protect against dissemination of tuberculosis in the mouse and the guinea pig model (9). Independent but complementary studies revealed that ESAT-6 and CFP-10 are secreted via the ESAT-6 system-1 (ESX-1), a dedicated secretion apparatus encoded by genes *esxA* and *esxB*. The surrounding RD1 region (9–11) harbors genes coding for the 6-kDa early secreted antigenic target (ESAT-6), and several putative membrane proteins identified by genes flanking *esxA* and *esxB* in the extended RD1 region (12–14). Among the proteins predicted to be involved in this process are a member of the AAA-family of ATPases (Rp3868), which can form a 1:1 complex in *vitro* (15), and several putative membrane proteins with 1, 3, or 11 transmembrane domains (Rp3869, Rp3870, Rp3877) or ATP binding sites (Rp3871), which could be involved in forming a transmembrane channel for the translocation of the effector molecules.

Although several publications have recently addressed the function of this secretion system (7, 9–14), many questions about the effector proteins remain unanswered. ESAT-6 and CFP-10, which have been shown to form a 1:1 complex in *vitro* (15), belong to a large family of small proteins identified in Gram-positive bacteria. Proteins of this family have a size of ~100 amino acids and are characterized by a conserved motif Trp-Xaa-Gly (WXXG) (16). To further elucidate the structure-function relationship in members of the ESAT-6, or WXG, family, a strategy has been developed to study variants of ESAT-6 in its natural bacterial host. The residues to be modified were selected according to their potential importance for secretion, immunogenicity and virulence. Among them were several amino acids that are highly conserved among ESAT-6 orthologs from different bacterial species. Other regions of interest were the N and C termini of the protein. We were also interested in identifying modifications that would allow the specific binding of biologically active ESAT-6 to selected matrices and modifications that may be used to localize ESAT-6 in cell biological experiments. Here we describe the behavior of these mutant strains in immunogenicity and virulence tests relative to control strains and show how the modifications allowed new features of ESAT-6 and the corresponding secretion system to be discovered.

The abbreviations used are: RD1, region of difference 1; ESAT, early secreted antigenic target; NFκB, nickel-nitrilotriacetic acid; IFN, interferon; GFP, green fluorescent protein.
Experimental Procedures

Genetic Constructs and Mycobacterial Strains—In the present study our aim was to introduce extra sequences or base substitutions into previously used cosmids pRD1–2F9 and amino acid conservation in C. diphtheriae. B. Immunoblot analysis of the 13 different H37RvΔRD1::2F9EsxA mutant strains, showing the cytosol-membrane (CM) and the supernatant (S) fraction using monoclonal anti-ESAT-6 antibody HYB 076-08 (Statens Serum Institute) or polyclonal antibodies. Note that mutation F8I has apparently changed the recognition site for the anti-ESAT-6 monoclonal antibody.

Bioinformatics—Selected sequences taken from the Tuberculist server at the Institut Pasteur were screened for the presence of putative coiled-coil motifs using the MultiCoil software available at the Massachusetts Institute of Technology. Based on the coiled-coil prediction and available experimental evidence, we used the structure of the four-helical-bundle structure of the cytoplasmic domain of a serine chemotaxis receptor (18) to build a model of the ESAT-6 and CFP-10 complex. In this model, the amino acids in position “a” and “d” of the helices in the template were occupied as far as possible by hydrophobic amino acids. In agreement with reported NMR data the two resulting helical hairpins for CFP-10 and ESAT-6 were put side-by-side and antiparallel, with the WXG motif at opposite ends of the complex structure. Structures were refined with a short molecular dynamics/simulated annealing protocol (19), restraining the experimentally known helical elements (Ala8–Gln40 and Ala96–Gly60 for CFP-10; Phe8–Trp43 and Glu49–Ala84 for ESAT-6) (20).

Protein Extraction, Column Purification, and Immunoblotting—Cell-free protein extracts were prepared from early log phase cultures of recombinant strains grown in 7H9 or Sauton’s medium and processed by standard procedures. Culture filtrates were concentrated using a Millipore filter with a 3-kDa cutoff. Western blots and antigen detection were performed as described previously, using anti-ESAT-6 monoclonal antibodies (HYB 076-08) purchased from the Statens Serum Institute (Denmark) and in-house polyclonal antibodies directed against CFP-10 or PPE-68 (12). His-tagged proteins were purified using mini-columns containing 80 μl Ni2+-NTA-agarose. Cell lysates or culture filtrates were passed through the mini-columns then washed with phosphate-buffered saline (pH 7.3) with 300 mM NaCl and 20 mM imidazole. Bound protein was eluted with phosphate-buffered saline containing 300 mM NaCl and 250 mM imidazole. Samples were precipitated with 10% trichloroacetic acid, dissolved in phosphate-buffered saline and separated by SDS-PAGE (15%), before blotting onto a nitrocellulose.
membrane. Monoclonal anti-ESAT-6, anti-HIS or polyclonal CFP-10 antibodies were used for immune detection.

**Virulence Studies and Immunological Assays**—Virulence and immunological assays were done using SCID mice (IFFA Credo, France) for intravenous infection and/or C57BL/6 mice for aerosol infection or subcutaneous vaccination as described previously (12–14).

**RESULTS**

**Mutation of Conserved Residues of ESAT-6 Identifies Biologically Important Amino Acids**—Sequence alignment of ESAT-6 orthologs from *M. tuberculosis* and *M. leprae* revealed that 33 amino acids are conserved between the 2 ESAT-6 molecules, and 13 of these are also conserved in ESAT-6 like proteins from the phylogenetically more distant species *Corynebacterium diphtheriae* (Fig. 1A). Thirteen residues scattered along the *M. tuberculosis* ESAT-6 molecule were selected for mutation in pRD1–2F9 (Fig. 1A), and the resultant cosmids were integrated into a *M. tuberculosis* ΔRD1 strain (H37RvΔRD1) (7). Using our cloning strategy, recombinant *M. tuberculosis* strains that expressed ESAT-6 with one or two amino acid replacements were obtained and screened for secretion of ESAT-6, immunogenicity, and virulence (TABLE ONE).

Among the 13 mutants tested, 7 showed enhanced in vivo growth after 3–4 weeks of infection in SCID mice, as witnessed by splenomegaly and high bacterial counts in lungs and spleen, whereas 5 mutant strains showed characteristics similar to the H37Rv/ΔRD1 strain (H37RvΔRD1) (7). Using our cloning strategy, recombinant *M. tuberculosis* strains were isolated for further analysis of these strains in SCID mice as shown in Fig. 1B. We had previously noticed that an enhanced in vivo growth phenotype was only observed when ESAT-6 was secreted. Thus, these findings suggest that loss of virulence in 4 of the 5 attenuated mutants is not caused by defective secretion but rather because of potential loss of interaction with protein partners or putative receptors. For further analysis of these results we used bioinformatic tools. Most interestingly, when we screened ESAT-6 and CFP-10 for the presence of coiled-coil motives using the MultiCoil program (21), a high probability of multimeric coiled-coil formation was found (supplemental Fig. S1).

These observations together with the results from the virulence assay of mutant strains and structural data from the available literature (15, 20, 22), allowed us to build a model of the ESAT-6/CFP-10 protein complex, which is crucial for the interpretation of the observed effects of ESAT-6 mutations on virulence. As shown in Fig. 2, mutations that abolish virulence concern residues Leu19–Leu20, important for the stability of the hairpin/coiled-coil structure of the molecule, residues Trp83 and Gly85 that constitute the loop and contain the conserved WXG motif, as well as residues Val89 and Phe92 in the extreme C-terminal tail. Similarly, M93I, also located at the extreme C terminus, showed reduced, intermediate virulence. The other tested mutants (T2H, Q4L, M83I) did not abolish the virulence of the recombinant *M. tuberculosis* strains in SCID mice as shown by splenomegaly and different degrees of enhanced in vivo growth rates (TABLE ONE). As shown in Fig. 2, these mutations affect residues in the N- or C-terminal regions (T2H, Q4L, M83I), are predicted to be at the outer surface of the four-helical bundle structure (A14R, Q56A, N67A, M83I), or are conservative in the sense that the mutated residue could probably be accommodated by small rearrangements in the structur...
Together, these results suggest that correct ESAT-6-CFP-10 complex formation is an important requirement for ESX-1-induced increased virulence of M. tuberculosis. To obtain experimental evidence of the protein-protein interaction involved in this process, we genetically engineered recombinant BCG strains that expressed and secreted HIS-tagged ESAT-6 and CFP-10 molecules.

Tagged ESAT-6 and CFP-10 Molecules in BCG Allow Co-purification of Protein Partners—When concentrated culture filtrates of recombinant BCG expressing ESAT-6-HIS at the C terminus were applied to a Ni²⁺-affinity column, two bands of low molecular weight were visible after elution (Fig. 3A). As shown in Fig. 3B, Western blot analyses identified the upper band as CFP-10, and the lower band as ESAT-6-HIS. The 2 molecules, overexpressed as recombinant proteins by Escherichia coli have previously been shown to interact, forming a 1:1 protein complex in in vitro experiments (15, 23). Our results confirm these observations and further demonstrate that in mycobacteria, ESAT-6 and CFP-10 are indeed present as a protein complex. To our knowledge this is the first experimental evidence that these 2 molecules form a tight complex even after being secreted by their proper secretion machinery. We also observed the same binding behavior using samples of the cytosolic fraction of BCG::2F9-EsxA-HIS (Fig. 3B), indicating that the 2 molecules form a tight complex even after being secreted by their proper secretion machinery.

In contrast to the results obtained with the C-terminal HIS-tagged ESAT-6, an N-terminal HIS tag for ESAT-6 was not effective for purification of the protein from BCG by Ni²⁺ affinity chromatography (data not shown), as the N-terminal HIS tag had been removed or cleaved from the ESAT-6 molecule in the cytosol, possibly by the action of an enzyme.

When CFP-10 carrying an N-terminal HIS tag was purified via Ni²⁺ affinity chromatography, ESAT-6 was obtained after elution of bound CFP-10 (Fig. 3A), although the amounts of ESAT-6 co-purified with CFP-10 were lower, probably because of weaker expression of ESAT-6:HIS-CFP-10 in this mutant strain relative to BCG::2F9-EsxA-HIS and/or interference of the N-terminal tag with the binding capacity of CFP-10. However, this experiment confirmed the strong interaction of these 2 molecules both in and outside the mycobacterial cell and the utility of the attached HIS tag for purification of proteins produced by slow growing mycobacteria. Like the ESAT-6-W43R mutant, a double band was observed for the HIS-tagged molecule, induced by the tag.

In contrast to the results obtained with the C-terminal HIS-tagged ESAT-6, an N-terminal HIS tag for ESAT-6 was not effective for purification of the protein from BCG by Ni²⁺ affinity chromatography (data not shown), as the N-terminal HIS tag had been removed or cleaved from the ESAT-6 molecule in the cytosol, possibly by the action of an enzyme.
whether they were exported. One of them, EsxA-EsxR, ESAT-6 fused at its C terminus with EsxR (Rv3019c), was secreted into the supernatant in sufficient amounts to be detected by Western blotting (supplemental Fig. S3B). For this construct, CFP-10 was also found in the supernatant. However, because of the larger size and different structure, much of the fusion protein was retained in the cell wall fraction, where wild type ESAT-6 was not present (supplemental Fig. S3B). Another recombinant protein, ESAT-6 fused to the ESAT-6 orthologue of M. leprae (MLEsxA, ML0049), was strongly expressed, but only very little amounts of the protein were detected in the culture supernatant, suggesting that the structural conformation of this fusion protein did not match the requirements of the secretion machinery. As with EsxA-EsxR, the EsxA-MLEsxA fusion protein was present in the cell wall fraction (data not shown). We also constructed an ESAT-6-C-terminal GFP fusion and obtained recombinant BCG that were characterized by green fluorescent colonies. Western blot analysis of the culture supernatant of this recombinant BCG strain showed a large fusion protein that reacted with the ESAT-6 monoclonal antibody, and infection of native macrophages revealed that the ESAT-6-GFP fusion protein was expressed in the engulfed bacteria (supplemental Fig. S3, C and D). However, this ESAT-6-GFP fusion protein, which is several times larger than ESAT-6-alone, did not induce ESAT-6-specific T-cell responses in C57BL/6 mice and did not increase virulence in SCID mice (data not shown), indicating that ESAT-6 fused to GFP had lost its biological function. For this reason, constructs that only contain small tags are better suited to preserve the biological function of the ESAT-6 molecule and still allow specific purification and detection procedures to be employed.

Analysis of IFN-γ Responses and Virulence Defines Three Groups of Mutant Strains—To monitor the biological effects of the various genetic modifications of ESAT-6, intravenous infection of SCID mice and recording of IFN-γ responses from splenocytes of immunocompetent C57BL/6 mice were used. First, we observed that the tested strains that induced an IFN-γ response to ESAT-6 were also found to cause splenomegaly and enhanced in vivo growth in SCID mice. However, variation in virulence occurred, as for some strains (e.g. BCG::2F9-EsxA-HIS) growth in SCID mice was less pronounced (TABLE ONE, Fig. 4A). A few selected strains were also tested in immunocompetent C57BL/6 mice by aerosol infection. This approach revealed that strain BCG::2F9-EsxA-HIS was able to multiply in the lungs of these mice but to a lesser degree than the BCG::RD1-2F9 control and the BCG::2F9-EsxA-HIS (Fig. 4B). As such, the fusion of a HIS tag to the C-terminal end of ESAT-6 (BCG::2F9-EsxA-HIS) resulted in intermediate virulence, also observed after intranasal infection (Fig. 4C). The strain, however, retained its capacity to induce IFN-γ responses upon restimulation with ESAT-6 peptides or proteins in C57BL/6 mice (Fig. 4D).

In contrast, the opposite was not always true, as mutants of the second group were virulent in the SCID mouse model, but did not elicit ESAT-6-specific T-cell responses, probably because of changes in the T-cell epitope or conformational changes, possibly interfering with protein processing. Examples of such strains are BCG::2F9-HIS-EsxA, H37RvΔRD1::2F9EsxA-F81 or H37RvΔRD1::2F9EsxA-A14R (TABLE ONE). It is noteworthy that ESAT-6 with the F81 mutation was not recognized by the monoclonal anti-ESAT-6 antibody, whereas a polyclonal anti-ESAT-6 antibody reacted strongly with the mutant protein (Fig. 1B). Studies are underway to determine whether peptides with these changes also fail to stimulate IFN-γ responses in mice.

The third group of strains was the one in which mutation of ESAT-6 abolished both virulence and immunogenicity. This phenomenon was observed for modified H37RvΔRD1 strains containing constructs...
Functional Analysis of ESAT-6

2F9EsxA-L28A/L29S, 2F9EsxA-W43R, 2F9EsxA-G45T, 2F9EsxA-V90R, and 2F9EsxA-F94Q. Among these were strains that had ESAT-6 mutated at the extreme C terminus (H37RvRD1::2F9EsxA-V90R, H37RvRD1::2F9EsxA-F94Q). Similar results were obtained with a recombinant BCG strain secreting a C-terminally truncated ESAT-6 protein lacking the last 12 residues (esxAΔ84–95, supplemental Fig. S3A), which did not induce ESX-1 enhanced virulence nor ESAT-6 specific T-cell responses (data not shown).

DISCUSSION

The combined efforts of this study using bioinformatics, protein engineering, as well as immunological and virulence studies allowed us to pinpoint regions of the ESAT-6 molecule that are critical for its biological activity. The study has clearly shown that it is indeed ESAT-6, in combination with CFP-10 that mediate ESX-1-associated virulence. We have demonstrated that mutation of only one or two selected residues in ESAT-6 can abolish the virulent phenotype of the recombinant M. tuberculosis strain and have linked this phenomenon in part to the destruction of the α-helical/coiled-coil motifs present in ESAT-6 and its protein partner CFP-10. The sequences of coiled-coils in proteins consist of heptad repeats denoted “abcdefg,” harboring two characteristic hydrophobic amino acids at positions “a” and “d” of an α-helix (24). Amino acids in other positions, in particular in “e” and “g,” are often polar residues important for the water solubility of the protein complex and the specificity of interactions between neighboring helices at different pH levels (supplemental Fig. S1B). It has been shown that short stretches of 30–40 residues may be sufficient to account for such interaction of proteins (25). It is interesting to note that many proteins involved in type III secretion systems of Gram-negative bacteria contain coiled-coil motifs, which enable or stabilize protein-protein interactions (26). In the case of ESAT-6 and CFP-10 the presence of multimeric, but not dimeric coiled-coil motifs (supplemental Fig. S1A), suggests that three or four helices may be involved in such an interaction.

As described under “Results,” the effects of the mutations introduced into ESAT-6 on virulence of recombinant strains can be rationalized from our model. The data confirm and extend predictions by Pallen (16), who described a large family of small proteins from Gram-positive bacteria, including ESAT-6 and CFP-10 from M. tuberculosis or EsxA/B from Staphylococcus aureus (27), which are all characterized by the WXG motif and have a size of ~100 residues. The Trp43 residue could have a functional role in interacting with other proteins. However, in our model it is turned inward (Fig. 2) and helical in the published NMR results (20). It could therefore be important for structural reasons, interacting with another conserved hydrophobic amino acid, Tyr25 in ESAT-6. Also the almost perfect conservation of a tryptophan at this position in the whole protein family points to a structural role rather than a purely functional role. This hypothesis is supported by the observation that EsxA-W43R-HIS secreted by recombinant BCG did not retain CFP-10 by Ni2+ affinity chromatography (Fig. 3C). The finding that replacement of a single residue severely disturbs binding to CFP-10 emphasizes the specificity of the ESAT-6-CFP-10 interaction and may explain why ESAT-6 did not bind to other members of the Ess protein family (22, 23). The neighboring mutation G45T seems to be important for the folding of ESAT-6, as glycine shows more flexibility and can adopt different conformations than other amino acids. It is therefore often found in short loops or turns. Another attenuating mutation, L28A/L29S, can be explained by the prediction that Leu28 is at position “a,” which is critical for the stability of the coiled-coil structure and also affecting secretion. Finally, three mutations showing attenuating effects, V90R, M93T, and F94Q are all in the extreme C-terminal end, outside the helical region. As the extreme C terminus of ESAT-6 represents a floppy, structurally not well defined region of the protein (20), it seems that mutation of residues at the C terminus may inhibit a specific interaction of ESAT-6 with host proteins rather than affecting the structure or stability of the protein itself. It was recently shown that the C terminus of ESAT-6 was apparently not involved in the protein interaction with CFP-10 (23), as is also suggested by our model. Moreover, based on our model, amino acids that are important for the correct function of CFP-10 can now be predicted and experimentally verified.

Research on ESAT-6 and the ESAT-6 system-1 has become an important topic for studies on the pathogenesis and immunogenicity of M. tuberculosis infections because of the outstanding role of ESAT-6 in these processes. However, to date many groups have used ESAT-6 molecules expressed by E. coli, and there are several examples in the literature that recombinant proteins may show properties that differ from the naturally occurring mycobacterial proteins (28). For this reason, one of our main goals was to establish an experimental system in the M. tuberculosis complex that allows ESAT-6 to be studied while retaining the characteristics of the naturally occurring molecule. In this respect, the C-terminal HIS construct of ESAT-6 was particularly interesting, as it fulfilled all these desired features. In contrast to predictions based on E. coli-expressed ESAT-6 (15), proteolytic cleavage of the 11 C-terminal residues did not occur for ESAT-6 expressed by BCG and M. tuberculosis. So far, in all ESAT-6 constructs that contained C-terminal tags or fusions we observed a size shift and did not detect molecules that had lost their tag (supplemental Fig. S3, B and C). The results obtained with co-purification assays, indicating that CFP-10 was eluted together with the HIS-tagged ESAT-6 protein from a Ni2+-affinity column after passage of supernatant from recombinant BCG is the first proof that indeed, in a biologically relevant, non-denatured state, ESAT-6 and CFP-10 form a protein complex even after secretion. As the BCG::2F9-EsxA-HIS strain retained intermediate virulence and specific immunogenicity, short tags added at the C terminus of the ESAT-6 protein do not seem to alter greatly the biological activity of ESAT-6 produced by recombinant BCG. With this knowledge it should now be possible to create recombinant BCG strains secreting ESAT-6 with small C-terminal tags applicable to cell biological experiments that are needed to investigate targeting and trafficking of ESAT-6 in macrophages or dendritic cells.

Certain isoforms of the ESAT-6 molecule from M. tuberculosis, separated by two-dimensional electrophoresis, were identified as carrying a post translational modification, namely an acetylation of the threonine residue at position 2, and it was observed that only the unacetylated form of ESAT-6 interacted with CFP-10 (29). Acetylation affects diverse protein functions like enzyme activity, stability, and protein-protein interactions (30) but the role of the acetylation of ESAT-6 during infection with M. tuberculosis is not known. This was one of the reasons why we constructed mutant strain H37RvRD1::2F9EsxA-T2H, which had the threonine replaced by histidine. However, as the mutant strain secreted ESAT-6 normally and retained the enhanced in vivo growth in SCID mice, we concluded that this particular posttranslational modification of the Thr2 residue in ESAT-6 does not have a major effect upon its function.

The observation that mutations in ESAT-6 that diminish or abolish virulence also inhibit ESAT-6-specific T-cell responses in mice clearly demonstrates that optimal recognition of ESAT-6 by the immune system not only requires secretion of the antigen but also needs a biologically active form of ESAT-6. The finding that a mutation at the C terminus ablates immunogenicity upon stimulation with the N-terminal ESAT-6 peptide 1–20 raises interesting questions for future studies on
antigen processing and innate and/or adaptive immune responses. It is tempting to speculate that the immune recognition depends on the proper trafficking of ESAT-6, and this process possibly requires the interaction of ESAT-6 with, as yet unknown, host proteins. From a more practical perspective, the use of the ESAT-6-HIS constructs expressed by BCG opens up new ways to identify such proteins. Furthermore, ESAT-6-HIS and/or ESAT-6-GFP constructs should enable high throughput screening procedures to be established, to identify potential inhibitors of the ESAT-6 system-1, which in turn could lead to the identification of new anti-tuberculous drugs. As another application, certain strains secreting mutated or tagged ESAT-6 with intermediate virulence are promising candidates for new, potentially more effective vaccines that combine lower virulence than the previously tested BCG::RD1–2F9 strain (12), with the beneficial effects of strong T-cell responses against the immunodominant proteins ESAT-6 and CFP-10 (9).

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