Targeting Mycobacterium tuberculosis Tumor Necrosis Factor Alpha-Downregulating Genes for the Development of Antituberculous Vaccines

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ABSTRACT Tumor necrosis factor alpha (TNF) plays a critical role in the control of Mycobacterium tuberculosis, in part by augmenting T cell responses through promoting macrophage phagolysosomal fusion (thereby optimizing CD4+ T cell immunity by enhancing antigen presentation) and apoptosis (a process that can lead to cross-priming of CD8+ T cells). M. tuberculosis can evade antituberculosis (anti-TB) immunity by inhibiting host cell TNF production via expression of specific mycobacterial components. We hypothesized that M. tuberculosis mutants with an increased capacity to induce host cell TNF production (TNF-enhancing mutants) and thus with enhanced immunogenicity can be useful for vaccine development. To identify mycobacterial genes that regulate host cell TNF production, we used a TNF reporter macrophage clone to screen an H37Rv M. tuberculosis cosmid library constructed in M. smegmatis. The screen has identified a set of TNF-downregulating mycobacterial genes that, when deleted in H37Rv, generate TNF-enhancing mutants. Analysis of mutants disrupted for a subset of TNF-downregulating genes, annotated to code for triacylglycerol synthases and fatty acyl-coenzyme A (acyl-CoA) synthetase, enzymes that concern lipid biosynthesis and metabolism, has revealed that these strains can promote macrophage phagolysosomal fusion and apoptosis better than wild-type (WT) bacilli. Immunization of mice with the TNF-enhancing M. tuberculosis mutants elicits CD4+ and CD8+ T cell responses that are superior to those engendered by WT H37Rv. The results suggest that TNF-upregulating M. tuberculosis genes can be targeted to enhance the immunogenicity of mycobacterial strains that can serve as the substrates for the development of novel anti-TB vaccines.

IMPORTANCE One way to control tuberculosis (TB), which remains a major global public health burden, is by immunization with an effective vaccine. The efficacy of Mycobacterium bovis BCG, the only currently approved TB vaccine, is inconsistent. Tumor necrosis factor alpha (TNF) is a cytokine that plays an important role in controlling TB. M. tuberculosis, the causative agent of TB, can counter this TNF-based defense by decreasing host cell TNF production. This study identified M. tuberculosis genes that can mediate inhibition of TNF production by macrophage (an immune cell critical to the control of TB). We have knocked out a number of these genes to generate M. tuberculosis mutants that can enhance macrophage TNF production. Immunization with these mutants in mice triggered a T cell response stronger than that elicited by the parental bacillus. Since T cell immunity is pivotal in controlling M. tuberculosis, the TNF-enhancing mutants can be used to develop novel TB vaccines.

Tuberculosis (TB) is one of the deadliest infectious diseases worldwide (1). It has been estimated that there were 9 million new cases of TB globally in 2013 and that 1.5 million persons died from diseases caused by the tubercle bacillus in that year (1). The propensity of Mycobacterium tuberculosis to persist in an infected host is conducive to the development of persister organisms that are difficult to treat (2). As a result, it takes, on average, 6 to 9 months of multidrug chemotherapy to effectively treat tuberculous infection (3). This requirement causes problems concerning compliance as well as drug toxicity issues, rendering treatment of TB a highly challenging task (1, 3). The emergence of multidrug-resistant and extensively drug-resistant strains of M. tuberculosis presents yet another obstacle to effective TB treatment (1, 3). This hindrance is further complicated by the increased susceptibility to
*M. tuberculosis* of individuals infected with the human immunodeficiency virus (HIV), a pathogen that continues to be a public health threat, as evidenced by the prevalence of HIV/TB coinfection (1, 3). Thus, more effective anti-TB intervention is urgently needed.

Immunization can be an efficacious and cost-effective measure to control infectious diseases (4). For example, the measles vaccine, which is highly efficacious and costs about $17 per disability-adjusted life year, represents a most cost-effective intervention against an infectious agent in developing countries (5). A vaccine of such quality is, however, lacking for the prevention of TB. The difficulty in developing an effective anti-TB vaccine despite the urgent need for one is at least partially due to our lack of understanding of the correlates of protection in tuberculous infection in molecular and biochemical terms (6). The efficacy of *Mycobacterium bovis* BCG, the only approved TB vaccine in use today, is inconsistent (7).

Proper containment of *M. tuberculosis* requires the development of optimal innate and adaptive immune responses, and most healthy individuals can control a tuberculous infection upon exposure to the tubercle bacillus (8–10). The mechanisms by which an infected host controls *M. tuberculosis* are, however, not clearly defined (6, 8–11). Tumor necrosis factor alpha (TNF), a cytokine with a diverse cellular source, has been shown to play a critical role in mice and nonhuman primates in host defense against *M. tuberculosis* during both the acute phase and the chronic persistent phase of infection (12–14). The enhanced risks for TB observed in individuals receiving anti-TNF therapies for a variety of inflammatory diseases have provided strong evidence that this cytokine plays an important role in mediating host defense mechanisms to prevent reactivation of latent TB (15, 16). Excessive TNF production can, however, result in the development of tissue-damaging immunopathology (12–14). Thus, it is generally thought that TNF production during *M. tuberculosis* infection is tightly controlled in order to attain optimal expression of this cytokine so as to contain the tubercle bacillus without collateral damage (14).

Although the precise mechanisms by which TNF mediates antimycobacterial activity remain to be elucidated, evidence exists that this cytokine can enhance phagosome-lysosome maturation (17), a process that promotes antimycobacterial activity, as well as antigen presentation, the latter process capable of enhancing CD4+ T cell response (18). Additionally, TNF can promote apoptosis in mycobacterium-infected macrophages (19, 20), an event that can lead to cross-priming of CD8+ T cells (21). Since T cell responses to *M. tuberculosis* and to immunization play an important role in the control of TB and in the development of vaccine-engendered protective immunity, respectively (6, 8–11, 22), TNF, via its ability to promote phagosome-lysosome maturation and macrophage apoptosis, can potentially enhance T cell-dependent antimycobacterial host defense mechanisms as well as vaccine immunogenicity and efficacy. A corollary of this notion is the possibility that, as a most adept intracellular pathogen, *M. tuberculosis* may downregulate host cell TNF production in order to evade the host antituberculous immune mechanisms. Indeed, it has been demonstrated that specific *M. tuberculosis* genes encode mycobacterial components that can modulate host cell TNF expression, including those that downregulate macrophage production of the cytokine (14, 23–25). Of note, certain mutant *M. tuberculosis* strains deficient in such downregulating elements have been shown to be attenuated for virulence (14, 23–25).

Together, the above-described observations prompted us to hypothesize that targeting TNF-downregulating mycobacterial genes can lead to the generation of mutant strains with enhanced immunogenicity that can be exploited to develop effective vaccine candidates. We have developed a genetic screen that has identified a set of mycobacterial genes whose disruption resulted in H37Rv deletion mutants that could stimulate macrophage TNF production at levels higher than that elicited by WT *M. tuberculosis*. In line with the property of TNF, these mutants, compared to parent bacilli, displayed an enhanced capacity to promote macrophage phagolysosomal fusion and apoptosis. Importantly, mice immunized with these TNF-enhancing mutants engendered CD4+ and CD8+ T cell responses superior to those elicited by WT H37Rv. These studies have provided evidence supporting the notion that targeting TNF-attenuating components in mycobacteria can produce strains with enhanced immunogenicity and therefore that this approach represents a viable approach for the rational design of efficacious TB vaccines.

**RESULTS**

**Generation of a TNF reporter macrophage screening system.** In order to comprehensively identify *M. tuberculosis* genes that mediate functions that enable the tubercle bacillus to downregulate host cell TNF production, we chose a nonbiased genetic approach, using a platform comprising two components: (i) a macrophage system capable of reporting TNF expression and (ii) an *M. tuberculosis* cosmid library in the heterologous *M. smegmatis* strain that enables a gain-of-function screen in infected macrophages. The macrophage was chosen as the surrogate *in vitro* host to study TNF expression upon interaction with the tubercle bacillus because well-studied robust cell lines exist for this immune cell and are amenable to genetic manipulation (26). In addition, the macrophage is a preferred niche for the tubercle bacillus *in vivo* and has been used extensively to study phagosome maturation, antigen presentation, and apoptosis (17–20), processes that are relevant to the present study. Green fluorescent protein (GFP)-based signal was chosen to assess TNF production as this approach enables an expeditious readout. Further, we have previously used a similar reporter system to study how *M. tuberculosis* modulates macrophage interleukin-12 (IL-12) production (26).

To generate a TNF-reporter macrophage system, a J774.16 mouse macrophage line was stably transfected with a TNF promoter–humanized recombinant GFP (hrGFP) II-1 fusion (TNFp-hrGFP II-1) (Fig. 1A). The ability of the transfectants to report TNF expression was assessed by fluorescence microscopy as well as by flow cytometric analysis upon treatment with lipopolysaccharide (LPS), a potent TNF inducer (27) (Fig. 1B and D, left panel). The latter observation demonstrated that the TNF promoter of this clone could be activated in response to mycobacterial infection, as assessed by the production of GFP signals, a prerequisite for the reporter macrophage system of the proposed genetic screen (Fig. 1C). Stable transfectants were subjected to limiting dilution, and one clone, designated C10, was chosen for further testing based on its level of responsiveness to LPS, as assessed both quantitatively and qualitatively (Fig. 1D, left panel). The functionality of clone C10 was assessed both *in vitro* and *in vivo* using a platform comprising two components: (i) a macrophage reporter system of the proposed genetic screen (Fig. 1C). Stable transfectants were subjected to limiting dilution, and one clone, designated C10, was chosen for further testing based on its level of responsiveness to LPS, as assessed both quantitatively and qualitatively (Fig. 1D, left panel). The functionality of clone C10 was assessed both *in vitro* and *in vivo* using a platform comprising two components: (i) a macrophage reporter system (ii) an *M. tuberculosis* cosmid library in the heterologous *M. smegmatis* strain that enables a gain-of-function screen in infected macrophages. The macrophage was chosen as the surrogate *in vitro* host to study TNF expression upon interaction with the tubercle bacillus because well-studied robust cell lines exist for this immune cell and are amenable to genetic manipulation (26). In addition, the macrophage is a preferred niche for the tubercle bacillus *in vivo* and has been used extensively to study phagosome maturation, antigen presentation, and apoptosis (17–20), processes that are relevant to the present study. Green fluorescent protein (GFP)-based signal was chosen to assess TNF production as this approach enables an expeditious readout. Further, we have previously used a similar reporter system to study how *M. tuberculosis* modulates macrophage interleukin-12 (IL-12) production (26).

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suscitation promoting factor A\(^1\) and \(rpfB\) alleles, genes that code for apparent peptidoglycan hydrolases \[28\], and the \(\Delta secA2\) mutant, deleted for the accessory secretion factor of the Sec-dependent protein export pathway) \[23\] that have previously been shown to induce macrophage TNF production at levels higher than that elicited by WT bacilli. The results demonstrated that the C10 macrophage clone could discriminate \(M.\) \(tuberculosis\) mutant strains with differential TNF-inducing capacities (Fig. 1D, right panel). C10 was used for the screen to identify TNF-regulating genes throughout this study.

Identification of \(M.\) \(tuberculosis\) genes involved in down-regulating macrophage TNF production. Certain relatively avirulent mycobacterial strains stimulate a higher level of expression of TNF in infected macrophage cultures than virulent pathogenic strains \[29–31\]. Relevant to the present study, it has been shown that the ability of the relatively avirulent \(M.\) \(smegmatis\) species to induce TNF production significantly exceeds that of the virulent \(M.\) \(tuberculosis\) H37Rv strain in both infected human and infected mouse macrophage cultures \[29, 30\]. In addition, virulent \(M.\) \(tuberculosis\), upon deletion of specific genes that lead to deficiency in certain bacterial components, exhibits an increased capacity to induce macrophage TNF production relative to the WT parental strain \[23–25\]. These observations have led us to posit that the relatively avirulent \(M.\) \(smegmatis\) species could be used as the sub-

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FIG 1 Generation of a C10 TNF reporter macrophage screen system. (A) The TNF promoter (TNFp) used for the generation of the GFP reporter was derived from a fragment released by KpnI and BamHI digestion of the “Pro-UTR” construct (kindly provided by Jiahuai Han \[27\]). The released TNFp was cloned into the modified phrGFP II-I vector (m-phrGFP II-I), whose CMV promoter has been deleted, upstream from the hrGFP II-I to generate the TNFp-hrGFP II-I fusion reporter construct contained in the m-phrGFP II-I vector (designated TNFp-hrGFP II-I). (B) The TNFp-hrGFP II-I fusion construct was stably transfected into J774.16 macrophages. G418 (1 mg/ml)-resistant transfectants were selected for and observed for GFP signals upon LPS treatment (1 \(\mu g/ml\)) by fluorescence microscopy. (C) J774.16 macrophages stably transfected with TNFp-hrGFP II-I respond to BCG infection (multiplicity of infection of 10:1). (D) Limiting dilution of stably transfected GFP-positive macrophages yielded clone C10, which responded well to LPS to generate GFP signal quantitatively and qualitatively (left panel). Importantly, C10 can differentiate between \(M.\) \(tuberculosis\) mutant strains with an enhanced capacity to induce macrophage TNF production (the \(\Delta rpfAB\) and \(\Delta secA2\) mutants) relative to WT bacilli (right panel). 10:1, multiplicity of infection of 10 bacilli to 1 macrophage.
strate for a nonbiased gain-of-function (attenuation of TNF production) screen to identify *M. tuberculosis* genes that mediate TNF-regulating functions. Accordingly, we initiated experiments to generate an H37Rv cosmid library in the heterologous *M. smegmatis* species for this screen. pYUB412-based H37Rv cosmids derived from a library built in *Escherichia coli* (32) were transformed into *M. smegmatis* mc²155 (33), resulting in the generation of 105 clones that cover ~50% of the *M. tuberculosis* genome. The pYUB412-based cosmids exist extrachromosomally in clones that cover ~50% of the *M. tuberculosis* genome. The pYUB412 vector (pYUB412 containing no H37Rv DNA) or *M. tuberculosis* genome containing no H37Rv DNA (Fig. 2). That TNF-downregulating clones 39 and 40 shared a three-gene overlapping region led us to directly clone the xylB-rv0730-rv0731c fragment into pMV361 to assess its TNF-modulating capacity. rv0729 (xylB) is a putative D-xylulose kinase that can participate in the synthetic pathway of arabinose (34, 35). rv0730 has been annotated as a GCN5-related N-acetyltransferase (GNAT family of N-acetyltransferases) that can potentially cross-link peptidoglycans (36). rv0731c has been annotated as an S-adenosylmethionine-dependent methyltransferase which may participate in mycolic acid synthesis (37). Therefore, all three genes located in the overlapping regions of clones 39 and 40 have been annotated to mediate functions that can have an impact on carbohydrate (xylB and rv0730) and lipid (rv0731c) metabolism.

Analysis of clone 40 revealed yet another subclone, named 40-5, that could mediate TNF-downregulating activity in infected C10 cultures (Fig. 3). Subclone 40-5 encompasses rv0755A (a fragment of a putative transposase), thrV (anticodon tRNA-Thr), and rv0756c (hypothetical protein). Subclone 164-2, which contains three genes from the acid-responsive *mymA* operon (38, 39), rv0730, rv0731, and rv0732 also displayed significant TNF-downregulating capacity relative to the controls, as assessed by
interaction between of the outer surface of the tubercle bacillus, thereby altering the production of the cytokine is by modifying the chemical composition of the cell envelope (40, 41), one possible way that these mycobacterial TNF-downregulating genes might modulate C10 production. Since lipids and carbohydrates are major constituents of the cell envelope (40, 41), one possible way that these mycobacterial TNF-downregulating genes might modulate C10 production is by modifying the chemical composition of the outer surface of the tubercle bacillus, thereby altering the interaction between M. tuberculosis and macrophages. The TNF-downregulating property of subclones 40-5 and 164-2, xylB-rv0730-rv0731c, and individual genes xylB and rv0370, as assessed by GFP signal of infected C10 macrophages (Fig. 3A), was confirmed by direct ELISA quantification of the amount of the cytokine in the corresponding culture supernatants (Fig. 3B). The only discordance was apparent in the analysis of rv0730, which displayed lower but statistically nonsignificant C10 fluorescence signal but was found to produce significant TNF downregulation based on ELISA analysis. The latter results, similarly to those presented in Fig. 2, again illustrate the reliability of the C10 reporter system.

M. tuberculosis mutants deleted for TNF-downregulating genes display an enhanced capacity to induce TNF production by infected macrophage cultures. The C10 screen of 105 M. smegmatis clones harboring H37Rv DNA identified a set of M. tuberculosis genes that could mediate functions involved in downregulation of the capacity of mc²155 to induce macrophage TNF production. Because these genes, when knocked into heterologous M. smegmatis, reduced TNF production of infected C10, we reasoned that the respective M. tuberculosis deletion mutants would be strains that would enhance the expression of the cytokine in infected macrophage cultures. To begin testing the possibility that such TNF-upregulating mutants could exhibit enhanced immunogenicity, we chose to focus on the two genes encoding triacylglycerol synthases (rv3087 and rv3088) and the gene encoding fatty acyl-CoA synthetase (fadD13). The choice to focus on analyzing the Δrv3087, Δrv3088, and Δrv3089 mutants was prompted by the fact that, while the validity of the functional annotation of xylB, rv0730, rv0731c, rv3087, and rv3088 has not been tested, results obtained from biochemical analysis of the products of the two genes encoding triacylglycerol synthases (rv3087 and rv3088) and of the gene encoding fatty acyl-CoA synthetase (rv3089) in in vitro systems support the validity of their function assignments (42–44). In addition, since the two putative triacylglycerol synthases can possibly affect lipid metabolism and biosynthetic pathways via overlapping or distinct functions, a mutant doubly deleted for rv3087 and rv3088 (the Δrv3087 Δrv3088 mutant) was generated to examine whether the resultant strain displayed an additive or a synergistic TNF-downregulating effect. Finally, since two of the TNF-downregulating genes have been annotated as encoding triacylglycerol synthases, we tested the Δrv3130c strain, a mutant deleted for Tgs1 (encoded by a gene located outside the mymA operon and reportedly the most enzymatically active of the 15 potential M. tuberculosis triacylglycerol synthases).
that virulent processing. Cytokine in infected macrophages. To induce macrophage TNF production can be generated by targets to arrest phagosomal maturation, thereby avoiding the hostile gene under the control of the conducted, and in each case, expression of the complementing was specific to the deleted alleles, complementation studies were ELISA study (Fig. 4). To show that this TNF-modulating effect macrophage TNF production upon infection, as assessed by ELISA. All deletion mutants induced significantly higher production of TNF by infected macrophage cultures than H37Rv. The TNF-upregulating phenotype of the deletion mutants can be reversed by complementation with the corresponding WT genes. Values are the means of the results of triplicates ± SD and are representative of three separate experiments.

***, P < 0.001.

FIG 4 Analysis of H37Rv mutants deleted for genes harbored in TNF-downregulating subclone 164-2. Identified specific M. tuberculosis alleles that mediates functions capable of attenuating C10 macrophage TNF production. Subclone 164-2 harbors rv3087 and rv3088 (both annotated to code for triacylglycerol synthase) and rv3089 (annotated to encode a fatty acyl-CoA synthetase), enzymes that are involved in lipid metabolic and synthesis pathways. J774.16 macrophages were infected at an MOI of 10 with H37Rv, deletion mutants, or complemented strains (designated by the suffix "c") or were left untreated. At 16 h postinfection, the amounts of TNF in culture supernatants were quantified by ELISA. M. tuberculosis rv3087 deletion mutants, which exhibited an enhanced capacity to induce macrophage TNF production upon infection, as assessed by ELISA study (Fig. 4). To show that this TNF-modulating effect was specific to the deleted alleles, complementation studies were conducted, and in each case, expression of the complementing gene under the control of the hsp60 promoter via the integrating pMV361 reversed the TNF-upregulating phenotype of the corresponding deletion mutant. These results essentially confirm that the TNF-upregulating phenotype of the various H37Rv knockouts studied was specific to the deleted genes and validate our hypothesis that M. tuberculosis strains with an enhanced capacity to induce macrophage TNF production can be generated by targeting genes that mediate downregulation of the expression of this cytokine in infected macrophages.

TNF-enhancing M. tuberculosis mutants affect endosomal processing. Ample experimental evidence supports the notion that virulent M. tuberculosis strains employ a wide array of strategies to arrest phagosomal maturation, thereby avoiding the hostile environment of the lysosome (46–49). One of the consequences of the ability of the tubercle bacillus to prevent phagosomal fusion could be suboptimal presentation of antigens to T cells. It has been previously demonstrated that TNF promotes phagosomal fusion (17) and, as a result, could enhance CD4+ T cell responses by optimizing antigen processing and presentation via the major histocompatibility complex class II (MHC-II) pathway (18). It is therefore possible that M. tuberculosis TNF-enhancing mutants can promote phagosomal fusion in infected macrophages, which, in turn, can lead to augmentation of T cell immunity. To begin testing this possibility, the level of fusion with lysosomes of phagosomes containing WT H37Rv was compared to the level seen with those harboring TNF-enhancing mutants. J774.16 macrophages were infected with WT M. tuberculosis H37Rv and the various TNF-enhancing deletion mutants labeled with fluorescein-5-isothiocyanate (FITC). Rab5 and LAMP1 (lysosome-associated membrane protein 1), the markers for early and late endosomal compartments, respectively, were tracked via the use of fluorescently tagged specific antibodies (Abs) against these surface molecules. Colocalization of FITC-tagged bacilli and the fluoroently labeled Rab5 and LAMP1 was assessed by confocal fluorescence microscopy in a blind fashion. The results revealed that WT H37Rv preferentially localized to the early endosomal compartment. The levels of localization of all five TNF-enhancing M. tuberculosis mutants to the Rab5-positive early endosomal compartments were significantly lower than those seen with WT H37Rv (Fig. 5A and C). Conversely, all five mutants localized to the LAMP1-positive late endosomal vesicles at a level higher than that observed with the WT parental strain (Fig. 5B and D). Together, these results lend support to the notion that targeting TNF-downregulating M. tuberculosis genes is a viable strategy to generate TNF-enhancing strains that, relative to WT bacilli, are attenuated for their capacity to block phagosome-lysosome fusion and, therefore, should promote antigen presentation, thereby augmenting the CD4+ T cell response.

TNF-enhancing M. tuberculosis mutants promote macrophage apoptosis upon infection. Apoptosis of M. tuberculosis-infected macrophages is beneficial to the host in that the process can kill tubercle bacilli and the apoptotic vesicles can be usurped to enhance T cell responses by cross-priming CD8+ T cells (21, 50). TNF can promote apoptosis in mycobacterium-infected macrophages (19, 20). We therefore proposed that M. tuberculosis bacteria that have been genetically engineered to upregulate TNF expression in infected macrophages represent strains with enhanced immunogenicity due to the effect of this cytokine on apoptosis. Adept in evading the host immune response, the tubercle bacillus has evolved means to counter this host defense mechanism. Indeed, antiapoptotic M. tuberculosis factors have been shown to impart tuberculous virulence (23, 51, 52). Relatively avirulent mycobacteria are superior to virulent strains in inducing macrophage apoptosis, and this process is dependent on the presence of TNF (53), a well-established inducer of the extrinsic pathway of apoptosis (50). Further, compared to avirulent mycobacteria, virulent M. tuberculosis has been shown to downregulate the expression of proapoptotic genes in infected macrophages (20). These observations have prompted us to speculate that the M. tuberculosis Δrv3087, Δrv3088, Δrv3087 Δrv3088, ΔfadD13, and Δigs1 deletion mutants, which exhibited an enhanced capacity to induce macrophage TNF production, can be more proapoptotic than WT H37Rv. Macrophage apoptosis was monitored by assessing the activity of effector caspases 3 and 7 using the caspase inhibitor-based FLICA (fluorochrome-labeled inhibitors of caspases) assay, in conjunction with laser scanning cytometry. The results depicted in Fig. 6 reveal that, indeed, TNF-enhancing M. tuberculosis strains stimulated significantly higher levels of activated caspases than the WT strain, demonstrating the proapoptotic property of these mutants.
Correlation between the phagosome maturation and apoptosis phenotypes of the TNF-upregulating M. tuberculosis mutants and enhanced TNF production in infected macrophages. Apoptosis and phagolysosomal fusion are highly regulated processes that involve complex mechanisms, including those that are TNF independent (46–50). Consequently, we initiated studies to probe the TNF specificity of the apoptosis- and phagolysosomal fusion-promoting phenotypes of the TNF-upregulating mutants using the TNF-neutralizing monoclonal antibody (MAb) MP6-XT22. For these studies, the TNF-downregulating rv3089 (fadD13) and rv3130c (tgs1) alleles were evaluated using the corresponding single deletion mutants, while rv3087 and rv3088 were assessed in the context of the \( \Delta^{H37Rv}rv3087/\Delta^{H37Rv}rv3088 \) double knockout strain. The results of these experiments have shown that the phagolysosomal fusion-promoting capacity of the three deletion mutants examined, as assessed by colocalization of FITC-labeled bacilli with late endosomal compartments, can be attenuated by the TNF neutralization (Fig. 7A). However, the MP6-XT22-mediated attenuation did not cause the level of colocalization to revert to that observed in TNF-neutralized, WT H37Rv-infected macrophage cultures. This partial TNF dependency of the colocalization of the mutant strains with LAMP1-positive compartments in infected macrophages suggests that TNF-independent mechanisms are operative in regulating phagosome maturation by the \( \Delta^{H37Rv}rv3087/\Delta^{H37Rv}rv3088 \), \( \Delta^{H37Rv}rv3089 (\Delta^{H37Rv}fadD13) \), and \( \Delta^{H37Rv}rv3130c (\Delta^{H37Rv}tgs1) \) mutants (Fig. 7A). The MP6-XT22 \textit{in vitro} infection system was also utilized to examine the relationship between the apoptosis-promoting attribute of the various mutant strains and their TNF-enhancing capacity. The results of these experiments have revealed that TNF neutralization resulted in a decrease in the level of apoptosis in macrophages infected with the \( \Delta^{H37Rv}rv3087/\Delta^{H37Rv}rv3088 \) mutant, the \( \Delta^{H37Rv}rv3089 (\Delta^{H37Rv}fadD13) \) mutant, or the \( \Delta^{H37Rv}rv3130c (\Delta^{H37Rv}tgs1) \) H37Rv mutant (Fig. 7B). This decrease, however, reached statistical significance only in cultures infected with the \( \Delta^{H37Rv}rv3087/\Delta^{H37Rv}rv3088 \) mutant or the \( \Delta^{H37Rv}rv3089 (\Delta^{H37Rv}fadD13) \) mutant and not in those infected with the \( \Delta^{H37Rv}rv3130c (\Delta^{H37Rv}tgs1) \) strain (Fig. 7B). As in the phagosome maturation study, TNF neutralization fell short of attenuating the apoptosis seen in cultures infected with the \( \Delta^{H37Rv}rv3087/\Delta^{H37Rv}rv3088 \) mutant or the \( \Delta^{H37Rv}rv3089 (\Delta^{H37Rv}fadD13) \) mutant to a level comparable to that observed in MP6-XT22-treated macrophages harboring WT \( M. tuberculosis \) (Fig. 7B). Together, these results suggest that the ability of the TNF-upregulating mu-
tants to promote apoptosis and phagosome maturation is partially due to their ability to enhance macrophage production of this cytokine and that the TNF specificity of the phenotypes can be gene dependent.

Immunization with TNF-enhancing \textit{M. tuberculosis} mutants elicited a T cell response superior to that engendered by WT bacilli. The phagolysosomal fusion- and apoptosis-promoting properties of the TNF-enhancing \textit{M. tuberculosis} mutants predicted that these strains should exhibit enhanced immunogenicity, in part via optimization of antigen presentation to CD4$^{+}$/H11001 T cells through the MHC-II pathway (17) and cross-priming of CD8$^{+}$/H11001 T cells (21), respectively. To determine the immunogenicity of the TNF-enhancing strains, C57BL/6 mice were immunized subcutaneously with $1 \times 10^6$ CFU of WT H37Rv or the various deletion mutants. The vaccination-engendered T cell response was assessed by quantification of the level of gamma interferon (IFN-$\gamma$)-producing splenic CD4$^{+}$/H11001 and CD8$^{+}$/H11001 T cells at 1 month postinoculation using the enzyme-linked immunosorbent spot (ELISPOT) assay. The results shown in Fig. 8 demonstrate that all five TNF-enhancing \textit{M. tuberculosis} deletion mutants (the $\Delta$rv3087, $\Delta$rv3088, $\Delta$rv3087 $\Delta$rv3088, $\Delta$fadD13, and $\Delta$tgs1 strains) induced a significantly higher level of \textit{M. tuberculosis} ESAT-6 (6-kDa early secretory antigenic target)-specific IFN-$\gamma$-producing CD4$^{+}$ T cells than the WT H37Rv bacilli. Similarly, all TNF-enhancing mutants elicited a significantly higher level of splenic IFN-$\gamma$-positive CD8$^{+}$ T cells with respect to \textit{M. tuberculosis} GAPDH (glyceraldehyde 3-phosphate dehydrogenase) than the WT tubercle bacilli (Fig. 8). These immunization studies support the notion that targeting TNF-downregulating \textit{M. tuberculosis} genes is a viable strategy to produce strains with enhanced immunogenicity that can possibly serve as the substrates for developing effective anti-TB vaccines.

**DISCUSSION**

TNF is arguably the best-established antimycobacterial immunological factor in humans, as reflected by the enhanced susceptibility to \textit{M. tuberculosis} infection of individuals treated with TNF blockade therapy (15). This relevance in human TB has prompted extensive investigative efforts to study the role of TNF in shaping the immune response during \textit{M. tuberculosis} infection (13). As one of the most tenacious intracellular pathogens, virulent \textit{M. tuberculosis} is endowed with components of diverse biochemical properties that can attenuate host cell production of TNF (12–14). These mycobacterial countermechanisms are relevant in tuberculous pathogenesis, as evidenced by the observation that ablation of certain TNF-attenuating mycobacterial factors can lead to attenuation of virulence (14, 23–25). By virtue of its ability to promote phagosome maturation (17) and apoptosis (19, 20) in \textit{M. tuberculosis}-infected macrophages, TNF can enhance T cell responses (18, 21). The latter property has implications in TB vaccine development, since T cell immunity plays a significant role in engendering immunization-induced protective responses (22). Our objectives in this study were first to identify genetic loci involved in the attenuation of host TNF production, using a TNF reporter macrophage system to screen an
M. tuberculosis cosmid library, and then to test whether disruption of these genes resulted in strains of tubercle bacilli that can elevate host TNF production and enhance anti-TB immunity. Focusing on a subset of TNF-enhancing mutants, the results of these proof-of-concept experiments have provided strong evidence supporting the notion that targeted disruption of M. tuberculosis genes that mediate down-regulation of the production of TNF by infected macrophages can lead to the generation of mutant strains with enhanced immunogenicity that can therefore serve as the substrates for vaccine development.

FIG 7 Correlation between the phagosome maturation and apoptosis phenotypes of the various M. tuberculosis mutants and their capacity to enhance macrophage TNF production. (A) For the phagosome maturation study, 1 x 10^6 cells of J774.16 macrophages per well were cultured in eight-well chambered slides with or without the TNF-neutralizing MP6-XT22 MAb (final concentration, 10 μg/ml) for 16 h. The macrophages, in culture medium with or without MP6-XT22, were then synchronously infected with FITC-stained WT M. tuberculosis H37Rv or the various TNF-upregulating mutants. Macrophages were washed to remove extracellular bacilli after 4 h of infection, and cultures were replenished with medium with or without MP6-XT22. Cells were fixed with 4% paraformaldehyde after incubation for an additional 4 h, permeabilized, and allowed to react with primary antibodies against LAMP1, followed subsequently by staining with fluorescently tagged secondary antibodies. Analysis performed with confocal microscopy has revealed that colocalization of M. tuberculosis mutants with LAMP1 can be significantly attenuated by TNF neutralization but that the attenuation did not attain the level of that observed in MP6-XT22-treated, WT bacillus-infected macrophages. (B) For the apoptosis study, J774.16 cells were similarly cultured, in the presence or absence of MP6-XT22, in wells of 96-well plates (MatriPlate; Brooks Life Science System) at 1 x 10^6 macrophages per well. Cultures were infected with the WT or with the various mutant strains of M. tuberculosis for 4 h and were then incubated for 16 h after removal of extracellular bacilli before being subjected to analysis for evidence of apoptosis using a FAM-FLICA polycaspase kit. Analysis by laser scanning cytometry (iCys; Thorlabs) has revealed that, relative to the levels seen with the MP6-XT22-treated WT H37Rv-infected cultures, the apoptosis-promoting capacity of the Δrv3087 Δrv3088 and Δrv3089 (ΔfadD13) strains can be partially and significantly attenuated upon TNF neutralization. MP6-XT22 treatment had no significant effects on the apoptosis-enhancing capacity of the Δrv3130c (Δgs1) strain. Values are the means of the results of triplicates ± SD. *, P < 0.05; **, P < 0.005.

FIG 8 TNF-upregulating mutants enhance the IFN-γ response in CD4⁺ and CD8⁺ T cells. C57BL/6 mice were immunized subcutaneously with 10⁶ bacilli of either H37Rv or TNF-enhancing mutants. After 30 days, IFN-γ-producing splenic CD4⁺ and CD8⁺ T cells were quantified by ELISPOT assay. Mice immunized with TNF-enhancing mutants developed more IFN-γ-producing CD4⁺ T cells (A) and CD8⁺ T cells (B) than animals vaccinated with WT H37Rv. **, P < 0.005; ***, < 0.001 (unpaired Student’s t test). Values are the means ± SD of the results from experiments performed in triplicate and are representative of three separate experiments.
Screening approximately half of the H37Rv genome yielded a set of genes with TNF-downregulating attributes, the majority of which have been annotated to encode functions related to metabolism of lipids or carbohydrates, macromolecules that are major components of the \textit{M. tuberculosis} cell envelope (40, 41). It is thus possible that the cell surface of these TNF-enhancing mutants, which presents an interface for the interaction between the bacilli and host cells, is chemically distinct from that of WT H37Rv. The altered chemical nature of the mutant envelope can also lead to changes in its architecture that, in turn, can result in unmasking or concealing of components that interact with host cells. The altered cell envelope of the mutants can thus result in aberrant interaction of the bacilli with macrophages, perhaps via specific surface molecules such as the pattern recognition receptors (54--56), thereby leading to differential expression of TNF. Indeed, precedents exist that indicate that \textit{M. tuberculosis} lipid and carbohydrate moieties can alter macrophage TNF production and that this can lead to modulation of virulence (14, 23--25). Relevant to this notion, it has been reported that inactivation of the acid-responsive \textit{mymA} operon (38, 39), which harbors three of the TNF-enhancing genes, \textit{rv3087}, \textit{rv3088}, and \textit{rv3089} (\textit{fadD13}), results in the production of anomalous mycolic acid species as well as in aberrant cell colony morphology and envelope architecture (57--59). The commonality of \textit{rv3087} and \textit{rv3088} as encoding putative triacylglycerol synthases (Tgs) and, in addition, displaying Tgs activity \textit{in vitro} may shed light on the mechanisms by which the members of this class of molecules modulate macrophage TNF production. Indeed, disruption of \textit{rv3130c} (\textit{tgs1}), encoding the triacylglycerol synthase with the most robust enzymatic activity among the 15 putative \textit{M. tuberculosis} Tgs (42), resulted in an H37Rv mutant that also upregulated macrophage TNF production. \textit{Rv3130c} is perhaps the best-studied \textit{M. tuberculosis} Tg (42, 45, 60); it is the Tgs that is the most highly induced upon hypoxia and NO treatment, conditions which are likely encountered inside in the host (42). Deletion of \textit{Rv3130c} resulted in nearly complete loss of triacylglycerol (TAG) synthesis (60), and there is evidence that this Tgs plays an important role in regulating energy storage in the form of TAG, which may be required for tuberculous persistence and reactivation (45). Finally, TAG can also serve as a source of fatty acids for phospholipid synthesis, thus influencing the properties of membrane lipid bilayers and their associated components (61). Of note, while the double deletion mutant (the \textit{Δrv3087 Δrv3088} mutant) deficient for the two putative Tgs’s \textit{Rv3087} and \textit{Rv3088} displayed an apoptosis phenotype that was stronger than that of the single \textit{tgs} knockouts, its effect on phagosome maturation is comparable to that exhibited by the single deletion mutant. The latter observation suggests a complex role of Tgs in modulating host cell-bacterium interactions. It is also possible that \textit{Rv3087} and \textit{Rv3088} might possess yet-to-be-elicited biochemical functions other than that of triacylglycerol synthase. Thus, despite the putative assigned functions shared among \textit{Rv3087}, \textit{Rv3088}, and \textit{Tgs1}, the mechanisms by which these enzymes mediate TNF-enhancing properties remain to be determined.

The \textit{M. tuberculosis} FadD family members, which play an important role in activating fatty acids, a critical first step for the generation of acyl-CoA via an acyladenylate intermediate for lipid biochemical reactions, can be subdivided into two classes—the fatty acyl-CoA synthetases (fatty acyl-CoA ligases [FACL]) and the fatty acyl-AMP ligases (FAAL) (62). \textit{In vitro} biochemical analyses using recombinant protein and \textit{in silico} molecular modeling have shown that FadD13 preferentially activates long-chain fatty acids (C\textsubscript{24}/C\textsubscript{16}, versus C\textsubscript{16}/C\textsubscript{10}) via FACL activity (43, 44) and may thus play a role in the biosynthesis of the C\textsubscript{60, 80} mycolic acids (63, 64), whose derivative trehalose dimycolate (TDM) induces host cell TNF production through interaction with cell surface receptors MARCO and Mincle (54, 55, 65). Existing knowledge of the functions of FadD and Tgs suggests that these proteins contribute to modulating the chemical composition and architecture of the mycobacterial cell envelope. Characterization of the cell-envelope-related mechanisms by which the lipid mutants upregulate TNF production will likely help efforts to gain insight into processes underlying TB pathogenesis and host defense and may lead to the discovery of bacterial surface-associated molecules that can be exploited for use in the development of TB vaccines.

Importantly, disruption of genes encoding enzymes known to regulate lipid metabolism (\textit{rv3087}, \textit{rv3088}, \textit{rv3089} \textit{[fadD13]}, and \textit{rv3130c} \textit{[tgs1]}) individually (or doubly for \textit{rv3087} and \textit{rv3088}) in WT H37Rv produces deletion mutants that upregulate macrophage TNF production relative to the levels seen with parental bacilli, and complementation of the deletion mutants with the corresponding WT genes reverses the TNF phenotype, proving gene specificity. Relative to WT bacilli, these mutant strains are bestowed with an increased capacity to promote macrophage phagolysosomal fusion and apoptosis, lending support to our hypothesis that TNF-enhancing \textit{M. tuberculosis} mutants can be more immunogenic than the WT bacillus. Indeed, results of the immunization studies support this notion.

Worthy of note, the results of the TNF neutralization experiments have provided evidence suggesting that the phagolysosomal fusion- and apoptosis-promoting phenotypes observed in macrophages infected with the gene deletion mutants of \textit{M. tuberculosis} (the \textit{Δrv3087 Δrv3088}, \textit{Δrv3089} \textit{[fadD13]}, and \textit{Δrv3130c} \textit{[tgs1]} mutants) are not totally TNF dependent. Given the complexity of the mechanisms that regulate apoptosis (50) and phagosome maturation (46--49) in \textit{M. tuberculosis}-infected macrophages, including those that involve TNF-independent pathways, the lack of absolute correlation between the capacities of the TNF-upregulating mutants and their proapoptosis and phagolysosomal fusion phenotypes is not surprising. It is possible that the deletion mutants investigated in the present study can influence the expression of cytokines and/or other host factors, in addition to TNF, which can modulate apoptosis and phagosome maturation. The idea of the existence of such a possibility is supported by the observations derived from the study of a phenolic glycolipid (PGL)-deficient mutant of HN878, a virulent \textit{M. tuberculosis} strain of the Beijing family, which upregulates macrophage production of at least three cytokines, TNF, IL-6, and IL-12 (24), demonstrating that alteration of a single mycobacterial product could have diverse effects on macrophage responses upon infection. The effect on macrophage expression of cytokines other than TNF and/or other host factors upon infection by the mutants examined in the present study remains to be examined. Detailed characterization of the events that ensue following interactions of the TNF-upregulating \textit{M. tuberculosis} strains with macrophages should shed light on the precise mechanisms underlying the apoptosis and phagolysosomal fusion phenotypes of these mutants.

One major goal of the development of vaccines against pathogens is the attainment of a long-term robust memory response (22, 66). Observations derived from various immunological models have provided evidence suggesting that certain inflammatory
cytokines, including TNF, may promote CD4 T cell activation and proliferation during the priming phase of the immune response, which may lead to enhancement of T cell memory (67–69). Indeed, it has been reported recently that a vaccination protocol using BCG-infected macrophages in conjunction with IL-1, IL-6, and TNF administration engenders protection in mice that is superior to that elicited by the regimen without the cytokines or by BCG alone upon challenge with virulent bacilli 8 months postimmunization (70). Interestingly, the enhanced protection displayed by mice vaccinated with BCG-infected macrophages concomitant with IL-1, IL-6, and TNF is associated with an augmented CD4 and CD8 T cell memory response. These results suggest that TNF might modulate the initial phase of T cell activation, which, in turn, can promote the development of robust, long-lasting T cell memory. Whether immunization with the M. tuberculosis mutants evaluated in the present study, which possess the capacity to enhance TNF production in infected macrophages, can elicit a rigorous and long-term memory T cell response that can be recalled to mediate protection against challenge with virulent tubercle bacilli remains to be determined.

In sum, results generated from this study have provided strong evidence that targeting mycobacterial TNF-downregulating genes is a viable approach to augment the immunogenicity of potential vaccine candidates. While that was a major goal of the study, effort expended in this work has generated a set of tools that should prove useful beyond the present research. The C10 TNF reporter macrophage clone has proven effective in identifying TNF-modifying M. tuberculosis genes and will be used to screen the remaining half of the cosmid library, which is currently under construction. This cosmid library proved invaluable for the present study, but its utility can be exploited to identify genes responsible for any defined phenotype that can be assessed by an effective in vitro system. The set of TNF-enhancing mutants can be exploited for studies beyond vaccine development; for example, understanding the biology of these strains will likely be informative regarding the roles of TNF in shaping the host response during tuberculous infection in a system where the level of TNF expression is locally manipulated by the tubercle bacillus.

**MATERIALS AND METHODS**

**Bacterial strains, growth media, and preparation for macrophage infection.** M. smegmatis mc‘155 was cultured as previously described (32), with modification. Starter cultures of M. smegmatis clones of the H37Rv cosmid library were initiated by inoculating 5 μl of individual clones from frozen stocks into 1 ml of Middlebrook 7H9 medium supplemented with 0.5% glycerol, 10% oleic acid–albumin–dextrose–catalase (OADC) enrichment (Becton, Dickinson), 0.05% tyloxapol (Sigma), and 50 μg/liter hygromycin (Roche). Strain mc‘155 transformed with pYUB412 with no H37Rv genomic DNA (empty vector) was similarly cultured. WT bacteria were grown without antibiotics. Bacteria from starter cultures were reseeded into fresh media and grown to mid-log phase, pelleted, washed in phosphate-buffered saline (PBS)–tyloxapol (Sigma), and sonicated to disrupt clumps. A portion of the bacterial suspension was used for measurement of the optical density at 590 nm (OD590). The remaining bacterial preparation was adjusted to a concentration of 1 × 10⁶ CFU in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, and 1× nonessential amino acids (complete DMEM) for use in infection of C10 macrophages.

**M. tuberculosis H37Rv and various deletion mutants were cultured at 37°C with shaking in Middlebrook 7H9 broth containing 0.2% glycerol, 0.05% Tween 80, and 10% OADC enrichment medium (Becton, Dickinson).** Deletion mutants and complemented strains were grown in similarly supplemented 7H9 medium containing hygromycin (Roche) (50 μg/ml) and kanamycin (Roche) (20 μg/ml), respectively. In preparation for macrophage infection, various M. tuberculosis strains were grown to mid-log phase. Bacteria were washed in complete DMEM and sonicated, and the OD590 was measured. The bacterial suspension was adjusted to appropriate titers for infection of J774.16 macrophages.

**Generation of M. tuberculosis gene deletion mutants and complemented strains.** The various gene deletion mutants were generated in the WT H37Rv strain using the mycobacteriophage-mediated specialized transduction method as previously described (32, 71). Complementation of the WT gene of interest (driven by the hsp60 promoter) into the attB site of the corresponding M. tuberculosis knockout strain was achieved using the integrating vector pMV361 as previously described (32).

**M. tuberculosis cosmid library construction and subcloning analysis of TNF-downregulating hits.** The plasmid pYUB412 was linearized with XbaI, dephosphorylated with shrimp alkaline phosphatase (Promega), and then digested with Bcl to obtain 3,789-bp and 4,773-bp fragments. M. tuberculosis H37Rv genomic DNA was prepared from a 20-ml culture (OD600, ~3) grown in Middlebrook 7H9 supplemented with 10% OADC, 0.2% glycerol, and 0.05% Tween 80 as previously described (32), with the following modification: the genomic DNA, once precipitated in isopropanol, was spooled and immersed in ethanol and then was air-dried instead of being spun down. The genomic DNA was partially digested with Sau3A for 1 h at 37°C to obtain DNA fragments of around 20 to 30 kb in size and then ligated overnight at 4°C to the pYUB412-derived 3,789-bp and 4,773-bp fragments using T4 ligase (Promega). The ligation reaction mixture (5 μl) was mixed with 25 μl of MaxPlax Lambda Packaging Extract (Epipcentre, Madison, WI) at room temperature (RT) for 1 h. Another 2 μl of ligation reaction mixture was added to the packaging reaction, and the mixture was incubated for an additional hour at RT. LB medium (0.1 ml) was added to the packaging reaction along with 200 μl of HB101 cells grown in LB supplemented with 0.3% maltose and 10 mM MgSO4. The resultant mixture was incubated at 37°C for 30 min with no shaking and was then diluted with 0.5 ml of LB media and incubated for an additional 1 h at 37°C with shaking. The packaging reaction mixture was plated on LB-carbenicillin (LB-Carb; 100 μg/ml) plates; individual colonies were picked and grown in LB-Carb. Cosmid DNA was extracted from individual E. coli colonies and was then subjected to sequencing at the two ends of the H37Rv DNA insertion. In parallel, DNA of individual cosmids was analyzed by restriction mapping to validate the finding that the cosmid harbors the M. tuberculosis H37Rv genes (predicted by the sequence analysis of the two ends) in the correct contiguous sequence. pYUB412-based cosmid DNA procured from E. coli clones were used to transform mc‘155 to generate the H37Rv cosmid library in the heterologous M. smegmatis strain. This effort yielded 105 M. smegmatis clones whose genomes harbor distinct M. tuberculosis DNA fragments, covering about 50% of the H37Rv genome in total. Three individual colonies from the transformation reaction were picked and stored for each clone. As a result, the final product consisted of three sets (“a,” “b,” and “c”) of the 105 M. smegmatis H37Rv cosmid clones. Once a TNF-regulating clone of M. smegmatis harboring H37Rv DNA was obtained from the C10 macrophage screen, the validity of the hit was confirmed by studying the same clone from another set from the stored library.

Subclones of the original TNF-regulating hits were transformed into M. smegmatis mc‘155 via the use of the integrating pMV361. Similarly to the storage of stocks for the original 105 M. smegmatis H37Rv cosmid clones described above, three individual colonies from the transformation reaction were picked and stored for each subclone, resulting in three sets (“a,” “b,” and “c”) of subclones derived from the original four TNF-regulating cosmid hits. This allowed confirmation of the TNF-regulating property of a hit by examining whether this attribute was reproducible using the same subclone from another set of the stored stocks.

**Transfection of J774.16 macrophages with a TNF promoter-GFP construct.** The TNF promoter, whose function has been previously characterized using chloramphenicol acetyltransferase (CAT) as the reporter,
was derived from the construct Pro-UTR (a kind gift of Jiahuai Han), harboring the TNF promoter (TNFp) in tandem with the CAT coding sequence and the 3' untranslated region (3' UTR) of the TNF allele (27). Digestion of pro-UTR with KpnI and BamHI released a fragment containing the TNFp. The cytomegalovirus (CMV) promoter was deleted from phrGFP II-1 (Stratagene) to yield the modified phrGFP II-1 (m-phrGFP II-1) and then digested with KpnI and BamHI. The TNFp fragment was ligated into m-phrGFP II-1 (upstream of hrGFP II-1), yielding the TNFp-hrGFP II-1 fusion construct, which was transformed into E. coli HB101. Transformants were selected for resistance to kanamycin (50 μg/ml).

J774.16 mouse macrophages were transfected with the m-phrGFP II-1 vector containing the TNFp-hrGFP II-1 fusion by nucleofection using an Amaza Nucleofector (Lonza, Cologne, Germany) and were subjected to selection using G418 (1 mg/ml). G418-resistant transfectants were stimulated with lipopolysaccharides (LPS) (Sigma) (1 μg/ml; Escherichia coli 0127:B8) and examined by fluorescence microscopy. GFP-positive colonies were picked and transferred individually into wells of 24-well plates. Expanded GFP-positive colonies underwent a second round of inspection by fluorescence microscopy. Colonies with positive GFP signals were collected, expanded, and stored for further selection.

Macrophage infections and assessment of TNF expression. J774.16 macrophages were grown as previously described (72). C10 macrophages were cultured in 100-mm-diameter Optilux petri dishes (BD Falcon) in complete DMEM. Day 3 C10 cultures (80% to 90% confluent) were used to seed wells of 96-well tissue culture plates (Becton, Dickinson) at 10^5 cells per well and were allowed to adhere overnight at 37°C in a 5% CO₂ atmosphere prior to infection. The macrophages were infected with individual M. smegmatis bacteria harboring H37Rv M. tuberculosis DNA (prepared as described above) at a multiplicity of infection (MOI) of 10:1 (10 bacilli to 1 macrophage). Uninfected C10 or cultures infected with M. smegmatis transformed with the pYUB412 empty vector served as controls. After 4 h of infection, the cultures were washed twice with warm supplemented DMEM, replenished with the same medium containing 10 μg/ml of gentamicin. At 16 h later, the culture medium was replaced by warm PBS and GFP expression was quantified by the use of a Walac Viktor II plate reader (PerkinElmer) (excitation wavelength, 488 nm; emission wavelength, 530 nm). Culture supernatants were collected, expanded, and stored for further selection.

Apoptosis studies. J774.16 cells were cultured in complete DMEM and plated at 1 × 10^5 cells per well on a glass-bottom 96-well plate (MatriPlate; Brooks Life Science System). Cells were allowed to adhere overnight, and macrophage cultures were infected the next day with mid-log-phase WT H37Rv, deletion mutants, and complemented strains (prepared as described above) at an MOI of 10. After 4 hours, cells were washed with prewarmed culture media and incubated for an additional 16 h in growth media. Samples were then analyzed for GFP fluorescence in a Viktor II plate reader as described above. For measurement of TNF in culture supernatants, samples were aspirated and filtered to remove infectious material and then subjected to analysis by ELISA.

Mouse immunization and IFN-γ ELISPOT assay. Animal studies were conducted according to protocols that have been approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. C57BL/6 female mice (Jackson Laboratories) (8 to 10 weeks old) were immunized subcutaneously with 10^6 cells of M. tuberculosis H37Rv, deletion mutants, or complemented strains. After 30 days, mice were sacrificed and splenic T cells were isolated by magnetically activated cell sorting (MACS) using a Pan T cell isolation kit (Miltenyi Biotec) according to the manufacturer’s protocol. Detection of IFN-γ-producing T cells was carried out as previously described (75), using a mouse IFN-γ ELISPOT Ready-Set-Go kit (eBioscience) according to the manufacturer’s instructions. Briefly, T cells (1 × 10^5 and 3 × 10^5) were seeded in 96-well ELISPOT plates (Millipore) that had been coated previously with IFN-γ capture antibody overnight. Peptides of M. tuberculosis ESAT-6 (the first 20 amino acids of Rv3873) were used to stimulate CD4⁺ T cells, and that of M. tuberculosis GAP (amino acid GAPINSATAM of Rv0125) was used to stimulate CD8⁺ T cells in this assay. Splenocytes (2 × 10^⁵) from naive uninfected mice were used as antigen-presenting cells (APCs) and were incubated with ESAT-6 (6-kDa early secretory antigenic target) or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (10 μg/ml) for 1 h at 37°C. After two careful washes were performed, the APCs with or without peptides were added to the ELISPOT wells. After a 36-h incubation at 37°C and 5% CO₂, cells were removed, the plates were washed, and the captured cytokine was detected by incubating wells with a biotinylated anti-mouse IFN-γ antibody (clone XMG1.2; eBioscience) for 2 h at 37°C. Avidin-horseradish peroxidase (eBioscience) was added to the wells for 45 min at 37°C, and spots were detected using AEC substrate solution (Sigma). The substrate reaction was stopped by washing the plate with distilled water. Spots were enumerated using an automated ELISPOT reader (Autoimmun Diagnostika).

Statistical analysis. Statistical analysis was performed with Prism 5.0 software (GraphPad) using the unpaired t test. A P value of less than 0.05 was considered significant.
SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl?doi=10.1128/mBio.01023-15/-/DCSupplemental.

Table S1, TIF file, 0.03 MB.

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