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Methylation-dependent T cell immunity to *Mycobacterium tuberculosis* heparin-binding hemagglutinin

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Although post-translational modifications of protein antigens may be important componenets of some B cell epitopes, the determinants of T cell immunity are generally nonmodified peptides. Here we show that methylation of the *Mycobacterium tuberculosis* heparin-binding hemagglutinin (HBHA) by the bacterium is essential for effective T cell immunity to this antigen in infected healthy humans and in mice. Methylated HBHA provides high levels of protection against *M. tuberculosis* challenge in mice, whereas nonmethylated HBHA does not. Protective immunity induced by methylated HBHA is comparable to that afforded by vaccination with bacille Calmette et Guérin, the only available anti-tuberculosis vaccine. Thus, post-translational modifications of proteins may be crucial for their ability to induce protective T cell-mediated immunity against infectious diseases such as tuberculosis.

Except for CD1-restricted T cell responses to glycolipids, the determinants of cellular immune responses are generally nonmodified peptides, usually derived from protein antigens, that are presented to T cells by major histocompatibility complex (MHC) class I or class II molecules. Post-translationally modified peptides do not usually constitute T cell epitopes, although many viral and eukaryotic antigens may be heavily glycosylated. Post-translational modifications of protein antigens from bacterial pathogens are rare and little is known about their role in T cell immunity. Responses mediated by $CD4^+$ and $CD8^+$ T cells are essential for protection against some of the most terrible infectious diseases, such as tuberculosis[1], which remains one of the world's leading causes of death due to a single infectious agent[2]. A better definition of the cellular immune responses to bacterial antigens such as those produced by *M. tuberculosis*, therefore, may have an important impact on vaccine development.

M. tuberculosis produces on its surface modified protein antigens such as HBHA[3]. This protein is produced by all the members of the *M. tuberculosis* complex including the vaccine strain *Mycobacterium bovis* bacille Calmette et Guérin (BCG)[3], as well as by other pathogenic mycobacteria such as *Mycobacterium leprae*[4] and *Mycobacterium avium*[5]. It is not produced by the nonpathogenic *Mycobacterium smegmatis*[6]. In pathogenic mycobacteria, HBHA is an adhesin for nonphagocytic cells[3,5,7] and is involved in extrapulmonary dissemination of the tubercle bacillus[7]. T lymphocytes from healthy human individuals infected with *M. tuberculosis* produce large amounts of HBHA-specific interferon-γ (IFN-γ), whereas the T cells from individuals affected with active tuberculosis do not[8], suggesting that T cell responses to HBHA may participate in the protection against active disease.

Native HBHA (nHBHA) is post-translationally modified[9]. This modification consists of a complex methylation pattern of the C-terminal domain of nHBHA[10]. The methylated domain contains several lysine-rich repeats that are functionally important for interaction of the mycobacteria with heparan sulfate–containing receptors on the surface of epithelial cells[11,12]. During the biosynthesis of nHBHA, most but not all of the lysine residues in these repeats undergo mono- or dimethylation catalyzed by methyltransferases that are most probably located in the mycobacterial cell wall[10]. Detailed biochemical analyses have indicated that nHBHA contains 20–26 methyl groups on residues 159–199 (ref. 10). These methyl groups are not present in the recombinant form of HBHA produced by *Escherichia coli* (rHBHA). We show here that this post-translational modification of HBHA is important for the induction of both T cell antigenicity and protective immunity to *M. tuberculosis* infection.

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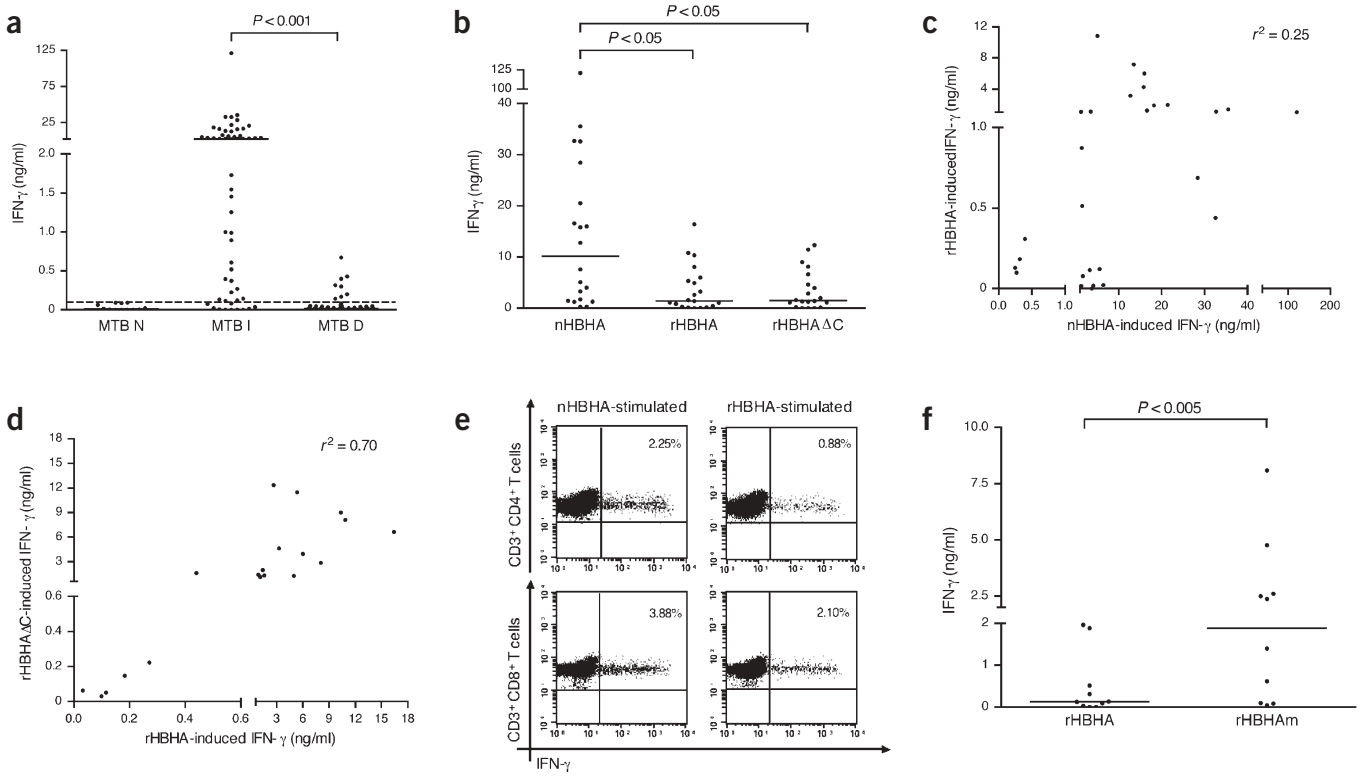

Figure 1 Human IFN-γ responses to nHBHA and rHBHA. (**a**) IFN-γ secreted by PBMCs from negative controls (MTB N), infected healthy individuals (MTB I) and individuals with tuberculosis (MTB D) stimulated with nHBHA. (**b**) IFN-γ secreted by PBMCs from infected healthy individuals stimulated with nHBHA, rHBHA or rHBHAΔC. (**c**,**d**) Correlation of nHBHA- and rHBHA-specific (**c**), and rHBHA- and rHBHAΔC-specific (**d**) IFN-γ secretion by PBMCs from infected healthy individuals. (**e**) PBMCs from an infected healthy individual were stimulated with nHBHA (left) or rHBHA (right), and the percentages of IFN-γ-producing $CD3^+$ cells were determined among the $CD4^+$ (top) and the $CD8^+$ (bottom) cells. (**f**) IFN-γ secretion by PBMCs from infected healthy individuals stimulated with nHBHA or rHBHAm. Horizontal bars (**a**,**b**,**f**) indicate the medians.

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RESULTS

HBHA methylation and human T cell responses

To determine the role of HBHA methylation in naturally induced cellular immune responses, peripheral blood mononuclear cells (PBMCs) from 55 healthy individuals infected with *M. tuberculosis*, 46 individuals affected with active tuberculosis, and 12 controls were stimulated *in vitro* with purified nHBHA or rHBHA. The PBMCs of 81.8% of the infected healthy individuals secreted more than 0.1 ng/ml of IFN-γ after stimulation with nHBHA, as compared with 17.3% of the individuals with tuberculosis and none of the negative controls (**Fig. 1a**). Although similar to the general trend observed previously in smaller numbers of individuals[8], our results from greater numbers of individuals showed a much stronger discrimination between the two groups.

As compared with nHBHA stimulation, rHBHA stimulation of the PBMCs from infected healthy individuals resulted in substantially less secretion of IFN-γ (**Fig. 1b**), suggesting that methylation of HBHA is important for the production of HBHA-specific IFN-γ in these individuals. When increasing amounts of antigen (up to 10 g/ml) were used for *in vitro* stimulation, the secretion of IFN-γ increased more in response to nHBHA than in response to rHBHA, resulting in an even greater discrimination between the two forms of the antigen at high concentrations. Therefore, the difference between the two forms of the antigen is not simply dose related, as confirmed by pair-wise analyses indicating that the IFN-γ production obtained on stimulation with nHBHA correlated poorly with that obtained on stimulation with rHBHA ($r^2 = 0.25$; **Fig. 1c**).

These observations suggest that most infected individuals recognized T cell epitopes that are present in nHBHA but absent in rHBHA. By contrast, the responses to rHBHAΔC correlated well with those to rHBHAΔC ($r^2 = 0.70$, $P < 0.0001$), a truncated form of HBHA lacking the C-terminal lysine-rich domain (**Fig. 1b**,**d**), indicating that the IFN-γ response of the few individuals who recognized rHBHA is directed against the N-terminal moiety of rHBHA.

Previous studies have indicated that both $CD4^+$ and $CD8^+$ T cells from infected healthy individuals produce IFN-γ after stimulation with nHBHA[8]. To determine whether rHBHA also stimulates these two T lymphocyte subsets, we compared the phenotypes of nHBHA-specific and rHBHA-specific IFN-γ-producing cells from individuals who recognized both forms of the molecule. Flow cytometry analysis indicated that both $CD4^+$ and $CD8^+$ T cells produced IFN-γ after stimulation with either nHBHA or rHBHA (**Fig. 1e**), suggesting that the presentation of either form of HBHA is restricted by MHC class I and II molecules. We confirmed this finding by showing that IFN-γ production was inhibited after incubation with antibodies to class I or class II molecules[8] but not by antibodies to CD1 (data not shown).

T cell reactivity to *in vitro*–methylated rHBHA

When rHBHA was methylated *in vitro*, increased amounts of HBHA-specific IFN-γ were produced (**Fig. 1f**), confirming the role of HBHA methylation in T cell responses. The chemically methylated protein (rHBHAm) was characterized by mass spectrometry analysis of both the full-size protein and proteolytic fragments, coupled with amino-

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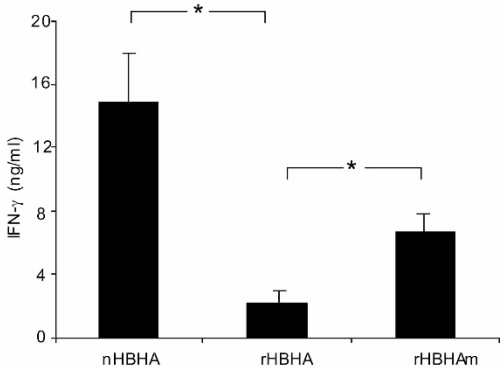
Figure 2 IFN-γ responses to nHBHA, rHBHA and rHBHAm in BCG-infected mice. Five BALB/c mice were infected with 5 10^5 c.f.u. BCG, and their spleens were isolated after 2 months. Spleen cells were stimulated with nHBHA, rHBHA or rHBHAm, as indicated, and the concentration of IFN-γ in the culture supernatants was determined. Results are expressed as the mean ± s.d. $^*P < 0.05$.



acid sequencing, as described[10]. These analyses indicated that all of the lysine residues were dimethylated and the other residues were not modified in rHBHAm. This methylation pattern differs substantially from the natural methylation pattern of nHBHA[10]; thus, wild-type levels of HBHA-specific IFN-γ secretion would not be expected on stimulation with rHBHAm.

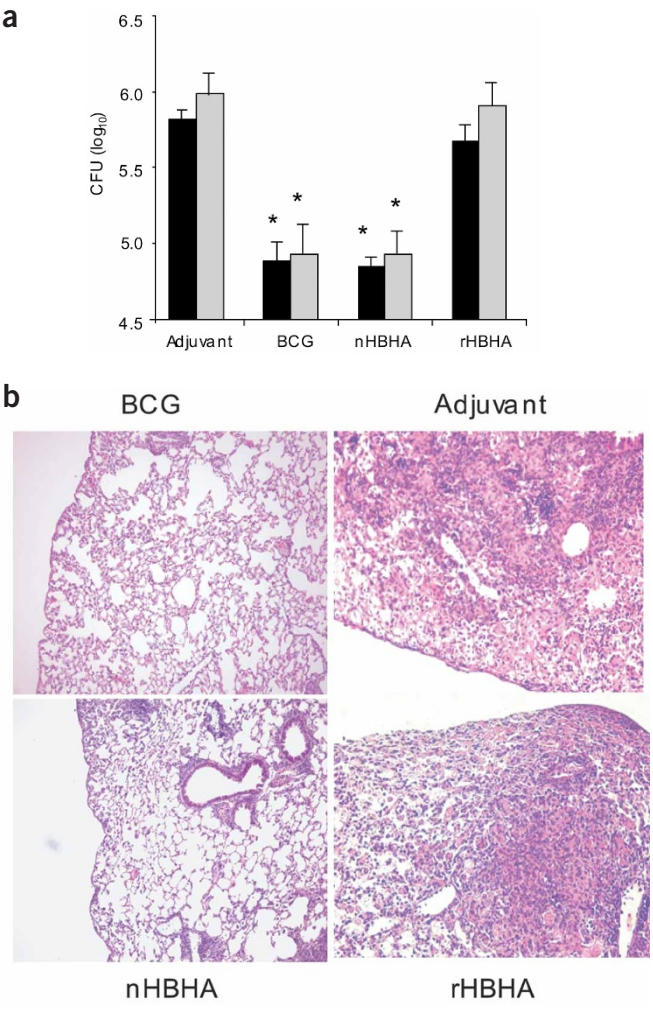
To determine whether the role of HBHA methylation in T cell antigenicity could be observed in animal models, BALB/c mice were infected with *M. bovis* BCG. After 2 months, their splenocytes were isolated and stimulated with nHBHA or rHBHA. Stimulation by nHBHA resulted in the secretion of significant amounts of IFN-γ, whereas stimulation by rHBHA induced about a 90% reduction in IFN-γ production (**Fig. 2**). Intermediate levels of HBHA-specific IFN-γ secretion were obtained for rHBHAm.

Protective immunity induced by methylated HBHA

The importance of IFN-γ in protection against mycobacterial diseases has been documented extensively in humans and in animal models[1,13]. IFN-γ has been used successfully as adjunctive therapy, especially in the treatment of multidrug-resistant tuberculosis[14]. The identification of IFN-γ-inducing antigens is therefore relevant to the development of new anti-mycobacterial vaccines.

To assess the protective potential of HBHA and to determine the role of HBHA methylation in protective immunity, BALB/c mice were immunized subcutaneously with nHBHA or rHBHA in the presence of dimethyl-dioctadecyl ammonium bromide (DDA) and monophosphoryl lipid A (MPL) used as adjuvant. The negative controls received adjuvant alone, and the positive controls were immunized with 5 10^5 c.f.u. live BCG. Ten weeks after the first injection, the mice were intravenously infected with *M. tuberculosis* MT103, a virulent clinical isolate. Six weeks after challenge, comparable numbers of mycobacteria present in the lungs and spleens of the infected mice showed that the level of protection offered by nHBHA was similar to that obtained after vaccination with BCG (**Fig. 3a**). By contrast, rHBHA induced no significant protection.

Similar results were obtained in an aerosol challenge model using C57Bl/6 mice and *M. tuberculosis* Erdman. Whereas nHBHA-immunized mice showed a 0.74-log reduction in bacterial counts in the lungs as compared with the adjuvant control, the numbers of bacteria recovered from the lungs of the rHBHA-immunized mice did not differ significantly from those recovered from control mice. Histological examination of lung sections from these mice was consistent with the bacteriological findings (**Fig. 3b**). Mice immunized with nHBHA and challenged with *M. tuberculosis* showed much less lung pathology than did mice immunized with either rHBHA or adjuvant, as assessed by the reduced inflammation and the areas of healthy lung tissue.



T and B cell immunogenicity of nHBHA and rHBHA

To determine whether the variation in protection offered by the two HBHA forms was due to a difference in their ability to induce cellular or humoral immune responses, HBHA-specific antibody titers and the HBHA-specific production of IFN-γ in BALB/c mice were measured 6 weeks and 1 week, respectively, after the last immunization with either rHBHA or nHBHA. The two groups of mice produced

Figure 3 HBHA-mediated protection against *M. tuberculosis* challenge. BALB/c (**a**) and C57Bl/6 (**b**) mice were vaccinated with nHBHA, rHBHA, BCG or adjuvant and challenged intravenously with *M. tuberculosis* MT103 (**a**) or by aerosol with *M. tuberculosis* Erdman (**b**). Bacterial counts were determined in spleens (black bars) and lungs (gray bars) 6 weeks after challenge (**a**). $^*P < 0.05$ for BCG-vaccinated or nHBHA-vaccinated mice versus the adjuvant control. Alternatively, 4 weeks after challenge lung sections were prepared, stained with hematoxylin and eosin and examined by light microscopy (**b**). Original magnification, 200.

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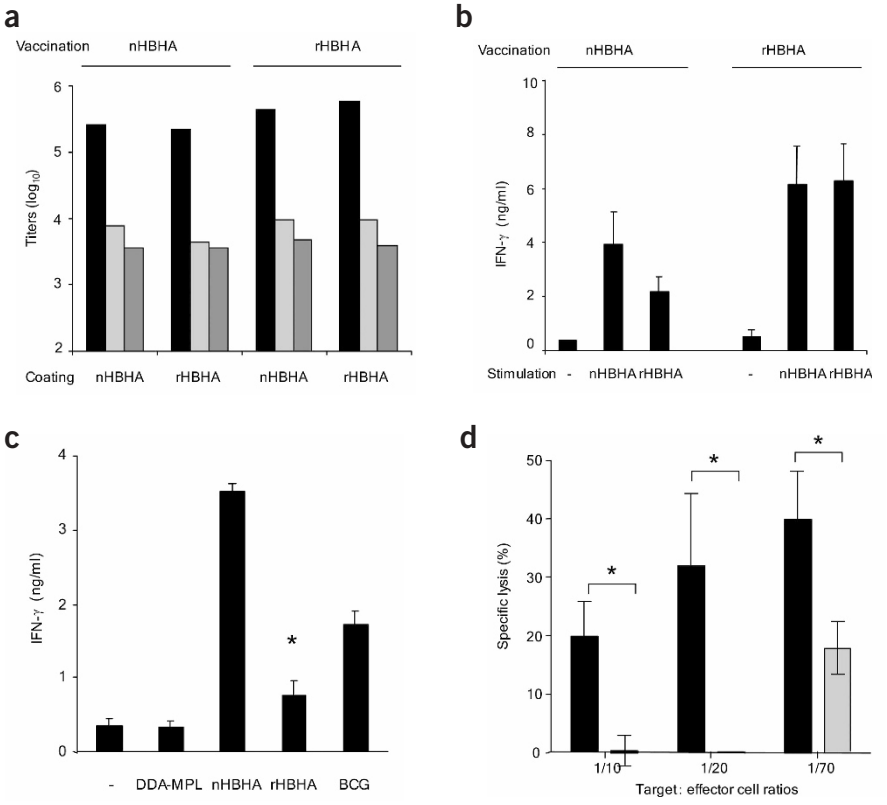

Figure 4 HBHA-mediated humoral, cellular and cytotoxic immune responses. BALB/c (**a**,**b**,**d**) and C57Bl/6 (**c**) mice were vaccinated with nHBHA, rHBHA, adjuvant or BCG. (**a**) Anti-nHBHA and anti-rHBHA IgG1 (black columns), IgG2a (light gray bars) and IgG2b (dark gray bars) titers were determined on pooled sera ($n = 10$ mice per group). (**b**) IFN-γ production by n-HBHA-, rHBHA-stimulated or unstimulated splenocytes was measured. Results are the mean ± s.d. ($n = 4$–5 mice per group). (**c**) *M. tuberculosis*–infected mouse macrophages were incubated with splenocytes from immunized mice, and IFN-γ production was measured. Results are the mean ± s.d. ($n = 3$ wells containing pooled splenocytes). (**d**) ^{51}Cr-pulsed, BCG-infected macrophages were incubated with antigen-expanded splenocytes from mice immunized with nHBHA (black bars) or rHBHA (gray bars) at the indicated effector:target cell ratios, and the percentage of HBHA-specific ^{51}Cr release was determined. Results are the mean ± s.d.($n = 5$ mice per group). $^*P < 0.05$.

comparable amounts of antibodies to HBHA, and these antibodies recognized nHBHA and rHBHA equally well (**Fig. 4a**). Methylation of HBHA also had no influence on the isotype profiles of the HBHA-specific antibodies.

When the splenocytes of the immunized mice were stimulated with either rHBHA or nHBHA, comparable amounts of IFN-γ were produced, regardless of which form of the antigen was used for vaccination (**Fig. 4b**). Similarly, when purified human dendritic cells isolated from a naive individual were loaded with 10 g/ml of rHBHA or nHBHA and incubated in the presence of purified autologous naive T cells, comparable amounts of IFN-γ were measured in the culture supernatants (data not shown). These results indicate that both forms of HBHA can induce primary immune responses in mice and humans.

When the splenocytes of the immunized mice were incubated with bone marrow–derived macrophages that had been pulsed with *M. tuberculosis*, significantly more IFN-γ was secreted by the splenocytes from mice immunized with nHBHA than by those from rHBHA-immunized mice (**Fig. 4c**). The splenocytes from the nHBHA-immunized mice also lysed BCG-loaded macrophages much more efficiently than did the splenocytes from rHBHA-immunized mice. Cells from the rHBHA-immunized mice lysed BCG-loaded macrophages at a target:effector cell ratio of 1:70, whereas specific lysis by cells from the nHBHA-immunized mice was observed at a target:effector cell ratio of 1:10 (**Fig. 4d**).

Similar results were obtained when the bactericidal activity of the splenocytes toward BCG-loaded macrophages was analyzed (data not shown), although the difference between the two groups of mice was less significant ($P = 0.28$) than that observed for the cytotoxic activity ($P = 0.03$). The splenocytes from nHBHA-immunized mice incubated with BCG-loaded macrophages also secreted larger amounts of tumor-necrosis factor-α (TNF-α; median 115.3 pg/ml) than did the

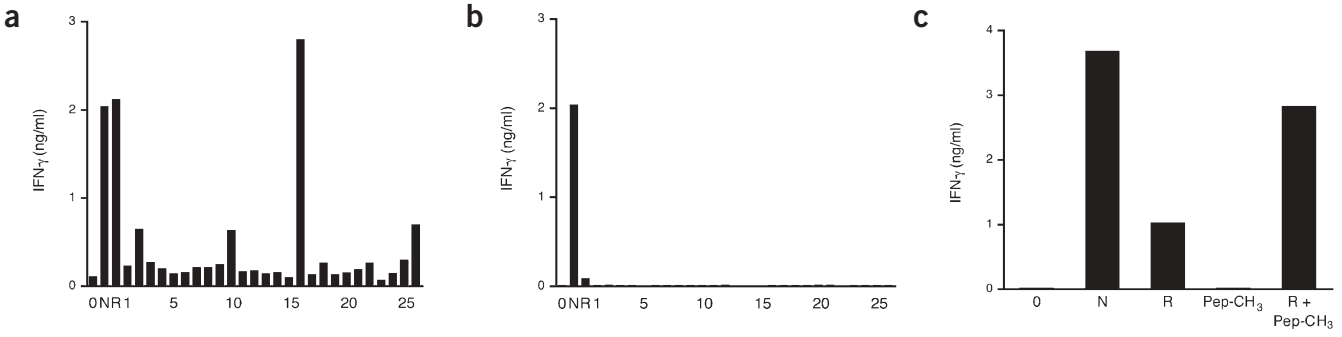

Figure 5 Peptide scan analyses. PBMCs from *M. tuberculosis*-infected healthy individuals were incubated in the absence (0) or the presence of nHBHA (N), rHBHA (R) or synthetic HBHA peptides labeled 1–26 (**a**,**b**), or in the presence of the methylated peptide (Pep-CH_3) with or without rHBHA (**c**). IFN-γ concentrations in the culture supernatants were determined by ELISA. Results are representative of multiple experiments ($n = 8$, **a**; $n = 2$, **b**; $n = 5$, **c**) on individuals recognizing both nHBHA and rHBHA (**a**) or recognizing nHBHA much better than rHBHA (**b**,**c**).

splenocytes from rHBHA-immunized mice (median below the detection limit; data not shown).

Peptide scan analysis of HBHA

Considering the mechanism by which methylation of HBHA might influence T cell antigenicity, we proposed that either the methylated portion of the protein forms part of the T cell epitopes or methylation alters the processing of HBHA in antigen-presenting cells to produce unique peptide epitopes that are not presented when nonmethylated HBHA is processed. These antigenic peptides would not necessarily be methylated. To distinguish between these two possibilities, two groups of individuals infected with *M. tuberculosis* were analyzed by HBHA peptide scanning using the PBMC IFN-γ assay: the first group responded to both forms of HBHA, whereas the second group responded strongly to nHBHA and poorly to rHBHA.

Twenty-six sequential peptides of 15 residues, overlapping by 7–8 residues and spanning the whole HBHA sequence, were incubated in the presence of PBMCs from these individuals. We considered that if the methylated portion of HBHA were part of the T cell epitopes, then the peptides would not induce the production of IFN-γ by PBMCs from individuals of the second group, whereas T cell–reactive peptides would induce IFN-γ in individuals from the first group. By contrast, if the methylation altered antigen processing, then T cell–reactive peptides might be detected in both groups, although they would be expected to differ between the two groups.

Several peptides induced the production of IFN-γ by PMBCs from individuals who recognized both forms of HBHA (**Fig. 5a**), whereas no peptide induced IFN-γ secretion by PBMCs from individuals who recognized only nHBHA (**Fig. 5b**). These results suggest that methylation does not induce alternative antigen processing, but rather the methylated portion of HBHA forms part of the T cell epitopes. To confirm this finding, we synthesized the methylated form of a C-terminal HBHA peptide and incubated it with PBMCs from individuals who recognized nHBHA much better than rHBHA. The methylated peptide induced the production of IFN-γ by PBMCs from these individuals, but only when it was incubated in the presence of rHBHA, which most probably acted as a carrier protein (**Fig. 5c**).

DISCUSSION

Post-translational modifications of protein antigens, which are frequently observed in viral and eukaryotic antigens, but are rare in bacterial antigens, are not generally considered to be important T cell immunogenicity. Although formylation is not a true post-translational modification, some bacterial proteins are formylated at their N terminus as a result of the initiation of prokaryotic protein synthesis by formylated methionine. In general, this residue is subsequently removed, but some proteins retain their formylated N-terminal methionine and formylated peptides may bind specifically to MHC class Ib molecules and induce cytotoxic T lymphocyte responses to intracellular pathogens such as *Listeria*[15] and *M. tuberculosis*[16].

In contrast to antigens with N-terminal formylation, HBHA from *M. tuberculosis* is methylated in its C-terminal domain by true post-translational modifications involving protein methyltransferases that are most probably localized in the mycobacterial cell wall[10]. We have shown here that methylation of HBHA is important for the induction of protective T cell immunity against tuberculosis. HBHA induced the production of IFN-γ by both splenocytes from BCG-infected mice and PBMCs from *M. tuberculosis*–infected healthy individuals, but only in its natural methylated form and not in its nonmethylated recombinant form, although some individuals recognized both forms.

The IFN-γ induced by nHBHA and rHBHA was produced by $CD4^+$ and $CD8^+$ T cells and was inhibited by antibodies to MHC class I or class II molecules, but not by antibodies to CD1, indicating that both nHBHA and rHBHA are presented through the classical antigen presentation pathways. Those individuals who responded to both forms, however, did not recognize the same epitopes as those who responded only to nHBHA, indicating that different T cell populations are activated by exposure to the two antigens. These populations differ by their epitope specificity rather than by their phenotypes, which may be relevant to the mechanism of protection because it suggests that, within the same antigen, some epitopes may be protective and others may not. As previously observed[8], nHBHA induced much greater secretion of IFN-γ from the PBMCs of infected healthy individuals than from the PMBCs of individuals with active disease. Because most individuals infected by *M. tuberculosis* do not develop disease but may be protected by their immune response to natural infection, antigens that are recognized by T cells from infected healthy individuals may be promising candidates for vaccines.

Methylated HBHA was indeed found to provide strong protection in mouse models, as shown by the reduction of bacterial counts in lungs and spleens of vaccinated mice challenged with *M. tuberculosis*. The protection provided by three injections of 5 g of nHBHA was similar to that offered by BCG vaccination in an intravenous challenge model. A similar protective effect was found in an aerosol challenge model, and histological examinations indicated that the lung pathology in nHBHA-immunized mice was much less than that in adjuvant control mice. Similar to the antigenicity observed in *M. tuberculosis*–infected healthy individuals or in BCG-infected mice, the protective effect of HBHA depended on its methylation, because effective immunity was observed with nHBHA but not with rHBHA.

Nevertheless, rHBHA induced primary T cell responses, as shown by the production of IFN-γ by naive human T cells incubated with HBHA-loaded dendritic cells, or by the induction of T cell responses in mice immunized with rHBHA. Quantitative evaluation of the immune responses in BALB/c mice immunized with rHBHA or nHBHA showed that both antigens can induce comparable titers and isotypes of antibodies to HBHA, as well as comparable IFN-γ responses. Therefore, the lack of anti-rHBHA T cell immune responses after infection and the lack of protective immunity induced by immunization with rHBHA are not due to an intrinsic inability of rHBHA to induce these responses. This finding is consistent with the observation that DNA vaccination with the *hbhA* gene provides no protection even though both HBHA-specific antibodies and IFN-γ are produced[17].

These observations indicate that the mere induction of T or B cell responses to protective antigens may not be sufficient to induce protective immunity and suggest that HBHA-mediated protection relies on specific methylation-dependent epitopes that are exposed by the mycobacteria during infection. Peptide scan analyses indicated that the methylated portion of the protein forms part of the epitopes recognized by healthy individuals infected with *M. tuberculosis*. This region is the only portion of nHBHA that is surface exposed by the mycobacteria, because antibodies directed to the C-terminal end of HBHA bind to *M. tuberculosis* and BCG[3], whereas antibodies directed to the N-terminal domain of the protein do not (data not shown).

Immunization with purified nHBHA probably induces immune responses to several nonmethylated epitopes that are not protective, as well as to the exposed methylated epitopes that are protective, whereas immunization with rHBHA induces only immune responses to the nonmethylated nonprotective epitopes. Consistent with this notion are the observations that splenocytes from nHBHA-immu-

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nized mice secreted large amounts of IFN-γ on *in vitro* incubation with *M. tuberculosis*–loaded macrophages, whereas much less IFN-γ was secreted by splenocytes from rHBHA-immunized mice. Similarly, splenocytes from nHBHA-immunized mice showed higher TNF-α secretion and cytotoxic and bactericidal activity toward BCG-loaded macrophages than did splenocytes from rHBHA-immunized mice. The role of specific antigenic determinants in protective immunity is suggested by the observation that some mycobacterial antigens are powerful inducers of IFN-γ responses, but do not protect against challenge and instead exacerbate disease and even abolish the protective effect of BCG[18].

The natural methylation of a protein antigen may thus have profound effects on its recognition by the cellular immune system and may be part of protective T cell epitopes. In addition, and in light of the fact that very few individual mycobacterial antigens have been shown to offer protection that approaches that afforded by BCG vaccination[19–21], the strong protection induced by methylated HBHA suggests that this antigen may be considered as a possible component of future acellular vaccines against tuberculosis.

METHODS

Human subjects. Blood was obtained from 46 individuals with culture-positive tuberculosis, 55 healthy individuals infected with *M. tuberculosis* (>10 mm induration after intradermal tuberculin injection, normal chest radiographs, no clinical sign of tuberculosis, no history of BCG vaccination) and 12 controls (tuberculin-negative skin test, no known history of either contact with individuals with tuberculosis or BCG vaccination). All individuals live in the Brussels area, a region with very low incidence of atypical mycobacterial infections. We excluded anergic individuals on the basis of the lack of IFN-γ responses to purified protein derivative or phytohemagglutinin stimulation. The study was approved by the Ethical Committee of the Université Libre de Bruxelles Medical Faculty, and informed consent was obtained from all individuals.

Antigens. nHBHA was purified from BCG or *M. tuberculosis* H37Ra by heparin-sepharose[3], followed by high-pressure liquid chromatography[8]. rHBHA and rHBHAΔC were purified from *E. coli* BL21(DE3)(pET-HBHA)[9] and *E. coli* BL21(DE3)(pET-HBHAΔC)[11], respectively, as described[11]. We produced and analyzed rHBHAm by mass spectrometry and amino acid sequencing, as described[10]. The peptides used for peptide scan analyses were purchased from BioSource. The methylated peptide was synthesized by the Fmoc/tert.butyl strategy[22] (**Supplementary Methods** online). Mono- and dimethylated lysine residues were incorporated at the ratios determined for nHBHA[10].

Antigen-specific cytokine determination. Isolated human PBMCs suspended at 2 10^6 cells per ml in RPMI 1640 medium (BioWhittaker) supplemented with 40 g/ml of gentamicin, 50 M β-mercaptoethanol, 1 nonessential amino acids, 1 sodium pyruvate, 2 mM glutamine (Life Technologies) and 10% fetal calf serum were cultured with 1 g/ml of nHBHA, rHBHA, rHBHAm or rHBHAΔC, or with 10 g/ml of synthetic peptides for 4 d. Concentrations of IFN-γ were measured in the culture supernatants by enzyme-linked immunosorbent assay (ELISA) using anti–human IFN-γ IgG1 (clone 350B 10G6, BioSource) for coating and biotin-labeled anti–human IFN-γ IgG1 (clone 67F 12A8, BioSource) for detection. The human primary immune response induced by HBHA was measured *in vitro* on cells from a naive individual (no IFN-γ secretion by PBMCs in response to purified protein derivative or HBHA). Isolated T cells were cocultured for 6 d with monocyte-derived dendritic cells[23] loaded with 10 g/ml of rHBHA or nHBHA. To analyze the phenotype of the IFN-γ producing cells, we used the BD FastImmune (BD Biosciences) protocol (**Supplementary Methods** online).

To analyze murine IFN-γ secretion, splenocytes were incubated in RPMI medium supplemented with 10 U/ml of penicillin, 10 g/ml of streptomycin, 50 M β-mercaptoethanol and 10% fetal calf serum at 10^6 cells per ml in the presence or absence of 5 g/ml of nHBHA, rHBHA or rHBHAm. After 72 h at 37 °C, the IFN-γ concentrations were measured by the OptEIA mouse IFN-γ kit (PharMingen). To measure the HBHA-specific IFN-γ response to antigen presented by live *M. tuberculosis*, bone marrow–derived macrophages were isolated as described[24] and infected for 2 h with *M. tuberculosis* Erdman at a multiplicity of infection of 3:1. Four weeks after immunization, splenocytes from five immunized and five control mice were pooled and incubated with *M. tuberculosis*–infected bone marrow–derived macrophages for 72 h. IFN-γ was determined by capture ELISA as described above. We measured TNF-α concentrations by the OptEIA mouse TNF-α (mono/mono) kit (PharMingen).

Murine antibody responses. To analyze the murine anti-HBHA responses, microtiter plates were coated overnight with 50 l of 0.05 M carbonate buffer (pH 9.6) containing 5 g/ml of nHBHA or rHBHA. After blocking with PBS-Tween containing 1% bovine serum albumin, 50 l of serum was added in twofold serial dilutions. After the plates were washed, horseradish peroxidase-conjugated anti–mouse IgG1, IgG2a or IgG2b was added, followed by ABTS (Boehringer Mannheim).

Cytotoxic and bactericidal activities. To measure cytotoxic and microbicidal activities, antigen-specific murine splenocytes were expanded by incubation with 3 g/ml nHBHA or rHBHA for 5 d and then incubated with BCG-infected bone marrow–derived macrophages. We measured cytotoxicity by the ^{51}Cr release method (**Supplementary Methods** online).

Vaccination and challenge. Mouse experiments were done according to the Institut Pasteur de Lille and National Institutes of Health (NIH) guidelines for laboratory animal husbandry and experimentation. Female BALB/c (for intravenous challenge) or C57Bl/6 (for aerosol challenge) mice aged 8 weeks were immunized subcutaneously three times at 2-week intervals with 5 g of nHBHA or rHBHA in the presence of DDA and MPL[19], or once with 5 10^5 c.f.u. BCG. Ten weeks after the first injection, the mice were challenged intravenously with 10^5 c.f.u. *M. tuberculosis* MT103 or by aerosol with 200 c.f.u. *M. tuberculosis* Erdman[17]. After 6 (intravenous challenge) or 4 (aerosol challenge) weeks, the lungs and spleens were collected and plated onto Middlebrook 7H11 agar. Bacterial counts were scored after 3–4 weeks of incubation at 37 °C (ref. 7). For histological examination, lung samples were fixed in 10% paraformalin and embedded in paraffin, and sections were stained with hematoxylin and eosin.

Statistical analyses. Differences between two and three groups were assessed by the nonparametric Mann-Whitney *U*-test (the Wilcoxon test for paired values) and the Kruskal-Wallis test followed by the Dunn's post-test (GraphPad Prism program), respectively. A value of $P < 0.05$ was considered to be significant. Significance of correlations was tested by the nonparametric Spearman test.

Note: Supplementary information is available on the Nature Medicine website

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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