in B cells (Ogata et al., 2000). Miyake and colleagues (1998) also identified MD-1 as a molecule that associates with the extracellular portion of RP105. Similarly to RP105-deficient mice, MD-1-deficient mice showed impairment in LPS-induced B-cell proliferation, antibody production, and CD86 upregulation (Nagai et al., 2002). Furthermore, surface expression of RP105 was abolished in MD-1-deficient B cells, indicating that MD-1 is essential for the responsiveness to LPS and surface expression of RP105 in B cells. It remains unclear whether RP105/MD-1 is involved in the LPS recognition in other cells that express RP105/MD-1, such as DCs and macrophages.

MD-2. Miyake and colleagues further identified MD-2, which is structurally related to MD-1 (Shimazu et al., 1999). Expression of both MD-2 and TLR4, but not TLR4 alone, conferred LPS-induced NF-κB activation in LPS-nonresponsive Ba/F3 cells, indicating that MD-2 associates functionally with TLR4. Physical association of MD-2 and TLR4 on mouse peritoneal macrophages was also shown using a monoclonal antibody against the TLR4/MD-2 complex (Akashi et al., 2000; Nomura et al., 2000). The importance of MD-2 in the LPS responsiveness was further demonstrated in genetic studies. Chinese hamster ovary cell lines that showed an impaired response to LPS have been shown to be mutated in the *MD-2* gene (Schromm et al., 2001). MD-2-deficient mice displayed severely impaired responses to LPS, and the phenotype was very similar to that of TLR4-deficient mice (Nagai et al., 2002). Analysis of the MD-2-deficient mice further demonstrated that the surface expression of TLR4 was abolished in these mice, indicating that MD-2 is required for the surface expression of TLR4 (Nagai et al., 2002).

Thus, several molecules which associate functionally with TLR4 have been identified. However, no molecules have been reported to associate with the other TLRs. It is very intriguing how TLRs, which have the conserved LRR domains in the extracellular portion, recognize quite distinct types of microbial components such as the lipid moiety, peptides, and nucleic acids. In this regard, we can hypothesize that, although molecules that associate with individual TLRs have not yet been reported, they might exist and directly recognize the microbial components. Indeed, there is a report that indicates that MD-2 directly regulates the species-specific recognition of the lipid moiety by TLR4 (Akashi et al., 2001).

SIGNALING PATHWAYS VIA TLRs

The signaling pathways via TLRs originate from the TIR domain. MyD88 harboring the TIR domain in the carboxy-terminal portion associates with the TIR domain of TLRs. Upon stimulation by TLR ligands, MyD88 recruits a family of IL-1 receptor-associated kinases (IRAKs) to TLRs. IRAKs then activate tumor receptor-associated factor 6 (TRAF6), thereby inducing activation of mitogen-activated protein (MAP) kinases and NF-κB (Fig. 3). Important roles for each molecule have been elucidated through the generation of gene-targeted mice.

MyD88 is a Common Adaptor in TLR Signaling

MyD88-deficient mice showed impaired responses to the IL-1 family of cytokines, whose receptors have the cytoplasmic TIR domain (Adachi et al., 1998). Subsequent studies of MyD88-deficient mice demonstrated that these mice did not produce any inflammatory cytokines in response to LPS, peptidoglycan, lipoproteins, CpG DNA, flagellin, dsRNA, or the imidazoquinolines (Kawai et al., 1999; Takeuchi et al., 2000b, 2000c; Hayashi et al., 2001; Alexopoulou et al., 2001; Hemmi et al., 2002; Hacker et al., 2000; Schnare et al., 2000). These findings indicate that MyD88 is an essential adaptor in the signaling pathways activated via all the TLR family members that lead to the production of inflammatory cytokines. Indeed, macrophages from MyD88-deficient mice showed no activation of NF-κB or JNK in response to

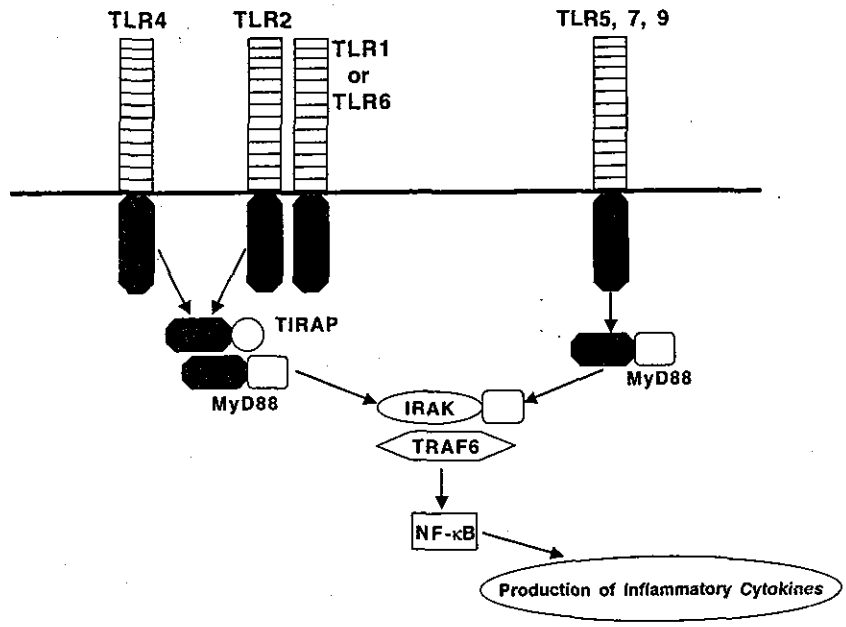

FIGURE 3 MyD88-dependent signaling pathway. A TIR domain-containing adaptor molecule, MyD88, associates with the cytoplasmic TIR domain of TLRs and recruits IRAKs to the receptor upon receptor activation. IRAKs then activate TRAF6, leading to the activation of MAP kinases and NF-κB. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4.

peptidoglycan, lipoprotein, CpG DNA, or the imidazoquinolines.

TRAF6-deficient mice also exhibited impaired responses to both IL-1 and LPS, indicating that TRAF6 is also a critical component of both the IL-1 receptor- and the TLR4-mediated signaling pathways (Lomaga et al., 1999; Naito et al., 1999). The IRAK family has four members: IRAK-1, IRAK-2, IRAK-M, and IRAK-4 (Li et al., 2002). Physiological roles for these family members have been elucidated, except for IRAK-2. IRAK-1-deficient mice were partially impaired in their responses to IL-1 and LPS (Kanakaraj et al., 1998; Thomas et al., 1999; Swantek et al., 2000). IRAK-4-deficient mice showed almost no response to IL-1 and TLR ligands (Suzuki et al., 2002). IRAK-4 associates with IRAK-1 in response to IL-1 stimulation, and the introduction of the dominant negative form of IRAK-4 resulted in impaired IRAK-1 activation in response to IL-1. Thus, IRAK-4 presumably acts as a central mediator in the IL-1 receptor and TLR signaling, upstream of IRAK-1 (Li et al., 2002).

Additional molecules that are involved in the TLR signaling have been reported. Receptor interacting protein-2 (RIP2), harboring a carboxy-terminal CARD domain, was originally identified as a serine/threonine kinase that associates with TRAF family members and with TNF receptor family members, such as the type I TNF receptor and CD40, and induces NF-κB activation and apoptosis (McCarthy et al., 1998; Inohara et al., 1998). RIP2-deficient mice were partially impaired in their response to LPS, peptidoglycan, and dsRNA, indicating that RIP2 is involved in the TLR signaling pathways (Kobayashi et al., 2002; Chin et al., 2002). It remains to be elucidated how RIP2 is connected to the TLR signaling. Toll-interacting protein (Tollip) has

been identified as a molecule present in a complex with IRAK (Burns et al., 2000). Upon stimulation with IL-1, the Tollip–IRAK complex is recruited to the IL-1R complex by the association of Tollip with IL-1RAcP. IRAK is then activated by phosphorylation, which in turn leads to dissociation of IRAK from Tollip. Tollip is seemingly involved in the negative regulation of the TLR signaling pathway because overexpression of Tollip blocked activation of NF-κB in response to TLR2 and TLR4 ligands (Bulut et al., 2002; Zhang and Ghosh, 2002). However, the physiological roles of Tollip remain to be elucidated through the generation of gene-targeted mice.

Signaling Pathways That Are Independent of MyD88

MyD88 is essential for the production of inflammatory cytokines in response to all the TLR ligands, as described above. However, unlike the case for stimulation with other TLR ligands, LPS stimulation resulted in the activation of NF-κB and JNK in MyD88-deficient macrophages, although the kinetics were delayed compared to wild-type macrophages (Kawai et al., 1999). This finding indicates that the LPS-induced inflammatory cytokine production is completely mediated by the MyD88-dependent signaling pathway, but that a pathway exists that is independent of MyD88 in the LPS response (see Fig. 4). Indeed, some LPS responses were observed in MyD88-deficient mice. DCs from MyD88-deficient, but not from TLR4-deficient, mice matured in response to LPS (Kaisho et al., 2001). Kupffer cells from MyD88-deficient mice showed caspase-1-dependent cleavage of the IL-18 precursor into the mature form after LPS stimulation (Seki et al., 2001). MyD88-deficient macrophages showed LPS-induced expression of several genes, such as those

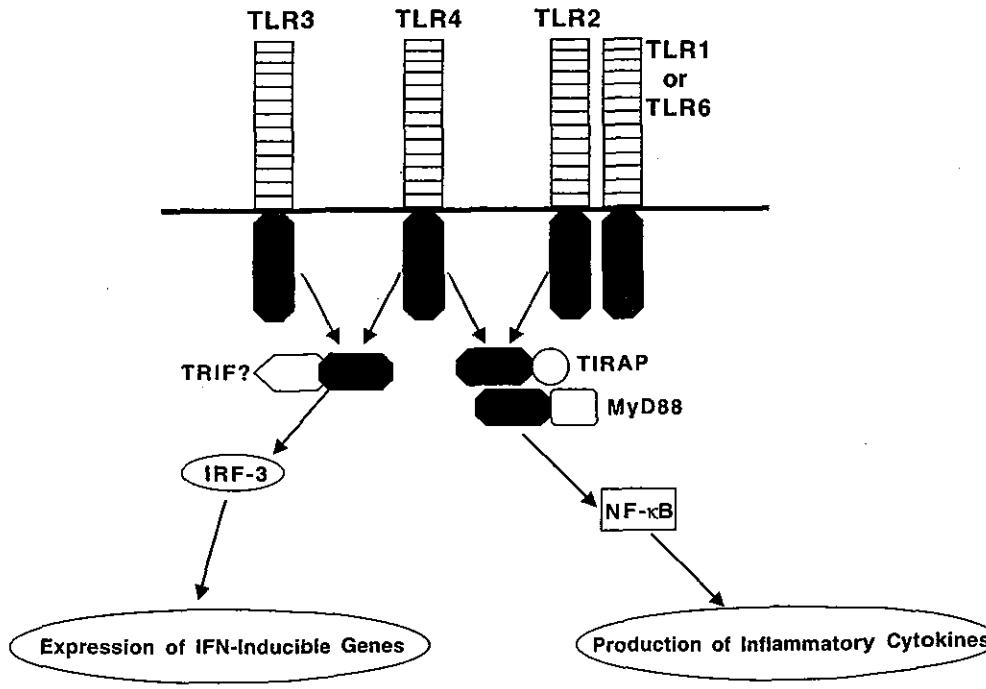

FIGURE 4 MyD88-independent signaling pathway. In the TLR3- and TLR4-mediated signaling pathways, LPS-induced activation of IRF-3 is observed in MyD88-deficient mice, indicating the presence of a MyD88-independent pathway. It remains unclear how IRF-3 is activated. Recently, a TIR domain-containing adaptor, TRIF, was found to associate with IRF-3 and TLR3, indicating a possible role for TRIF in the MyD88-independent pathway.

encoding IP-10 and GARG16, all of which are known as IFN-inducible genes (Kawai et al., 2001). In addition, MyD88-deficient macrophages showed NF-κB activation in response to dsRNA, as is the case in LPS stimulation (Alexopoulou et al., 2001). Thus, TLR4 and TLR3 utilize the MyD88-independent pathway, although it remains unclear whether the pathways activated by TLR4 and TLR3 are equivalent.

Stimulation with dsRNA or viral infection results in the activation of interferon regulatory factor 3 (IRF-3), a member of the IRF family of transcription factors, and thereby induces IFN-α/β and the IFN-inducible genes (Weaver et al., 1998; Yoneyama et al., 1998). IRF-3-deficient mice were impaired in the viral infection-induced expression of IFN-α/β, demonstrating the essential role of IRF-3 in virus-induced IFN-α/β expression (Sato et al., 2000). LPS has also been shown to activate IRF-3 (Kawai et al., 2001; Navarro and David, 1999). Thus, IRF-3 is activated in the TLR3 and TLR4 signaling pathways. Furthermore, LPS stimulation induced the activation of IRF-3 in MyD88-deficient mice, indicating that IRF-3 activation occurs in a MyD88-independent manner (Kawai et al., 2001). Subsequent studies demonstrated that LPS-induced activation of IRF-3 induced the MyD88-independent expression of IFN-β and then IFN-β induced Stat1-dependent expression of the IFN-inducible genes in macrophages and DCs (Toshchakov et al., 2002; Hoshino et al., 2002; Doyle et al., 2002). Thus, IRF-3 presumably plays an important role in the MyD88-independent pathway of TLR3 and TLR4 signaling. Analysis of the role of IRF-3 in the TLR signaling pathways would be of great interest.

IFN-α has been shown to be induced in response to the activation of TLR7 as well as TLR4 (Ito et al., 2002; Hemmi et al., 2002). However, unlike TLR4, TLR7-dependent induction of IFN-α was not observed in MyD88-deficient mice (Hemmi et al., 2002). In addition, a certain type of CpG DNA that activates TLR9 also induced IFN-α in plasmacytoid DCs (Krug et al., 2001). TLR7 and TLR9 are structurally related and presumably utilize a similar signaling cascade, thereby leading to similar biological outcomes. Therefore, we presume that an unknown signaling cascade that is dependent on MyD88 induces IFN-α in the TLR7- and TLR9-mediated signaling pathways.

TIR Domain-Containing Adaptors

In attempts to characterize the MyD88-independent signaling pathway, a second adaptor molecule containing the TIR domain was identified and designated TIRAP or MyD88-adaptor-like (Horng et al., 2001; Fitzgerald et al., 2001). The initial in vitro studies suggested that TIRAP specifically associates with TLR4 and acts as an adaptor in the MyD88-independent signaling pathway. However, TIRAP-deficient mice have recently been generated, and analysis of these mice has revealed an unexpected role for TIRAP in the TLR signaling (Yamamoto et al., 2002; Horng et al., 2002). TIRAP-deficient mice showed no production of inflammatory cytokines in response to the TLR4 ligand. However, TIRAP-deficient macrophages showed delayed activation of NF-κB and MAP kinases in response to LPS, as is the case in MyD88-deficient macrophages. Furthermore, TIRAP-deficient mice were not impaired in the LPS-induced expression of IFN-inducible genes and maturation of DCs. Even in TIRAP/ MyD88 double-deficient mice, these LPS responses were normal. These findings indicate that TIRAP is essential for the TLR4-mediated MyD88-dependent, but not for the MyD88-independent, signaling pathway. In addition, although TIRAP-deficient mice showed normal responses to the TLR3, TLR7, and TLR9 ligands, they were defective in their response to the TLR2 ligands. Thus, TIRAP has been demonstrated to be essential for the MyD88-dependent signaling pathway via TLR2 and TLR4, but not for the MyD88-independent signaling. These studies further indicate that the TIR domain-containing molecules provide the specificity for individual TLR-mediated signaling pathways.

The phenotype of the TIRAP-deficient mice encouraged us to search for new TIR domain-containing adaptors, and led to the identification of a third TIR domain-containing adaptor (Yamamoto et al., 2002). Overexpression of this molecule, as well as MyD88 and TIRAP, resulted in activation of the NF-κB reporter gene in 293 cells. Furthermore, its overexpression led to the activation of the IFN-β promoter, which was not observed when MyD88 or TIRAP was overexpressed. Therefore, this molecule was named TRIF for TIR domain-containing adaptor inducing IFN-β. The dominant negative form of TRIF inhibited TLR3-dependent activation of the IFN-β promoter. Association between TRIF and IRF-3 was also shown. These findings indicate that TRIF may act in the MyD88-independent pathway leading to the induction of IFN-β. Analysis of TRIF-deficient mice will reveal its precise role in TLR signaling in the near future.

CONCLUDING REMARKS

We are now aware that innate immunity detects microbial invasion through the recognition of specific patterns of microbial components by TLRs. However, it is still elusive how TLRs recognize them. In the signaling pathways via TLRs, MyD88 is a common adaptor leading to the induction of inflammatory cytokines. However, additional adaptors, such as TIRAP and TRIF, exist that are expected to specify the TLR signaling pathway. Elucidation of the signaling pathway that is specific to each TLR will provide us with an important clue to understanding the molecular mechanisms by which innate immunity is activated and finally lead to the development of antigen-specific adaptive immunity.

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Differential Immune Responses and Protective Efficacy Induced by Components of a Tuberculosis Polyprotein Vaccine, Mtb72F, Delivered as Naked DNA or Recombinant Protein[1]

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Key Ags of *Mycobacterium tuberculosis* initially identified in the context of host responses in healthy purified protein derivative-positive donors and infected C57BL/6 mice were prioritized for the development of a subunit vaccine against tuberculosis. Our lead construct, Mtb72F, codes for a 72-kDa polyprotein genetically linked in tandem in the linear order $Mtb32_C$-Mtb39-$Mtb32_N$. Immunization of C57BL/6 mice with Mtb72F DNA resulted in the generation of IFN-γ responses directed against the first two components of the polyprotein and a strong $CD8^+$ T cell response directed exclusively against $Mtb32_C$. In contrast, immunization of mice with Mtb72F protein formulated in the adjuvant AS02A resulted in the elicitation of a moderate IFN-γ response and a weak $CD8^+$ T cell response to Mtb32c. However, immunization with a formulation of Mtb72F protein in AS01B adjuvant generated a comprehensive and robust immune response, resulting in the elicitation of strong IFN-γ and Ab responses encompassing all three components of the polyprotein vaccine and a strong $CD8^+$ response directed against the same $Mtb32_C$ epitope identified by DNA immunization. All three forms of Mtb72F immunization resulted in the protection of C57BL/6 mice against aerosol challenge with a virulent strain of *M. tuberculosis*. Most importantly, immunization of guinea pigs with Mtb72F, delivered either as DNA or as a rAg-based vaccine, resulted in prolonged survival (>1 year) after aerosol challenge with virulent *M. tuberculosis* comparable to bacillus Calmette-Guérin immunization. Mtb72F in AS02A formulation is currently in phase I clinical trial, making it the first recombinant tuberculosis vaccine to be tested in humans. *The Journal of Immunology*, 2004, 172: 7618–7628.

Globally, approximately two billion people are infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB),[4] and an estimated three million deaths due to this disease occur annually (1, 2) Although combination chemotherapy is generally effective in the treatment of TB, the treatment is arduous and requires stringent compliance to avoid the development of multidrug resistant strains of *M. tuberculosis* (3, 4).

With respect to prophylaxis against TB, the attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guérin (BCG), is currently the only available vaccine (5, 6). However, the prophylactic use of BCG has demonstrated varying levels of efficacy in different clinical trials and geographically distinct populations (6, 7). It has been proposed that the variable efficacy in BCG vaccine trials may be due to interference caused by previous exposure to environmental mycobacteria (8, 9). A further deficiency of BCG is that it can cause disseminated disease in immunocompromised individuals (10, 11). Thus, whereas BCG has a protective effect in children, particularly against forms such as meningeal TB, it does not consistently prevent the development of pulmonary TB in adults. Consequently, there is need for the development of more effective vaccines against TB.

M. tuberculosis is an intracellular pathogen, and, as such, cell-mediated immunity plays a key role in the control of bacterial propagation and subsequent protection against TB. In animal studies, acquired resistance against TB is mediated by sensitized T lymphocytes, in particular, IFN-γ-secreting $CD4^+$ T lymphocytes are critical in mediating protection against TB in the murine model of this disease (12–16). The central role of IFN-γ in the control of TB has been further demonstrated by the high susceptibility to mycobacterial infections in mice with a disrupted IFN-γ gene and in humans with a mutated IFN-γ receptor (10, 11, 17–19). In addition, MHC class I-restricted $CD8^+$ T cells may be required for resistance to *M. tuberculosis* as an alternative source of IFN-γ. Recent studies have suggested that whereas $CD8^+$ T cells do not appear to be critical during acute TB, they may play an important role in preventing the reactivation of latent TB infection (10, 11, 17–20).

The identification of mycobacterial Ags that preferentially activate T cells to proliferate and secrete IFN-γ by both CD4 and CD8 T cells, is critical to the development of subunit vaccines against TB. We have identified several *M. tuberculosis* Ags in the context of controlled infection in humans and C57BL/6 mice characterized by their ability to elicit T cell and Ab responses (21–28). Of these, two proteins, Mtb32 (generated as overlapping amino and carboxyl fragments) and Mtb39 (Rv0125 and Rv1196, respectively) (23,

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[4] Abbreviations used in this paper: TB, tuberculosis; BCG, bacillus Calmette-Guérin; 3D-MPL, 3-deacylated monophosphoryl lipid A; IPTG, isopropyl-β-D-thiogalactoside; ORF, open reading frame; PPD, purified protein derivative.

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26), were developed further, and their open reading frames (ORFs) were expressed as a single recombinant polyprotein with a predicted size of 72 kDa (Mtb72F). We report in this study that immunization of C57BL/6 mice with Mtb72F DNA or recombinant protein formulated in two different adjuvant systems, AS01B and AS02A, resulted in the elicitation of differential immune responses (both qualitative and quantitative) to the components of Mtb72F, with the AS01B formulation eliciting the broadest range of immune response. Despite these immunogenicity differences, all three forms of immunization protected mice against TB infection. Most significantly, immunization of guinea pigs with Mtb72F, delivered either as DNA or as a rAg-based vaccine, prolonged the survival of the animals after aerosol challenge with virulent *M. tuberculosis*.

Materials and Methods

Generation of a tandemly linked ORF encoding Mtb72F

Mtb72F was generated by the sequential linkage in tandem of the ORFs of the ~14-kDa C-terminal fragment of mtb32 (26) (residues 192–323; 132 aaa) to the full-length ORF of mtb39 (23), followed at the C terminus with the ~20-kDa N-terminal portion (residues 1–195) of mtb32. These two genes correspond to the ORFs Rv0125 and Rv1196, respectively, as defined in the TubercuList H37Rv database (http://genolist.pasteur.fr/Tuberculist/). This was accomplished using sequence-specific oligonucleotides containing unique restriction sites (*Eco*RI and *Eco*RV) and devoid of the authentic stop codons at the C-terminal ends (in the case of Mtb32-C and Mtb-39) by PCR using genomic DNA from the *M. tuberculosis* strain H37Rv as template. The details of the process were as follows.

Generation of Mtb32c construct devoid of a stop codon

The 5′and 3′ oligonucleotides to the C-terminal portion of mtb32 (mtb32c) were designed as follows: 5′ (5′-CAA TTA CAT ATG *CAT CAC CAT CAC CAT CAC* **ACG GCC GCG TCC GAT AAC TTC**-3′) and 3′ (5′-CTA ATC GAA TCC **GGC CGG GGG TCC CTC GGC CAA**-3′). The 5′ oligonucleotide contains an *Nde*I restriction site (underlined) preceding an ATG initiation codon, followed by nucleotide sequences encoding six histidine residues (italics) and sequences derived from the first seven amino acid residues of Mtb32c (bold). The 3′ oligonucleotide contains an *Eco*RI restriction site (underlined), followed immediately by sequences comprising the last seven amino acid residues (bold) and devoid of the termination codon. These oligos were used to amplify Mtb32c, the carboxyl 396-nt portion (aa residues 192–323; a 14-kDa 132 aa residues) of Mtb32 and the resulting PCR-amplified product digested with *Nde*I and *Eco*RI, followed by subcloning into the pET17b expression vector similarly digested with *Nde*I and *Eco*RI. Ligated products were then transformed into *Escherichia coli*, and transformants with the correct insert were identified by restriction digest and verified by DNA sequencing. The mtb32c-pET plasmid was subsequently linearized by digestion with *Eco*RI and *Eco*RV. The latter cuts within the polylinker sequence of the pET vector that is located downstream of the *Eco*RI site.

PCR amplification of the full-length coding sequences of Mtb39 and subcloning into the Mtb32c-pET plasmid

The 5′and 3′ oligos of Mtb39 contain the following sequences: 5′ (5′-CTA ATC GAA TTC **ATG GTG GAT TTC GGG GCG TTA**-3′) and 3′ (5′-CTA ATC GAT ATC **GCC GGC TGC CGG AGA ATG CGG**-3′). The 5′ oligonucleotide contains an *Eco*RI restriction site (underlined) preceding the first seven amino acid residues of Mtb39 (bold). The 3′ oligonucleotide contains an *Eco*RV restriction site (underlined), followed immediately by sequences comprising the last seven amino acid residues (bold) and devoid of the termination codon. These were used to amplify the full-length coding sequence of Mtb39 (1173 bp; a 391-aa stretch with a predicted size of ~39 kDa), and the resulting PCR-amplified product was digested with *Eco*RI and *Eco*RV, followed by subcloning in-frame with the predigested Mtb32c-pET plasmid. The ligated products were then transformed into *E. coli*, and transformants with the correct insert were identified by restriction digest and verified by DNA sequencing. For expression of the recombinant Mtb32c-Mtb39, the pET plasmid construct was transformed into the bacterial host (BL-21; pLysE), and expression of the protein resulted in a single recombinant.

Cloning of the N-terminal 195-aa sequence of mtb32 into the mtb32c-mtb39 pET construct

The 5′ and 3′ oligonucleotides of the N-terminal fragment of Mtb32 were designed as follows: 5′ (5′-CTA ATC GAT ATC **GCC CCG CCG GCC TTG TCG CAG GAC**-3′) and 3′ (5′-CTA ATC GAT ATC *CTA* **GGA CGC GGC CGT GTT CAT AC**-3′). Both sets of oligonucleotides contain an *Eco*RV restriction site (underlined) preceding the first eight amino acid residues of Mtb32 (bold) and immediately following the sequences upstream of the stop codon (italics). The 3′ oligonucleotide also includes sequences comprising the last 20 nt (bold) of Mtb32n. They were designed to amplify the N-terminal 585-bp (195-aa residue) portion of Mtb32. The resulting PCR-amplified product was digested with *Eco*RV, followed by subcloning into the Mtb32c-Mtb39 fusion pET plasmid (similarly digested with *Eco*RV). Ligated products were then transformed into *E. coli*, and transformants with the correct insert and orientation were identified by restriction digest and verified by DNA sequencing. The final construct, a 72-kDa polyprotein (Mtb72F), comprises a single ORF organized in the linear order, $Mtb32_C$-Mtb39-$Mtb32_N$.

Recombinant protein expression and purification of Mtb72F

The recombinant (His-Tag) Ag was purified from the insoluble inclusion body of 500 ml of isopropyl-β-D-thiogalactoside (IPTG)-induced batch cultures by affinity chromatography using the one-step QIAexpress Ni-NTA-agarose matrix (Qiagen, Chatsworth, CA) in the presence of 8 M urea. Briefly, 20 ml of an overnight-saturated culture of BL21 containing the pET construct was added to 500 ml of 2× YT medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and grown at 37°C with shaking. The bacterial cultures were induced with 2 mM IPTG at an OD 560 of 0.3 and grown for an additional 3 h (OD, 1.3–1.9). Cells were harvested from 500-ml batch cultures by centrifugation and resuspended in 20 ml of binding buffer (0.1 M sodium phosphate (pH 8.0) and 10 mM Tris-HCl (pH 8.0)) containing 2 mM PMSF and 20 μg/ml leupeptin. *E. coli* was lysed by adding 15 mg of lysozyme and rocking for 30 min at 4°C after sonication (four times, 30 s each time), then spun at 12,000 rpm for 30 min to pellet the inclusion bodies.

The inclusion bodies were washed three times in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate in 10 mM Tris-HCl (pH 8.0). This step greatly reduced the level of contaminating LPS. The inclusion body was finally solubilized in 20 ml of binding buffer containing 8 M urea, or 8 M urea was added directly to the soluble supernatant. Recombinant Ags with His-Tag residues were batch-bound to Ni-NTA-agarose resin (5 ml of resin/500 ml of inductions) by rocking at room temperature for 1 h, and the complex was passed over a column. The flow through was passed twice over the same column and the column washed three times with 30 ml each of wash buffer (0.1 M sodium phosphate and 10 mM Tris-HCl (pH 6.3)) also containing 8 M urea. Bound protein was eluted with 30 ml of 100 mM imidazole in wash buffer, and 5-ml fractions were collected. Fractions containing the rAg were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0), bound one more time to the Ni-NTA matrix, eluted, and dialyzed in 10 mM Tris-HCl (pH 7.8). The yield of purified recombinant protein varied from 50–75 mg/l induced bacterial culture with >98% purity. Endotoxin levels were typically <10 endotoxin U/mg protein (i.e., <1 ng of LPS/mg).

Mice, immunizations, and cytokine assays

Female C57/BL6 mice were obtained from Charles River and age-matched (4–6 wk) within each experiment. Mice were immunized three times (3 wk apart) with 8 μg of rMtb72F formulated with the adjuvants AS02A or AS01B or with a 100-μg dose of Mtb72F-DNA. For protein formulations, the required immunization dose of rMtb72F (typically 8 μg) was brought up to either 50 or 43 μl with 1× PBS (pH 6.8) and mixed with 50 μl of AS01B or 57 μl of AS02A, respectively. Mice were injected with a total volume of 100 μl/mouse via the i.m. (tibialis) route with 50 μl/leg. Three weeks after the last boost, animals designated for immunogenicity studies were killed, and spleen cells were obtained by conventional procedures. Mononuclear cells were cultured at 37°C in 5% CO_2 in the presence of either medium (containing 10% FBS, 50 μM 2β-ME, and 50 μg/ml gentamicin), or medium plus rAg. For cytokine analysis, spleen cells were plated in duplicate 96-well tissue culture plates at 2.5×10^5 cells/well and cultured with or without Ags for 72 h. Supernatants were harvested and analyzed for IFN-γ by a double-sandwich ELISA using specific mAb (BD PharMingen, San Diego, CA) as previously described (22, 27).

ELISPOT assay

An ELISPOT assay was used to determine the relative number of IFN-γ-expressing cells in the single-cell spleen suspensions. A MultiScreen 96-well filtration plate (Millipore, Bedford, MA) was coated with 10 μg/ml rat anti-mouse IFN-γ capture Ab (BD PharMingen) and incubated overnight at 4°C. Plates were washed with PBS, blocked with RPMI 1640 and 10% FBS for at least 1 h at room temperature, and washed again. Spleen cells were plated in duplicate at 1×10^5 cells/well in 100 μl and stimulated with the specific rAg at a 10 μg/ml dose mixed with 0.2 ng/ml IL-2 for 48 h at 37°C. The plates were subsequently washed with PBS and 0.1% Tween and incubated overnight at 4°C with a biotin-conjugated, rat anti-mouse IFN-γ secondary Ab (BD PharMingen) at 5 μg/ml in PBS, 0.5% BSA, and 0.1% Tween. The filters were developed using the Vectastain ABC avidin peroxidase conjugate and Vectastain AEC substrate kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The reaction was stopped by washing the plates with deionized water, plates were dried in the dark, and spots were counted.

IgG isotype ELISA

Mice were bled 3 wk after the last immunization, and sera were stored at −20°C until use. The specific serum IgG isotype Ab response was measured by conventional ELISA. Recombinant Ags were coated onto 96-well Immulon-4 ELISA plates at a concentration of 100 ng/well and incubated overnight at 4°C. The plates were blocked with 200 μl/well PBS containing 0.05% Tween 20 and 1% BSA for 2 h at room temperature and washed with PBS-0.05% Tween, followed by the addition of 50 μl/well of diluent (PBS-0.05% Tween and 0.1% BSA). Sera were added at serial 2-fold dilutions (beginning at a 1/200 dilution) incubated in a humidified box on a rocking platform for 2 h at room temperature and washed (as described above), followed by the addition of 100 μl/well of biotinylated isotype-specific secondary Abs (rat anti-mouse IgG1 or IgG2a; Southern Biotechnology Associates, Birmingham, CA) diluted at 1/4000 in PBST-0.1% BSA. Plates were incubated for 1 h at room temperature, washed, and developed with for ~5 min with 100 μl/well of tetramethylbenzidine peroxidase substrate (Kirkegaarde & Perry Laboratories, Keene, NH) mixed 1/1 with peroxidase solution B. Reactions were stopped by the addition of 50 μl/well of 1 N H_2SO_4 and were read on an ELISA plate reader (Dynatech Laboratories, Chantilly, VA) at 450 nm with 570 nm as the reference wavelength.

DNA vaccine and retroviral constructs

The full-length coding sequence of Mtb72F was PCR amplified from the protein expression vector (pET-72F) using primer-specific pairs. Except for the N-terminal six histidine residues, the sequence of the entire ORFs of Mtb72F was identical in the protein and DNA constructs. The 5′ primer was designed to contain a *Hin*dIII recognition site and a Kozak sequence upstream of the initiator ATG codon. The resultant PCR product was digested subcloned into the eukaryotic expression vector pJA4304 (gift from J. I. Mullins and J. Arthors, University of Washington School of Medicine, Seattle, WA).

In addition, the three subcomponents of Mtb72F (mtb32-C, Mtb39, and Mtb32-N) were subcloned into the retroviral vector pBIB-X, a retroviral expression vector that contains a selectable marker (bsr) under translation control of an intraribosomal entry site sequence. This vector is under the control of the murine leukemia virus long terminal repeat promoter. The sequences of all three genes were obtained by PCR amplification using 5′ oligonucleotides designed with the initiating methionine. The 3′ oligonucleotide included the stop codon. The 5′ primers also included a Kozak consensus sequence (GCCGCCACC) upstream of the initiation codon to ensure efficient translational initiation in the pBIB-X vector.

CTL assay

Target cells were EL-4 cells retrovirally transduced with mtb32-C, mtb39, and $Mtb32_N$ essentially as previously described (27). Briefly, the retroviral constructs (described above) were used in transfections of Phoenix-Ampho, an amphotropic retroviral packaging line. Approximately 48 h post-transfection, supernatants containing recombinant virus were harvested and used to transduce EL-4 cells. Transduction efficiency was measured by flow cytometry using EL-4 transduced with pBIB-enhanced green fluorescent protein viral supernatants as a positive control. All transductants were selected with blastocidin-S (Calbiochem, San Diego, CA) at a concentration of 10 μg/ml. These cells were then used as targets in standard ^{51}Cr release CTL assays. For analysis of effector CTL, spleen cells from immunized mice were cultured in 24-well Costar (Corning Glass, Corning, NY) tissue culture plates at a density of 5×10^6/well and stimulated with 2.5×10^5/well of EL4 cells expressing Mtb-specific Ags in complete medium for 6 days, harvested, and tested in a standard 5-h ^{51}Cr release assay as previously described (27).

The Mtb32c-specific CTL line was generated from spleen cells from mice that were immunized with Mtb72F DNA and restimulated in vitro with EL4 cells transduced with mtb32c. The CTL line was maintained by weekly stimulation of 4×10^5 CTL with 2×10^5 irradiated EL4 transductants and 2.5×10^6 irradiated syngeneic spleen cells in 2 ml of medium containing 1 ng/ml IL-2 in 24-well plates.

CTL epitope mapping and MHC restriction analysis

Peptides used in this study were synthesized using a Symphony system (Rainin Instrument, Woburn, MA) according to the guidelines of the manufacturer. P815 ($H\text{-}2^d$) cells and $H\text{-}2D^b$ and $H\text{-}2K^b$ transfectants of P815 were gifts from M. Bevan (University of Washington, Seattle, WA). Either EL4 ($H\text{-}2^b$) or P815 (parental or $H\text{-}2D^b$ or $H\text{-}2K^b$ transfected) stimulator cells were pulsed with peptides at various concentrations for 1.5 h at 37°C and then incubated with Mtb32C-specific CTL. After 48 h, culture supernatants were assayed for IFN-γ by ELISA.

Protection experiment

Mice were immunized i.m., three times, 3 wk apart, with 8.0 μg of the rAg formulated in the indicated adjuvant or 100 μg of plasmid DNA containing the gene of interest. Positive control mice were immunized with BCG (5×10^4 CFU) in the base of the tail (once), and negative control animals were injected with saline, adjuvant alone, or DNA vector. Thirty days after the last immunization, mice were challenged by low dose aerosol exposure with *M. tuberculosis* H37Rv strain (ATCC 35718; American Type Culture Collection, Manassas, VA) using a Glas-Col (Terre Haute, IN) aerosol generator calibrated to deliver 50–100 bacteria into the lungs. Four weeks later, mice were euthanized, and lung and spleen homogenates were prepared in PBS/Tween 80 (0.05%). Bacterial counts were determine by plating serial dilutions of individual whole organs on nutrient Middlebrook 7H11 Bacto Agar (BD Biosciences, Cockeysville, MD) and counting bacterial colony formation after 21-day incubation at 37°C in humidified air.

Guinea pigs were similarly immunized via the i.m route, three times, 3 wk apart, with either a 200-μg dose of Mtb72F-DNA (brought up to 250 μl with 1× PBS, pH 7.0) or a 20-μg immunization dose of rMtb72F (or a mixture of the three components on a molar basis) formulated in AS02A in a final volume of 250 μl. The protocol for rMtb72F formulation in AS02A (Ag:adjuvant) was identical with those described for the mouse studies, but was adjusted for a 20-μg immunization dose in a final volume of 250 μl. Animals were immunized with 125 μl of the final formulation per leg. BCG (a single dose of 10^3 CFU) was used as the positive control and administered via the intradermal route. Negative control groups include adjuvant and saline alone groups. Thirteen weeks after the third immunization, the animals were challenged with the virulent H37Rv strain via the aerosol route by calibrating the nebulizer compartment of the Middlebrook airborne-infection apparatus (using the same Glas-Col aerosol generator used in the mouse experiments) to deliver ~20–50 bacteria into the lungs. All challenge studies reported in this paper were performed in the same laboratory. Animals were killed at the indicated time. At necropsy, all lung lobes were removed from the thorax individually to enable separate manipulations with each lobe. The number of viable bacteria in the lungs was determined by plating serial 10-fold dilutions of right cranial lung lobe homogenates onto nutrient Middlebrook 7H11 agar, and bacterial colony formations were counted after 21 days of incubation at 37°C under 5% CO_2. Data are expressed as $\log_{10}$ of the mean number of bacteria recovered.

Results

Generation, expression, and purification of recombinant Mtb72F

Two Mtb Ags, Mtb32 and Mtb39 (Rv0125 and Rv1196, respectively), previously identified by serological and T cell expression cloning and demonstrated to stimulate CD4 and CD8 responses in PBMC of healthy, purified protein derivative-positive (PPD^+) donors and in infected or immunized mice (23, 27), were selected for the development of a TB vaccine. An initial, straightforward approach was to link in tandem the ORFs of both genes such that the final construct would result in the generation of a single rAg comprising both Ags. However, attempts to express and purify Mtb32 in *E. coli* (either as the full-length or mature form) or as a fusion with Mtb39 (at either the N or C end) were unsuccessful. This was

attributed to the inherent serine protease activity of Mtb32 probably leading to toxicity of the expressing host cell (26). In contrast, it was relatively easy to express and purify high levels of two separate fragments of Mtb32 (N- and C-terminal portions). Therefore, we reasoned that it should be feasible to generate a chimeric construct by fusing at either end of Mtb39, the N- and C-terminal portions of Mtb32 (designated $Mtb32_N$ and $Mtb32_C$). Our initial experience with the expression level and purification profile of the C-terminal portion of Mtb32 ($Mtb32_C$) revealed that when expressed in *E. coli*, the recombinant protein partitions into the soluble fraction. In addition, $Mtb32_C$ has been demonstrated to drive the high level expression of heterologous proteins in *E. coli* when fused to their N-terminal end (29). We therefore engineered for expression in *E. coli* a polyprotein construct organized in the linear order $Mtb32_C$-Mtb39-$Mtb32_N$ coding for an ORF with a predicted molecular mass of 72 kDa (Mtb72F).

Fig. 1*A* is a diagrammatic representation of Mtb72F showing the physical organization and the restriction enzyme sites used to link the three subunits. The length of the predicted ORF of Mtb72F is 2187 nt, coding for a 729-aa polypeptide with a predicted molecular mass of ~72 kDa, an isoelectric point of 4.98, and a net charge of −15.88 at pH 7.0. Because of the way the construct was generated, the addition of the hinge sequences (*Eco*RI and *Eco*RV) resulted in the introduction of six nucleotides (two amino acid residues) at each of the junction sites. In addition, the construct was designed such that the N terminus contained six histidine residues for ease of purification by affinity chromatography over Ni-NTA matrix. After expression in *E. coli*, the recombinant protein was purified from inclusion bodies with yields ranging from 50–75 mg of purified protein/l induced culture. Fig. 1*B* shows a Coomassie Blue-stained SDS-PAGE gel of the *E. coli* culture before and after induction along with the final purified recombinant Mtb72F (migrating at its predicted size of ~72 kDa).

Immune responses in mice immunized with Mtb72F DNA

To investigate the specificity and breadth of the immune response to Mtb72F in C57BL/6 mice, we initially used the naked DNA approach because this form of immunization is known to stimulate both $CD4^+$ and $CD8^+$ T cell responses. The Mtb72F ORF was subcloned into the eukaryotic expression vector pJA4304, which is under control of the CMV promoter. Plasmid DNA was prepared with >95% migrating as a supercoiled form, as visualized by ethidium bromide staining (not shown). Mice (typically eight to 10/group) were immunized with three doses of 100 μg of Mtb72F-DNA via the i.m. route at 3-wk intervals. Three weeks after the third immunization, three mice from each group were killed for evaluation of anti-Mtb72F Ab and T cell responses ($CD4^+$ and $CD8^+$). IgG1 and IgG2a Ab responses were evaluated by ELISA

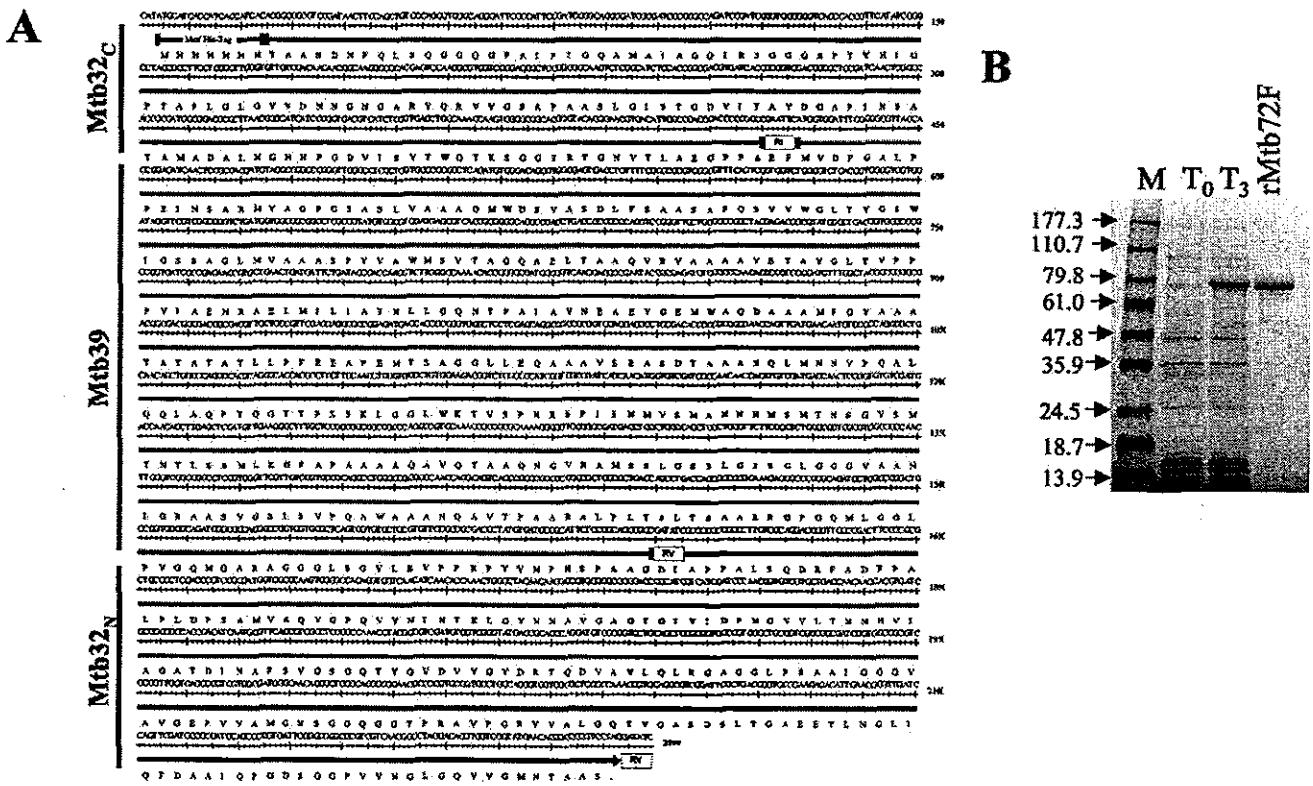

FIGURE 1. *A*, Generation of recombinant Mtb72F. Mtb72F was generated by the sequential linkage in tandem of the ORFs beginning at the amino end with Mtb32c linked to the full-length ORF of Mtb39 and terminating with sequences comprising $Mtb32_N$. The final construct comprises a single polyprotein in the linear order $Mtb32_C$-Mtb39-$Mtb32_N$. The size of the predicted ORF of Mtb72F is 2187 nt and codes for a 729-aa polypeptide with a predicted molecular mass of ~72 kDa. The figure depicts a diagrammatic representation of Mtb72F, showing the physical organization of the three components along with the hinge sequences (*Eco*RI and *Eco*RV), resulting in the generation of two amino acid residues each at the joining junction. The N-terminal sequence contains six histidine residues for use in purification of the recombinant protein by affinity chromatography over an Ni-NTA-agarose column. *B*, Expression and purification of recombinant Mtb72F. *E. coli* (BL-21/pLysE) transformed with the expression vector harboring Mtb72F was grown and induced with IPTG. The figure is a Coomassie Blue-stained 12% SDS-PAGE of the *E. coli* lysates before (*lane* T_0) and 3 h after (*lane* T_3) induction with IPTG. The resulting protein, Mtb72F (rAg), was purified from the inclusion fraction by affinity chromatography over Ni-NTA agarose matrix (*lane 3*). The faint and lower bands were verified by both N-terminal sequencing and immunoblot analysis to represent proteolytic breakdown products of the full-length Mtb72F. Molecular mass markers (*lane M*; in kilodaltons) are indicated on the *left*.

using specific mouse IgG isotype antibodies. The results revealed that mice immunized with DNA developed an Mtb72F Ab response of the IgG2a, but not the IgG1, subclass, and the response was exclusively directed against the N-terminal ($Mtb32_C$) portion of the molecule (Fig. 2*A*). No Mtb72F-specific Ab was detected in sera of mice immunized with the empty vector or injected with saline (data not shown).

$CD4^+$ T cell responses were evaluated by stimulating spleen cells in vitro with either the full-length rMtb72F protein or with each of the three separate protein components, $rMtb32_C$, rMtb39, and $rMtb32_N$, at 10, 2, and 0.4 μg/ml Ag. Supernatants were harvested and assayed for IFN-γ at 72 h poststimulation. The results revealed that immunization of C57BL/6 mice with Mtb72F DNA (but not the vector or saline control groups) induced the production of high amounts of IFN-γ (>20 ng/ml) after in vitro stimulation with rMtb72F (Fig. 2*B*). Stimulation of the same splenocyte culture with each of the three components of Mtb72F revealed that the IFN-γ response is elicited predominantly by sequences comprising the first two components (Mtb32c and Mtb39) of the polyprotein construct, with little or no response directed against the C-terminal portion ($Mtb32_N$) of the molecule. Finally, PPD (at a 10 μg/ml dose) also stimulated the splenocyte cultures from Mtb72F DNA-immunized mice to produce IFN-γ.

Because DNA immunization has been shown to be an effective method for the induction of a $CD8^+$ T cell response, we evaluated whether immunization with Mtb72F DNA could result in the generation of CTL responses. Splenocytes from immunized mice were stimulated for 6 days with EL4 cells that had been transduced with $Mtb32_C$, $Mtb32_N$, or Mtb39. The cells were subsequently washed and evaluated for cytotoxicity against specific targets or EL-4 cells transduced with enhanced green fluorescent protein as a negative control. Fig. 2*C* shows that immunization of mice with Mtb72F-DNA induced the generation of Mtb72F-specific CTL, and the CTL response was directed exclusively against the N-terminal portion ($Mtb32_C$) of the polyprotein. The lysis of Mtb32C targets was specific to immunized mice, because no lysis was observed in control groups immunized with either saline or the empty vector (data not shown).

Mapping of the CD8 T cell epitope of Mtb32C

We next analyzed the CTL response to $Mtb32_C$ in more detail. First, we generated an $Mtb32_C$-specific CTL line from spleen cells of mice immunized with $Mtb32_C$ DNA and stimulated in vitro with EL-4 cells retrovirally transduced with $Mtb32_C$. Epitope-mapping studies were performed by stimulation of the $Mtb32_C$-specific CTL with 23 peptides (15-mer overlapping by 10) spanning the entire length of $Mtb32_C$ (Fig. 3*A*). This analysis revealed that the Mtb32c-specific CTL made a strong IFN-γ response to peptide 18 (residues 92-DGAPINSATAMADAL-106) even at the low stimulation dose of 1 ng/ml (Fig. 3*A*). The adjacent sequence (peptide

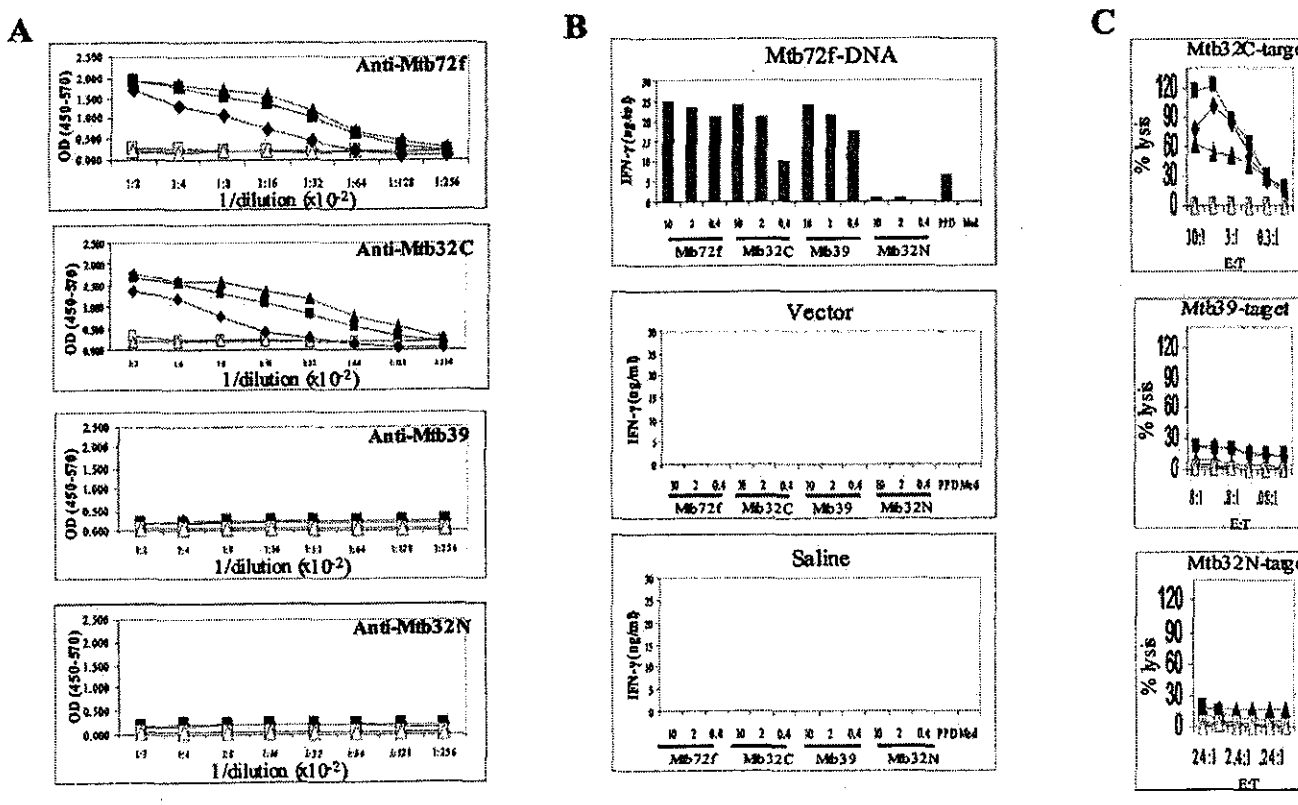

FIGURE 2. Immune responses in C57BL/6 mice immunized with Mtb72F-DNA. *A*, IgG1 and IgG2a Ab responses. Mice were immunized three times i.m. with 100 μg/dose of either Mtb72F-DNA or the empty vector or saline control (not shown). Three weeks after the third immunization, the animals were bled, and sera were tested for anti-Mtb72F and for each of the three components for Ab responses of both IgG1 (□ and △) and IgG2a (■, ▲, and ◆) *isotypes* by ELISA. *B* and *C*, For IFN-γ responses (*B*), spleen cells were obtained from the same mice and stimulated in vitro for 3 days with a 5-fold serial dilution (10, 2, and 0.4 μg) of either rMtb72F or each of the three components ($Mtb32_C$, Mtb39, and $Mtb32_N$) as indicated. As controls, splenocyte cultures were also stimulated with either PPD (10 μg/ml) or medium alone. IFN-γ production was subsequently measured by ELISA. For evaluation of CTL activity (*C*), the mononuclear spleen cells were stimulated for 6 days with $Mtb32_C$-, Mtb39-, or $Mtb32_N$-transfected EL-4 cell targets. Effector cells were washed and tested for cytotoxicity in a 4-h ^{51}Cr release assay against both control transduced EL-4 cells (EGPF; □ and △), and Mtb-transduced EL-4 cells (■, ▲, and ◆). Results are expressed as the percent specific cytotoxicity against the respective transduced targets. The curves express the results obtained for each individual mouse. These data are representative of five experiments.

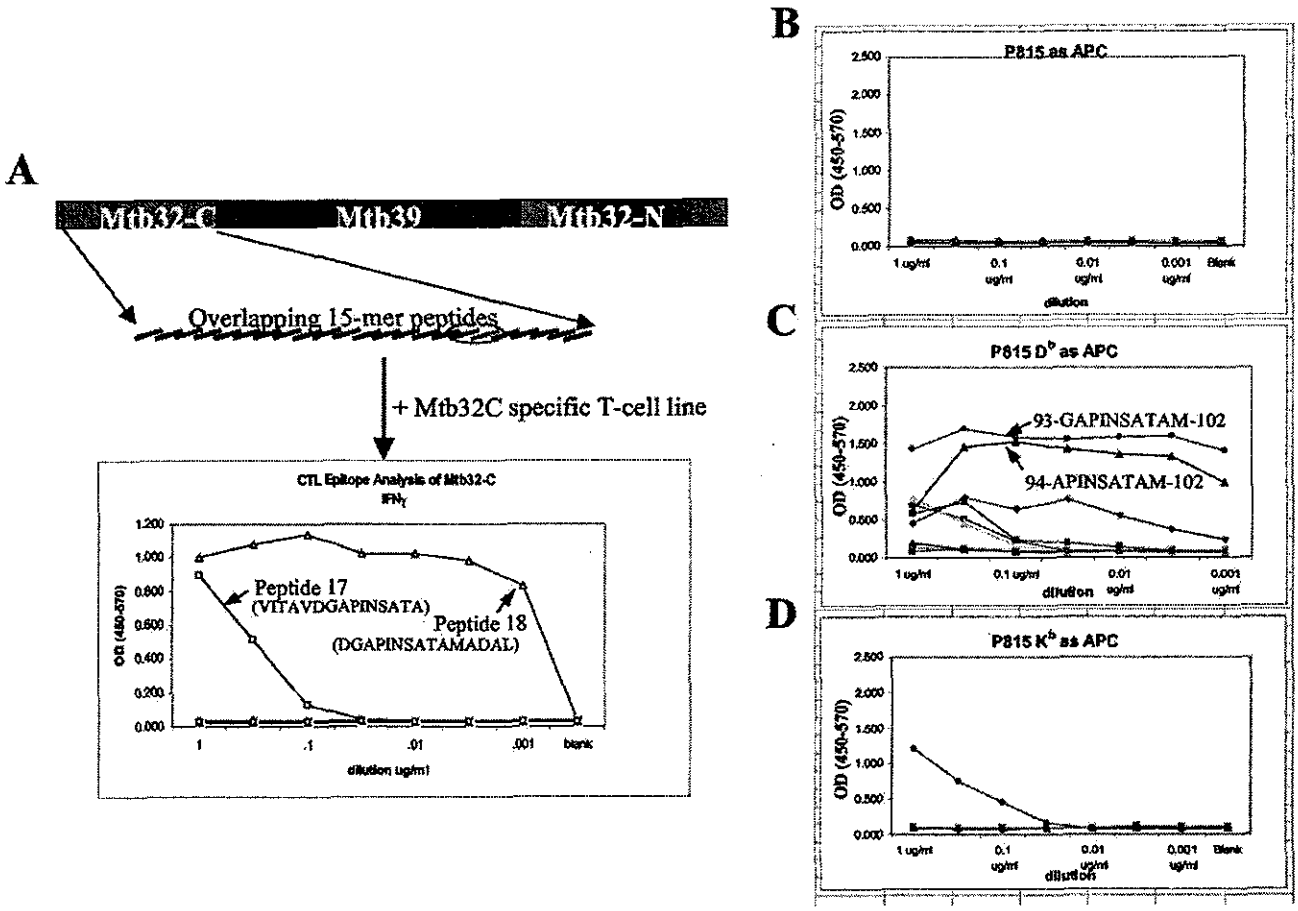

FIGURE 3. Mapping of the CTL epitope of Mtb32C and MHC restriction analysis. *A*, Stimulation of $Mtb32_C$-specific CTL with overlapping 15-mer peptides corresponding to the sequence of Mtb32. EL4 cells were pulsed with the peptides at the indicated concentrations for 1.5 h at 37°C and then incubated with Mtb32C-specific CTL. After 48 h, culture supernatants were assayed for IFN-γ by ELISA. *B*, Stimulation of Mtb32C-specific CTL with peptides derived from the peptide sequence DGAPINSATAM derived in *A*. P815 cells or P815 cells transfected with either D^b or K^b gene K41 cells were pulsed with the indicated peptides at the indicated concentrations and then incubated with MTB32C-specific CTL. After 48-h culture, supernatants were assayed for IFN-γ by ELISA.

17, residues 87-VITAVDGAPINSATA-101) read out at the high dose, but rapidly titrated out at 0.1 μg/ml. Thus, the sequence of overlap between the two positive peptides was identified as DGAPINSATAM. Given these results, the deduced sequence of the CD8 T cell epitope was further dissected into overlapping 9- and 10-mer peptides. In addition, we wanted to determine which MHC allele was responsible for presentation of the peptide to the Mtb32c-specific CD8 T cells. Therefore, we used P815 (H-2d-restricted) cells that had been transfected with either H-2Kb or H-2Db as APCs (Fig. 3, *B–D*). The data showed that only the peptide-pulsed H-2Db, not H-2Kb-transduced, P815 cells stimulated strong responses by the $Mtb32_C$-specific CTL line. The two peptides that stimulated the strongest IFN-γ responses were the 10-mer (93-GAPINSATAM-102) and 9-mer (94-APINSATAM-102) sequences (Fig. 3*C*). In agreement with these data, both peptides are predicted to bind with high affinity to Db, as determined by class I MHC prediction algorithms.

Immune responses to Mtb72F protein formulated in two adjuvant systems, AS02A and AS01B

The immune response to Mtb72F protein were subsequently evaluated in two adjuvants, AS01B and AS02A (GlaxoSmithKline Biologicals, Rixensart, Belgium). AS01B contains monophosphoryl lipid A (MPL) and QS-21 in a liposomal formulation, whereas AS02A has the same components formulated in an oil-in-water emulsion. Mice were immunized three times, 3 wk apart, with 8 μg of rMtb72F formulated in AS01B or AS02A via the i.m. route. Three weeks after the third immunization, three animals from each group were killed, and anti-Mtb72F Ab and T cell responses were evaluated. With regard to Ab responses, mice immunized with rMtb72F formulated in either AS01B or AS02A mounted strong and comparable IgG1 and IgG2a responses against Mtb72F (Fig. 4*A*) and to each of the three components comprising the construct (data not shown). No Mtb72F-specific responses were detected in the adjuvant-alone control groups.

$CD4^+$ T cell responses were evaluated by IFN-γ ELISA of the supernatant culture or by the more sensitive IFN-γ ELISPOT assay. Immunization of mice with Mtb72F formulated in AS02A resulted in the production of a strong IFN-γ response after in vitro stimulation of splenocyte cultures with rMtb72F or rMtb39, a relatively weaker response to $Mtb32_C$, and a low to undetectable response to $Mtb32_N$ (Fig. 4*B*). Immunization of mice with rMtb72F formulated in AS01B stimulated a robust production of IFN-γ (5- to 10-fold higher) against all three components of the polyprotein. Of particular interest, the adjuvant AS01B induced a strong IFN-γ response specific for the $Mtb32_N$ portion of the molecule, a response not readily observed after immunization with

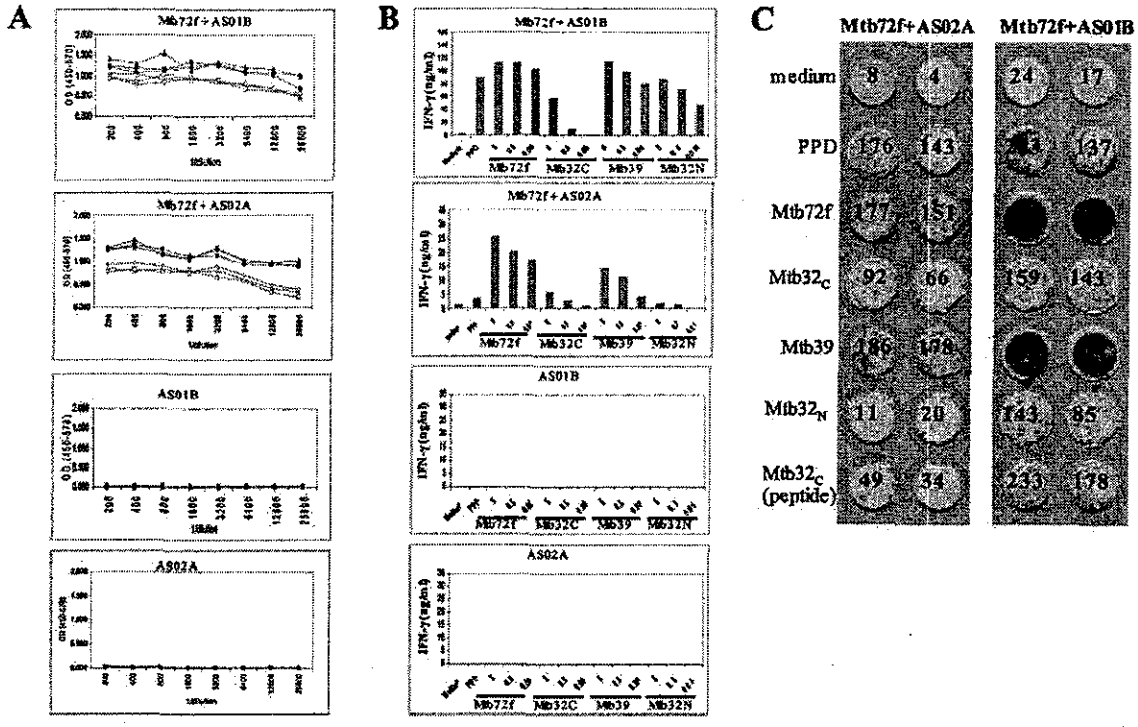

FIGURE 4. Immune responses in C57BL/6 mice immunized with rMtb72F formulated in either AS02A or AS01B adjuvants. *C57BL/6 mice were* injected three times (at 3-wk intervals) i.m. with 8 μg of rMtb72F formulated in either AS02A or AS01B. Control groups include animals immunized with adjuvant alone or saline. Three weeks after the third immunization the animals were bled and killed. Sera were obtained and tested for specific anti-Mtb72F (and all three components; not shown) Ab response of both IgG1 (■ and ▲) and IgG2a (□ and △) isotypes by ELISA (*A*). For cytokine production (*B*), mononuclear spleen cells were obtained from mice immunized with rMtb72F formulated in AS01B and AS02A or with the adjuvants alone (control groups) as indicated and stimulated for 3 days with the indicated dose of rMtb72F protein or each of the three components at 5, 0.5, or 0.05 μg/ml. Control groups were stimulated with either medium alone or PPD (10 μg/ml). Supernatants were harvested and assayed for the presence of IFN-γ by ELISA. *C*, ELISPOT assay. An ELISPOT assay was used to determine the relative numbers of IFN-γ-expressing cells in *single-cell spleen suspensions of mice* immunized with rMtb72F formulated in AS01B or AS02A and stimulated with the indicated rAg or the $CD8^+$ 10-mer peptide from $Mtb32_C$.

rMtb72F formulated in AS02A or with Mtb72F-DNA. Qualitatively, the corresponding IFN-γ ELISPOT is in agreement with the ELISA data, in that AS01B stood out as inducing the broadest and most robust T cell response to all three components of *Mtb72F* (Fig. 4*C*).

Formulation of Mtb72F in AS01B elicits a robust $CD8^+$ (CTL and IFN-γ) T cell response in C57BL/6 immunized mice

$CD8^+$ T cell responses are typically associated with DNA or viral delivery approaches. In contrast, rAg based formulations do not readily induce such responses. Given that the quality and strength of the immune response to rMtb72F were remarkably affected by the adjuvant system (AS01B or AS02A) used in the formulation, we extended our study to include $CD8^+$ T cell responses to rMtb72F in immunized C57BL/6 mice. Fig. 5*A* shows that immunization of C57BL/6 mice with rMtb72F formulated in AS01B resulted in a strong CTL response directed exclusively against $Mtb32_C$. As with Mtb72F-DNA immunization, this CTL response was directed against the same CD8 10-mer peptide (residues 92-GAPINSATAM-102; data not shown). In contrast, mice immunized with a formulation of Mtb72F in AS02A mounted a relatively weaker CTL response (Fig. 5*B*). Spleen cells from rMtb72F-immunized mice were also stimulated with the CTL peptide derived above and assayed for IFN-γ by ELISPOT. In agreement with the cytotoxicity data, strong IFN-γ responses to peptide GAPINSATAM were observed in mice immunized with rMtb72F formulated in AS01B (Fig. 4*C*). Responses in mice immunized with rMtb72F in AS02A were weaker, but stronger than those in the nonstimulated cultures.

Mtb72F protects C57BL/6 mice against M. tuberculosis *infection*

Given the results of the above immunogenicity experiments, we sought to determine whether the way in which Mtb72F is delivered (naked DNA or recombinant protein formulated in AS01B or AS02A) impacts the outcome of protection against infection of C57BL/6 mice with *M. tuberculosis*. Briefly, C57BL/6 mice were immunized three times i.m (at 3-wk intervals) with Mtb72F-DNA (100 μg/dose) or with three doses (8 μg/dose) of recombinant protein formulated in AS01B or AS02A. As controls, groups of mice were immunized with saline, adjuvant, or BCG. Four weeks after the last immunization, mice were challenged via the aerosol route with ~100 CFU of the virulent *M. tuberculosis* strain H37Rv. Bacteriological burden (CFU) was measured in the lungs of mice at 4 wk postchallenge. The results from three independent challenge experiments revealed that immunization of C57BL/6 mice with Mtb72F-DNA consistently led to a 0.7- to 1.0-log reduction in bacterial burden approaching the protective efficacy observed with BCG (Fig. 6*A*). We also compared the protective efficacy of rMtb72F formulated in AS02A with the DNA delivery approach in the same experiment (Fig. 6*A*). Mtb72F in AS02A has been evaluated exhaustively, and in six independent experiments we found that this immunization with this formulation resulted in a 0.4- to 0.6-log reduction of bacterial burden in the lung. This level of protection was comparable to that seen after immunization with a formulation comprising a mixture of the three components of Mtb72F (data not shown). Interestingly, despite the qualitative and quantitative differences observed in the immune responses to

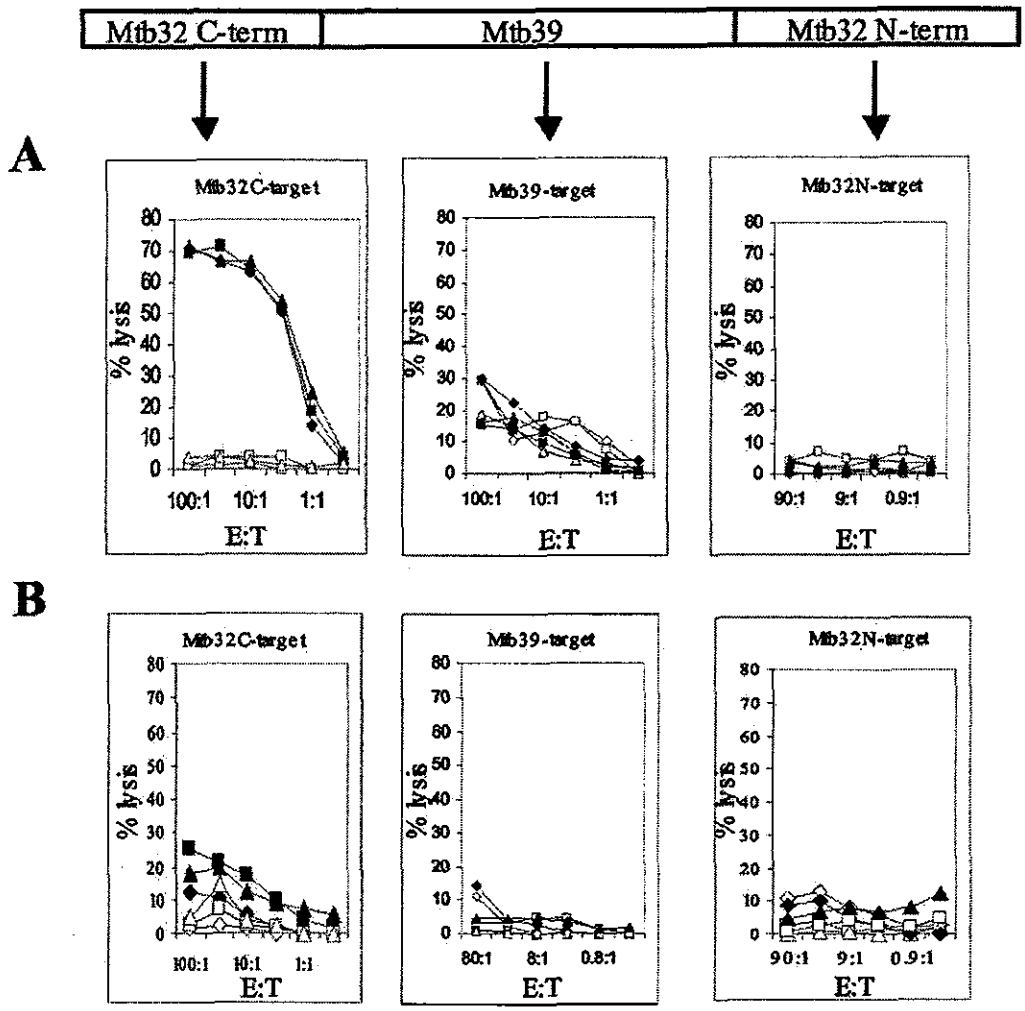

FIGURE 5. $CD8^+$ T cell responses to Mtb72F components after immunization of mice with rMtb72F formulated in either AS01B or AS02A. For evaluation of CTL activity, mononuclear spleen cells of mice immunized with rMtb72F formulated in AS01B (*A*) or AS02A (*B*) were stimulated for 6 days with EL4 cells transduced with $Mtb32_C$, Mtb39, or $Mtb32_N$. Effector cells were washed and tested for cytotoxicity in a 4-h ^{51}Cr release assay against both control transduced EL-4 cells (enhanced green fluorescent protein; □, △, and ◇), or against specific target cells (■, ▲, and ◆). Results are expressed as the percent specific cytotoxicity against Mtb32C-transfected cells for each of the three animals per group as indicated.

rMtb72F formulated in AS01B Vs AS02A, immunization of mice with three doses of 8 μg of rMtb72F in either of the two adjuvant systems resulted in a comparable reduction in bacterial burden in the lung (~0.6 log; Fig. 6*B*).

Mtb72F protects guinea pig against aerosol challenge with virulent M. tuberculosis

Having demonstrated that in the mouse model immunization with Mtb72F-DNA or rMtb72F formulated in AS02A protected against TB, we next evaluated whether these vaccination approaches would also protect against TB infection in the guinea pig model. Groups of five guinea pigs were immunized with three doses each of 200 μg of Mtb72F-DNA or 20 μg of rMtb72F formulated in AS02A. Control groups were immunized with AS02A adjuvant alone or saline via the i.m. route or with a standard dose of 10^3 CFU BCG administered via the i.d. route. Thirteen weeks after the third immunization, the animals were aerosol-challenged with 20–50 CFU of the virulent Mtb strain H37Rv. Protection was monitored by outward signs of infection (difficulty in breathing and weight loss), with survival as an end point. One of the five animals in the BCG control group died at ~7 wk postchallenge, but of causes not related to TB infection. Therefore, in the case of the BCG control group, the percent survival was based on $n = 4$. The results from this study revealed that at 30 wk postchallenge (Fig. 7) although all animals in the saline and adjuvant control groups were moribund and had to be euthanized, three of four (75%) of the guinea pigs immunized with BCG, four of five (80%) of those immunized with rMtb72F, and three of five (60%) of the animals immunized with Mtb72F-DNA were still alive. At 40 wk (~3 mo after all animals in the control groups succumbed), three of five (60%) of the animals in each of the Mtb72F-vaccinated groups were still alive. By 70 wk (~15 mo) postchallenge, two of five (40%) animals in the DNA-immunized groups and one of four (25%) animals in the BCG group were still alive. Taken together, the results revealed that in the guinea pig model, Mtb72F, delivered either as naked DNA or recombinant protein formulated in AS02A, protected guinea pigs against virulent TB challenge to an extent comparable to that seen with BCG and for periods lasting >1 year.

Discussion

The development of an efficacious subunit-based recombinant vaccine against TB would require a multivalent mixture of Ags for a broad coverage of a heterogeneous MHC population. Despite the fact that a single Ag could by itself induce protection in inbred

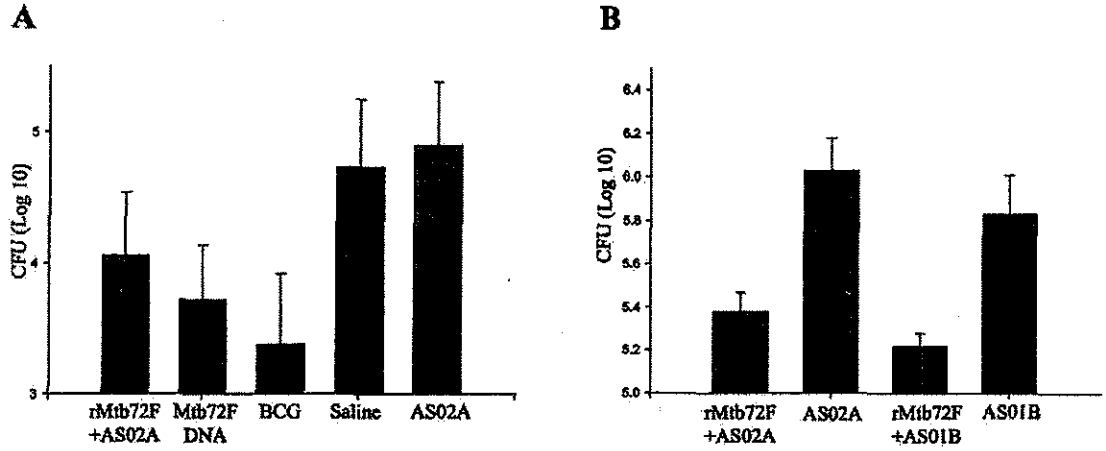

FIGURE 6. Mtb72F protects C57BL/6 mice against aerosol challenge with Mtb. C57BL/6 mice (five animals per group) were injected three times i.m. with 100 μg of Mtb72F-DNA or with 8 μg of rMtb72F formulated in AS02A (*A*) or AS02A vs AS01B (*B*). Control groups were injected once (s.c.) with 5×10^4 CFU of BCG (Aventis Pasteur, Lyon, France) or with saline or adjuvant alone. One month after the last immunization, the animals were challenged with 100 viable aerosolized *M. tuberculosis*, and CFU in the lungs were enumerated 3 wk later.

strains of mice, a mixture comprising several Ags is conceivably a better vaccine for applications in humans because it is less likely to suffer from MHC-related unresponsiveness in a heterogeneous population. In fact, using in vitro responses (proliferative and IFN-γ production) of PBMC from healthy PPD^+ donors as a measure of the extent of coverage of infected responders to a panel of defined TB Ags, a broad-range coverage could only be accomplished with Ag combinations. Therefore, although the murine model is an important first step to determine the nature of the immune responses as well as the protective capacity of Ags, this model does not necessarily predict the outcome in the context of human MHC restriction.

Although the nature of an effective immune response to TB is incompletely understood, particularly in humans, the most effective vaccination strategies in animal models are those that stimulate T cell responses, both $CD4^+$ and $CD8^+$, to produce Th1-associated cytokines. Therefore, formulations that induce the production of enduring Th1 responses are desirable and probably are an essential element of a successful vaccine. Of about a dozen T cell Ags initially identified in the context of host response to infection in infected donors and C57BL/6 mice (21–28), we prioritized Mtb32 (Rv0125) and Mtb39 (Rv1196) as our lead candidates for the development of a TB vaccine. Their potential was further corroborated in animal protection studies suggesting that the combination of Ags was more effective at protecting mice and guinea pigs than the individual subunits (not shown). However, from a practical standpoint for the development of a TB vaccine for developing countries, a vaccine consisting of multiple recombinant proteins would be too expensive to manufacture and formulate. For this reason, together with the difficulties encountered in the expression of a stable form of the full-length secreted version of Mtb32, we engineered for expression in *E. coli* a genetic fusion construct encoding a 72-kDa polyprotein in a contiguous ORF organized in the linear order $Mtb32_C$-Mtb39-$Mtb32_N$ (Mtb72F). In addition to simplifying the manufacturing process, we reasoned that immunization with a single construct may ensure equivalent uptake of the components by APCs and, in turn, generate an immune response that is broadly specific.

Because genetic delivery approaches of vaccine candidates are efficient methods for the elicitation of both $CD4^+$ and $CD8^+$ T cell responses and, as well, an effective vaccination protocol for conferring protection against several infectious disease targets, we used the naked DNA approach to initially determine the nature of the immune responses and the protective efficacy of Mtb72F in C57BL/6 mice. Indeed, we found that immunization of C57BL/6 mice with Mtb72F-DNA elicited both $CD4^+$ and $CD8^+$ IFN-γ responses. The $CD4^+$ T cell response was predominantly directed against Mtb39, whereas the $CD8^+$ T cell response (as assessed by both IFN-γ and CTL) was directed against a single epitope within $Mtb32_C$. We next evaluated the immune responses and protective efficacy of rMtb72F formulated in two novel adjuvant systems, AS01B and AS02A (30–34).

The active ingredients of both adjuvants are 3-deacylated MPL (3D-MPL) (35–38), a nontoxic derivative of LPS and QS21 (a triterpene glycoside purified from the bark of *Quillaja saponaria*, (39–41), and both components have a good clinical safety record (31, 32, 42, 43). The biological properties of MPL are attributed to its immunostimulatory effects on the innate immune system (via activation of the Toll-like receptor 4) and the direct activation of

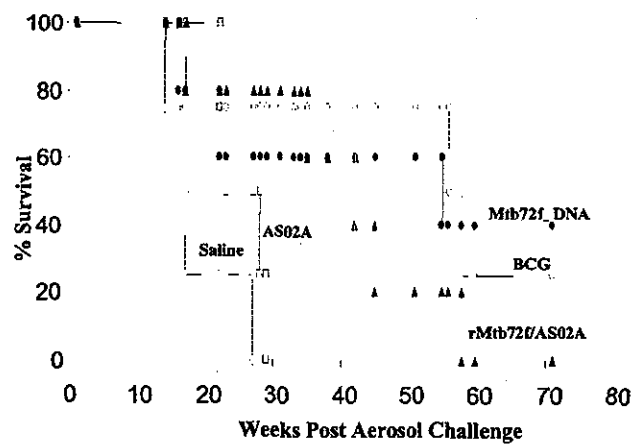

FIGURE 7. Mtb72F protects in the guinea pig model of TB. Guinea pigs were immunized three times i.m with 20 μg of rMtb72F formulated in AS02A or 200 μg of Mtb72F-DNA. Control groups include BCG, saline, and AS02A. Approximately 13 wk after the last immunization, animals were challenged with a virulent strain of Mtb via the aerosol route. Protection was assessed by weight loss and survival.

APCs resulting in enhanced phagocytosis and microbicidal activities as a consequence of the production of IL-12, TNF-α, GM-CSF, and IFN-γ (36, 38, 44, 45). The immunostimulant activity of QS21 extract resides in the saponic fraction and acylation appears to be critical for adjuvant activity (46). QS21 promotes both humoral and cell-mediated immunity when added to parenteral or mucosal vaccine formulations (46). Analysis of cytokine secretion by Ag-specific T cells demonstrated that QS21 augmented Th1 and Th2 responses, whereas addition of 3D-MPL resulted in preferential induction of type 1 T cells (46–49). The distinction between AS01B and AS02A formulations resides in their liposome or oil-in-water emulsion properties, respectively. Both adjuvants as well as their components are currently under clinical evaluation for various vaccines and has been tested in thousands of patients in several clinical trials, including infectious disease vaccines such as malaria (31, 33), hepatitis B (50, 51), and allergy desensitization (52–54). Furthermore, the use of MPL-stable emulsion as an alternate adjuvant to IL-12, known for its Th1-inducing properties, in conjunction with a polyprotein Ag was recently demonstrated as a safe and effective vaccine against *Leishmania* infection (55).

The immunogenicity studies revealed that immunization of C57BL/6 mice with rMtb72F formulated in AS01B elicited an immune response profile that is stronger and broader than those observed with AS02A formulation or with naked DNA delivery. AS01B stood out at eliciting a robust and comprehensive ($CD4^+$,$CD8^+$ T cells and Ab) immune response profile encompassing all three components of the vaccine. Therefore, with an appropriate adjuvant formulation, it is, in fact, possible to generate a $CD8^+$ T cell response (typically associated with DNA or recombinant viral delivery systems) using a recombinant-based vaccine. In addition, with AS01B, a relatively robust $CD4^+$ T cell IFN-γ response was generated against the N-terminal portion of Mtb72F ($Mtb32_C$). This was not detected after immunization with rMtb72F formulated in AS02A or with naked DNA or a recombinant Mtb72F adenovirus. Given this unique aspect of AS01B, experiments are currently underway to determine the strength of AS01B by varying the Ag dose and coupling the immunological readout with protection against TB challenge.

There is a general consensus that the guinea pig is currently the best animal model of human TB, and for this reason, it has been widely used as a model to evaluate new vaccines and vaccine delivery approaches. The central reason for this assumption is the fact that guinea pigs develop granulomas similar to those seen in humans with active TB. Guinea pigs can be used for both short term protection studies (CFU) or as longer term disease models (56–59). As lung tissue necrosis progresses, guinea pigs, like humans, begin to undergo weight loss and eventually die from TB. Successful immunization leads to reduced necrosis, with small lesions characterized by infiltrating lymphocytes, decreased weight loss, and prolonged survival (56, 60). In this model, BCG protects guinea pigs from disease and death for periods of >1 year compared with nonvaccinated animals (56, 58, 60, 61). Thus, more recent and rigorous vaccine evaluation in guinea pigs has focused on using disease and survival as end points instead of only short term bacterial counts, which are not always predictive of long term disease outcome. Of importance to the development of Mtb72F as a vaccine against TB, we report that in this model the delivery of Mtb72F as recombinant protein or DNA protected guinea pigs from death against infection with TB. The extent of protection with Mtb72F represents the longest documented survival end point reported to date for any defined subunit vaccine. In addition, given that the animals in this study were aerosol-challenged with TB at ~13 wk after the third immunization (instead of the standard 4–6 wk after the third immunization), the protective outcome confirms that the immune responses elicited by Mtb72F, delivered as either rAg formulated in AS02A or naked DNA, are long lasting. The protection with Mtb72F DNA immunization was comparable to that with BCG, whereas the efficacy of rMtb72F formulated in AS02A approached that of BCG. At the time these experiments were initiated, the adjuvant AS01B was not available for evaluation. Based upon more recent data obtained with AS01B, experiments are currently underway in the guinea pig model aimed at comparing the protective efficacy of rMtb72F formulated in either AS01B or AS02A.

Even though DNA and viral delivery approaches are good and effective first methods for the screening of vaccine candidates, particularly in small animal models, the apparent ineffectiveness of most DNA vaccines in humans and nonhuman primates and, from a regulatory standpoint, the lack of a sufficient safety record on DNA vaccines make the recombinant subunit-plus-adjuvant approach more desirable. Further, there is sufficient proof of concept for the latter approach given that it has already been demonstrated to be safe and efficacious in several human clinical trials. Therefore, the use of AS01B and AS02A in conjunction with the feasibility of manufacturing Mtb72F polyprotein under GMP conditions represent major developments toward the realization of an affordable and safe vaccine against TB. The rMtb72F formulated in AS02A adjuvant is currently in phase I clinical trial in the U.S., making it the first recombinant TB protein vaccine ever to be tested in humans.

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