HspX-mediated protection against tuberculosis depends on its chaperoning of a mycobacterial molecule

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New approaches consisting of ‘multistage’ vaccines against (TB) are emerging that combine early antigenic proteins with latency-associated antigens. In this study, HspX was tested for its potential to elicit both short- and long-term protective immune responses. HspX is a logical component in vaccine strategies targeting protective immune responses against primary infection, as well as against reactivation of latent infection, because as previously shown, it is produced during latency, and as our studies show, it elicits protection within 30 days of infection. Recent studies have shown that the current TB vaccine, bacilli Calmette-Guerin (BCG), does not induce strong interferon-γ T-cell responses to latency-associated antigens like HspX, which may be in part why BCG fails to protect against reactivation disease. We therefore tested HspX protein alone as a prophylactic vaccine and as a boost to BCG vaccination, and found that HspX purified from M. tuberculosis cell lysates protected mice against aerosol challenge and improved the protective efficacy of BCG when used as a booster vaccine. Native HspX was highly immunogenic and protective, in a dose-dependent manner, in both short- and long-term infection models. Based on these promising findings, HspX was produced as a recombinant protein in E. coli, as this would enable facile purification; however, recombinant HspX (rHspX) alone consistently failed to protect against aerosol challenge. Incubation of rHspX with mycobacterial cell lysate and re-purification following incubation restored the capacity of the protein to confer protection. These data suggest the possibility that the native form may chaperone an immunogenic and protective antigen that is mycobacteria-specific.

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Declared a global health emergency in the early 1990s, tuberculosis (TB) remains a major health problem today, with over two million deaths because of this disease each year, and one-third of the world’s population infected with Mycobacterium tuberculosis.¹ Most infected people initially control the infection by generating cell-mediated immunity; however, residual latent bacilli remain viable in healthy hosts for many years and can reactivate into contagious TB disease. In fact, latent infections that have reactivated into active disease may be the predominante source of contagious TB.²

The only vaccine currently available against TB is live attenuated M. bovis, bacilli Calmette-Guerin (BCG). BCG is effective in preventing severe forms of childhood TB, but its protective effect wanes in adulthood,³ and it does not protect against reactivation of latent TB, which partially explains why this vaccine has had little impact on the global TB epidemic.⁴ Therefore, concerted research efforts have focused on developing a TB vaccine that confers consistent, long-term protection against adult pulmonary TB, and ideally one that can protect against initial infection, as well as reactivation of latent infection.

It is well-established that T helper cell type 1 immunity is required for protection against M. tuberculosis infection,⁵ but mounting evidence suggests that interferon-γ (IFN-γ) induction alone does not guarantee long-term protection. Therefore, it is necessary to assess additional cytokines beyond IFN-γ, such as tumor necrosis factor (TNF)-α and interleukin (IL)-2 to generate a more complete functional assessment of effector and memory T cells.⁶,⁷ Forbes et al.⁸ showed that the induction of multifunctional T cells (that is, T cells that produced IFN-γ, TNF-α and IL-2) was associated with...
markedly reduced mycobacterial burden in the lungs of mice given a prime-boost vaccine with BCG followed by virally encoded *M. tuberculosis* antigen Ag85A compared with BCG vaccination alone.

Several conventional subunit protein vaccines have been shown to induce multifunctional T cells, and many of these subunit vaccines have been shown to confer protection rivaling that of BCG in several animal models. Until recently, the majority of subunit vaccines were based on antigens expressed during the early stages of *M. tuberculosis* infection; however, to target protection during latency, several current studies have incorporated the latency-associated antigen, 16-kDa α-crystallin protein (also called HspX, acr and Rv2031c) in heterologous prime-boost strategies using protein HspX subunit vaccines, DNA vaccines expressing HspX and recombinant BCG strains overexpressing HspX.

The HspX protein is associated with latency because it is the dominant antigen produced by *M. tuberculosis* during the latent stage of infection, accounting for up to 25% of the total protein expressed during static growth or under oxygen deprivation, as has been demonstrated in models aiming to mimic the granulomatous environment *in vitro*. The proposed role of HspX is to enhance long-term stability of proteins and cell structures, which in turn helps the bacilli maintain long-term survival. In addition, rapid and robust expression of HspX has been demonstrated upon entry into macrophages and under oxygen-deprivation *in vitro*, and during log-phase growth, low levels of HspX protein are produced as early as day four of incubation. As this antigen is associated with latency, several current studies have incorporated the latency-associated antigen, 16-kDa α-crystallin protein (also called HspX, acr and Rv2031c) in heterologous prime-boost strategies using protein HspX subunit vaccines, DNA vaccines expressing HspX and recombinant BCG strains overexpressing HspX.

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### RESULTS

**Native HspX vaccine generates short-term and long-term acquired IFN-γ responses specifically against HspX**

To investigate whether the concentration of HspX used to vaccinate mice affected the generation and the quality of long-term immunity, splenocytes from vaccinated mice were harvested at either 30 days or 6 months after the last vaccination, stimulated with HspX *in vitro*, and assayed by ELISpot assay for IFN-γ production. Cells from mice vaccinated with 1.0 μg and 10 μg of HspX produced significantly more IFN-γ-producing cells than BCG-vaccinated mice in response to HspX both 30 days and 6 months after the vaccination (Figures 3a and b, respectively), with over a 10-fold increase in the number of IFN-γ-producing cells after 6 months. Interestingly, and in line with previous reports, splenocytes from BCG-vaccinated mice recognized HspX but produced very little IFN-γ in comparison with mice vaccinated with the 1.0 μg and 10 μg concentrations of HspX.

**Native HspX subunit vaccination induces multifunctional CD4+ T cells**

We next looked at the ability of the nHspX subunit vaccine to stimulate multifunctional CD4+ T cells both in the short- and long-term models. Splenocytes, harvested from mice at 30 days and 6 months after the last vaccination, were stained for surface CD4 and intracellular IFN-γ, IL-2 and TNF-α cytokines. Before staining, the cells were incubated and stimulated with brefeldin A and anti-CD3/anti-CD28 antibodies as a means to assess what the cells were capable of producing *ex vivo*. As Figure 4 shows, the number of multifunctional T cells capable of producing IFN-γ, IL-2 and TNF-α increased in the BCG- and 1.0 μg nHspX-vaccinated mice at day 30. At 6 months after the last vaccination, however, only the BCG-vaccinated group had increased numbers of multifunctional T cells compared with the saline control group. While not statistically significant, there was an increase in the number of multifunctional T cells in the HspX-vaccinated groups that was concentration-dependent, with the highest concentration of nHspX (10 μg) eliciting...
the greatest number of multifunctional T cells at the 6-month time point. Interestingly, the 1.0 μg concentration of nHspX was able to induce multifunctional CD4+ T cells within the first 30 days after immunization.

Native HspX vaccine boosts the immunogenicity and protective efficacy of BCG

One shortcoming of the BCG vaccine is that it fails to induce protective T-cell responses against latency-associated antigens. As the nHspX subunit vaccine protected mice in both the short-term and long-term studies, we next wished to know whether it could boost the protective immune response of BCG vaccination, and potentially compensate for the lack of T-cell responses to dormancy antigens like HspX in BCG-vaccinated individuals. Mice were vaccinated once with BCG followed by one dose of nHspX 6 weeks later. The mice were exposed to a low-dose aerosol challenge, and their lungs assayed for viable M. tuberculosis bacilli 30 days post infection. Table 1 shows that BCG boosted with nHspX was highly effective (P < 0.0001 compared with the saline control mice), decreasing the bacterial load in both the lungs and the spleen better than BCG vaccination alone (P = 0.05). Accordingly, the number of IFN-γ-secreting spleen cells was significantly greater in the mice vaccinated with BCG and boosted with nHspX compared with the BCG vaccine alone (Figure 5).

Granulomatous response in the lungs varies depending on native HspX vaccine concentration

To examine if the concentration of native HspX subunit affected granuloma formation and lung architecture, mice were immunized with 0.1 μg, 1.0 μg, or 10 μg nHspX three times over a 6-week period, and then infected with M. tuberculosis by the aerosol route 30 days after the last vaccine dose. Lungs were harvested 30 days post infection and processed for microscopy. The inflammation in each group of nHspX-vaccinated animals (Figure 6, panels d: 0.1 μg; e: 1.0 μg; f: 10 μg) was quantitatively similar to that observed in the saline- and adjuvant-control animals (Figures 6a and c, respectively). The lesions in all of the groups were markedly dominated by lymphocytes bordered by activated macrophages, and no neutrophils were observed. BCG-vaccinated mice (Figure 6b) had quantitatively milder lung inflammation, but the composition of the infiltrates was similar to that of nHspX-vaccinated animals. Interestingly, the number and size of the granulomatous lesions in the 1.0 μg nHspX-vaccinated mice (Figure 6e) was most similar to those in the BCG-vaccinated mice. These results are not surprising considering the number of live bacilli was most reduced in the lungs of the groups vaccinated with either BCG or the 1.0 μg concentration of native HspX.

rHspX protein incubated with whole-cell lysate protects against M. tuberculosis

Our findings suggest that native HspX, not rHspX, confers protection in our model. This finding is unexpected, given that native HspX does not appear to be post-translationally modified. We further investigated the ineffectiveness of rHspX compared with native HspX to explore other rationale for this difference in protection. Given that one of the major roles of heat shock proteins is to chaperone other proteins, particularly during cell stress as is the case with α-crystallins, we hypothesized that native HspX protein conferred protection because of either its ability to chaperone...
another antigen or other unique attribute present only when the protein is produced by M. tuberculosis. To test this possibility, we treated rHspX with whole-cell lysate from a M. tuberculosis ΔhspX deletion mutant (ΔHspX) and then used it as a vaccine to determine if it could protect mice from infection with either wild type or ΔHspX. Whole-cell lysate from the ΔHspX strain was used because of binding of the rHspX to nHspX protein in the wild-type strain cell lysate (data not shown). The resultant protein, now termed rHspX-pulldown, was used to immunize mice. Mice were challenged 30 days later with either wild type or ΔHspX strains of M. tuberculosis. Lungs were assessed 30 days post infection for the number of viable bacilli. Consistent with that shown previously, rHspX did not protect against either strain of M. tuberculosis, but rHspX-pulldown protein significantly reduced the bacterial load in mice infected with either the wild type or ΔHspX strain of M. tuberculosis (Figure 7).

DISCUSSION

While most TB vaccines currently in clinical trials target early expressed antigens, targeting latency-associated antigens, like HspX, has recently gained momentum in the field of TB vaccine development. In the present study, we tested the ability of HspX protein to induce short- and long-term protective immunity both as a prophylactic vaccine and as a booster to BCG vaccination. We found that native HspX conferred both short- and long-term protection, with long-term protection superior to that conferred by BCG. In fact, native HspX boosted BCG both in terms of protective efficacy and immunogenicity. The protective effect of the native HspX subunit vaccine was concentration-dependent, which also affected the quality of the immune response and extent of immunopathology in the lungs. Interestingly, rHspX was ineffective against infection, possibly due to chaperoning and/or folding differences between the native and recombinant forms of the protein.

Owing to the nature of TB infection, an effective TB vaccine should protect prophylactically, as well as against reactivation of latent TB. HspX is a logical vaccine target antigen because it is produced both during latency and, as our results support, during the early phase of infection in vivo. Here, the subunit form of the vaccine induced protective immunity within the first 30 days of infection (Figures 1a and b), suggesting that HspX protein is produced by the bacilli and is detected by the immune response during the early stage of infection, thereby rendering HspX an effective prophylactic vaccine candidate. Furthermore, the concentration of native HspX protein used per dose to vaccinate the animals affected the ability of the vaccine to protect. These data suggest that the concentration of a vaccine antigen may determine its ability to protect and that higher concentrations do not provide better protection. As in the lungs, the 0.1 μg concentration
was ineffective in the spleen, indicating that the concentration of the vaccine affected the dissemination of bacilli, and that the optimal concentration for preventing dissemination was 1.0 mg. Compellingly, the native HspX vaccine was able to generate long-term protective immunity that rivaled that of BCG (Figure 2a, \( P = 0.05 \)).

The vaccinating dose of native HspX also affected the quality of the immune response. Induction of cell-mediated immunity is essential for protection against TB and CD4\(^+\) T cells are thought to impart protection by producing T helper cell type 1 cytokines.\(^{27}\) The native HspX vaccine used in the present study, therefore, is a promising

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**Table 1** Native HspX vaccine boosts the protective efficacy of BCG vaccination

| Vaccine group | Lung | Spleen | Lung | Spleen |
|---------------|------|--------|------|--------|
| Saline        | 7.27 ± 0.20 | 5.82 ± 0.08 | 7.27 ± 0.20 | 5.82 ± 0.08 |
| BCG           | 6.43 ± 0.21* | 4.98 ± 0.22** | 8.55** | 0.84** |
| Adjuvant      | 7.28 ± 0.23 | 5.80 ± 0.09 | 0.00 | 0.02 |
| BCG + Adjuvant| 6.57 ± 0.11** | 5.27 ± 0.22* | 0.71** | 0.55* |
| BCG + rHspX   | 6.57 ± 0.30** | 5.15 ± 0.27* | 0.70** | 0.66* |
| BCG + nHspX   | 6.17 ± 0.13*** | 4.59 ± 0.26** | 1.10 *** | 1.23** |

Abbreviations: BCG, bacilli Calmette-Guerin; CFU: colony forming units. ***\(P<0.0001\), **\(P<0.01\) and *\(P<0.05\) compared with the saline-vaccinated group, and *\(P<0.05\) compared with BCG alone using a 2-tailed Student's t-test.

\(\text{n} = 5\) mice in each group.

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**Figure 4** Native HspX vaccine induces multifunctional CD4\(^+\) T cells. Splenocytes were obtained from vaccinated mice at 30 days (white bars) and 6 months (gray bars) post vaccination. Single cell suspensions were treated with brefeldin A, anti-CD28 and anti-CD3 for 6h and then stained for surface CD4 and for intracellular IFN-\(\gamma\), IL-2 and TNF-\(\alpha\). (a) Gating strategy: lymphocytes were gated according to their scatter profile and then further gated upon CD4-positive, IFN-\(\gamma\)-positive cells that were double positive for TNF-\(\alpha\) and IL-2. (b) The data are expressed as the percentage of multifunctional CD4\(^+\) T cells within the lymphocyte gate. Data are expressed as the mean (\(n=5\) mice) s.e.m. **\(P<0.01\) and *\(P<0.05\) when compared with the saline-vaccinated group using a 2-tailed Student's t-test.
vaccine candidate because it induced IFNγ-producing cells both in the short- and long-term models (Figure 3). The marked increase in the number of HspX-specific spleen cells producing IFNγ 30 days post vaccination compared with almost 10-times as many cells observed after 6 months suggests that the native HspX vaccine induced T helper cell type 1 memory immunity that correlated with a

Figure 6 Native HspX induces anti-mycobacterial inflammation dominated by lymphocytes and activated macrophages. Mice were immunized with 0.1–10 μg native HspX (nHspX) three times, 2 weeks apart, and then infected with a low-dose aerosol of H37Rv M. tuberculosis 30 days after the last immunization. Mice were killed 30 days post infection and the lungs were processed for microscopy. While inflammation in the nHspX-vaccinated animals (d: 0.1 μg; e: 1.0 μg; f: 10 μg) did not differ quantitatively from that seen in the saline- and adjuvant-control animals (a, c, respectively), the lesions were notably dominated by lymphocytes and a rim of activated macrophages. Neutrophils were absent. BCG-vaccinated animals (b) had quantitatively milder lung inflammation, but the composition of the infiltrates were similar to that of nHspX-vaccinated animals. Hematoxylin and eosin; 40 x.

Figure 7 rHspX protein incubated with whole-cell lysate protects against M. tuberculosis. (a) Diagrammatic representation of the hspX deletion/hyg insertion presents in the ΔHspX M. tuberculosis mutant strain. Open reading frames and relative orientations are indicated (arrows), and the hspX region replaced with a hygromycin resistance gene (hyg) is indicated (dashed lines). Mice were immunized with 5.0 μg native HspX (nHspX), rHspX or rHspX-pulldown three times, 2 weeks apart, and then infected with a low-dose aerosol of (b) H37Rv or (c) ΔHspX strains of M. tuberculosis 30 days after the last immunization. BCG-vaccinated mice were given a single dose at the time of the third immunization. Mice were killed 30 days post infection and the bacterial burden was assessed in the lungs. Data are expressed as the mean (n = 5 mice) ± s.d. ***P < 0.001, **P < 0.01 and *P < 0.05 when compared with the saline-vaccinated group using a 2-tailed Student’s t-test.

HspX protects mice against pulmonary tuberculosis
JL Taylor et al
Immunology and Cell Biology
significant reduction in the bacterial load following immunization with the 1.0 μg dose. Expansion of fewer HspX-specific cells in the BCG-vaccinated animals may be explained by the fact that BCG, as a live replicating vaccine, induces a very diverse T-cell pool, and therefore the number of HspX-specific T cells in these mice may be diluted compared with vaccination with a single protein. Moreover, it has recently been shown that multifunctional T cells, that is, T cells capable of simultaneously producing IFN-γ, IL-2 and TNF-α are functionally superior to single cytokine-producing T cells, and are correlated with protection against TB. As shown in Figure 4, multifunctional CD4+ T cells could be detected in the spleens of BCG- and HspX-vaccinated mice after 30 days and their occurrence was concentration-dependent. Only the BCG group maintained these cells over time, which were not necessarily HspX-specific as the cells were stimulated ex vivo with antibodies against CD3 and CD28. As such, it is not unexpected that BCG would induce a greater pool of multifunctional T cells specific to a wide variety of different antigens.

Besides affecting the quality of the immune response, the concentration of native HspX protein used to vaccinate mice also affected the pulmonary pathology following infection with M. tuberculosis. Immunization with the 1.0 μg concentration of native HspX resulted in the least amount of pathology (Figure 6e). Although the composition of the infiltrates was similar in all the groups, the inflammatory response in the lungs of the mice vaccinated with the 0.1 μg and 10 μg concentrations (Figures 6d and e, respectively) was more severe than in the BCG- or 1.0 μg HspX-vaccinated mice, with larger lesions resembling those observed in the saline- and adjuvant-control groups (Figures 6a and c, respectively). These histological data may lend insight into the observation that, while both the 0.1 μg and 10 μg concentrations of native HspX conferred protection, only the 1.0 μg concentration decreased dissemination of the bacilli to the spleen.

Here, we tested the ability of the native HspX vaccine to boost the protective efficacy of BCG and found that this approach significantly increased the protective efficacy of BCG vaccination alone. Boosting BCG with the native HspX vaccine significantly increased the number of HspX-specific, IFN-γ-producing cells in mice, which may have been, in part, the reason the prime-boost approach worked better than BCG alone. Overall, our results confirm data in the literature indicating that HspX, particularly when used in conjunction with BCG, is a promising target for developing vaccine regimens effective against the multiple stages of M. tuberculosis infection. It has been proposed that the failure of BCG to sterilize the lungs of M. tuberculosis may be due to its inability to induce long-lived T-cell responses against latency-associated antigens like HspX. That said, replacing BCG seems unlikely at this point as it is the most widely distributed vaccine in the world, and it confers protection in children. Several investigators have recently tested different vaccine strategies aimed at inducing both prophylactic and therapeutic protection against M. tuberculosis. Aagaard et al. recently tested a ‘multistage’ vaccine regimen directed against both early-secreted and dormancy-associated antigens and found it to be protective prophylactically, as well as against reactivation TB disease. Others have employed HspX-based vaccines as a means to improve upon the protective effect of BCG in mice.

Based on these data combined with those from other laboratories, incorporating HspX into vaccine regimens may prove a powerful strategy. Our finding, however, that the rHspX was ineffective against pulmonary TB (Figure 1a and Figure 7), indicated that the engineering of this protein (and possibly others) must be considered in vaccine strategy design. The HspX protein is a member of the α-crystallin-like heat shock protein family and functions as a molecular chaperone, in part by preventing heat-induced aggregation of proteins in M. tuberculosis. Although it is unclear how this chaperoning function may contribute to the hypoxic response of the bacilli, it has been proposed that HspX may help to stabilize cell structures in the thickening cell wall, thereby allowing the bacilli to survive low oxygen tension within the granuloma. In this regard, we hypothesized that protective immunity is generated against a molecule the native HspX protein chaperones, and that this chaperoned molecule is produced by M. tuberculosis but not by E. coli. If this hypothesis is correct, the reason rHspX protein failed to protect was because E. coli did not produce the substrate molecule to which HspX binds, thereby preventing epitope exposure and subsequent generation of a protective immune response when rHspX alone is used as the vaccine. As shown in Figure 7, the fact that the recombinant protein, when incubated with whole-cell lysate from ΔHspX M. tuberculosis, was able to confer statistically significant protection against infection, suggested that this hypothesis could be true. Further investigation of potential molecular and functional differences among the native, recombinant and recombinant pull-down proteins is currently in progress.

In summary, the results of this study showed that native HspX subunit vaccination protected mice when used on its own and that protective immunity was long-lived. Perhaps more clinically relevant, though, is that native HspX boosted the protective effect of BCG vaccination, maintaining the positive aspects of BCG while improving upon its shortcomings, namely BCG’s inability to stimulate strong T-cell responses against latency-associated antigens. Another important finding of this study was that the native HspX subunit vaccine was protective, while the recombinant form was not, most likely because it chaperoned an immunogenic, mycobacterial molecule produced by M. tuberculosis. Based on the results presented here, the native HspX protein serves to be a promising multistage vaccine for use in both prophylactic and therapeutic approaches and to boost BCG vaccination.

**METHODS**

**Mice**

Specific pathogen-free female, 6–8-week-old C57BL/six mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All animals were maintained under barrier conditions with sterile mouse chow and water ad libitum. The specific pathogen-free nature of the mouse colonies was demonstrated by testing sentinel animals, which were shown to be negative for 12 known mouse pathogens. All experimental procedures were approved by the Colorado State University Animal Care and Use Committee.

**Bacterial strains and growth**

*M. tuberculosis* H37Rv (wild type) and *HspX* gene deleted (ΔHspX) were used. ΔHspX was made by allelic exchange using a modified protocol of Pelicic et al. Briefly, *hspX*-flanking regions from 825 bp to 107 bp upstream of *hspX* and from the second last codon of *hspX* to 644 bp downstream were amplified from genomic DNA of *M. tuberculosis* strain H37Rv with primer pairs hspXupF1[SpeI]/hspXupR1[PucI] and hspXdownF1[PucI]/hspXdownR1[BamHI], respectively, and inserted into *SpeI/BamHI*-digested plasmid pPR27. The cloned regions in the resulting plasmid (pPR27ΔhspX) were determined to be free of mutations by DNA sequencing. Plasmid was digested with *PvuI*, blunted and ligated with a ~1300 bp blunt *BspHI*/Smal hygromycin resistance gene from plasmid p16R1. Resulting plasmid, pPR27ΔhspX\_hyg, which contains the hygromycin resistance gene in the same orientation as the original *hspX* gene, was electroporated into *M. tuberculosis* strain H37Rv and the cells were plated on Middlebrook 7H11 agar supplemented with OADC (oleic acid, albumin, dextrose and catalase), 0.05% Tween-80 and 50 μg ml−1 hygromycin (7H11OADC\_CTH) and...
incubated at 30°C. Three transformants were cultured in 5 ml Dubos broth supplemented with OADC, 0.05% Tween-80, and 50 μg ml⁻¹ hygromycin for 6 weeks at 32°C. To obtain candidates that integrated the plasmid into the chromosome and lost the plasmid-encoded sacB gene, 100 μl of each culture was spread onto 7H11OADC medium supplemented with 2% sucrose and the plates incubated at 32°C for 6 weeks. To confirm plasmid loss by screening for loss of the plasmid-encoded gentamycin resistance gene, twenty colonies from each plate were patched onto 7H11OADC medium with 2% sucrose plates containing or lacking 5 μg ml⁻¹ gentamycin and incubated for 6 weeks at 32°C. Two of the 60 candidates were sensitive to gentamycin. Genomic DNA from both candidates was purified and screened by PCR with primers hspXoutF1 and hspXoutR1. Results demonstrated that only one of the strains (subsequently named AHspX) contained the single band corresponding to replacement of hspX with the hygromycin resistance gene. The mutant was confirmed by Southern analysis (data not shown).

Primers used:
hspXoutF1 forward: GGACTGATTGCGGATGATCGAGCA
hspXoutR1 reverse: ACCCTTTAATACCTTGCCGTTGCTGTG
hspXdownF1 forward: ACCCTTAATACCTTGACACCTTGCGGCT
hspXdownR1 reverse: CGGGATTCTCCCTGCTGGTGACCT
hspXoutF1: GCACCCGATCTTTCGAGGC
hspXoutR1: CAGCAGCTCTGATTCAGACC

One milliliter of each wild type and AHspX was transferred into a glass tube containing 9 ml of Proskauer-Beck (PB) medium39 and static cultures incubated at 37°C for 3 weeks, or until pellicle formation was visible on top of the media. Pellicles were harvested and used for sequential inoculation into 25 ml and 100 ml of PB medium and incubated as before. Pellicles of M. tuberculosis from the final passage were transferred to 20 ml of PB media containing 20% glycerol (v/v); cells were mixed by agitation, and suspensions bath sonicated at 4°C for 10 min. One milliliter seed stock vials were prepared and stored at -80°C. One vial of each strain of seed stock was added to 9 ml of 7H9 + OADC medium containing 0.1% Tween-80 and incubated at 37°C with shaking for 14 days. Cultures were inoculated into 45 ml of 7H9 plus OADC + 0.1% Tween-80 and incubated at 37°C with shaking, harvested, and 1 ml working stock vials were prepared and stored at -80°C. Three vials of each strain were randomly recovered, thawed, serially diluted in 7H9 + OADC medium and plated in triplicate for the determination of CFU.

Protein purification
Native HspX (nHspX) protein was purified from whole γ-irradiated Mtb cells following cell disruption using a French Press as described previously.39 The whole-cell lysate was centrifuged at 40,000 g, the supernatant discarded, and the pellet suspended in phosphate-buffered saline with 1% (v/v) n-octyl-D-thiogluopyranoside, and centrifuged at 27,000 g. The supernatant was collected, exchanged into 10 mM ammonium bicarbonate, and lyophilized. The dried material was suspended in 7,250 μl 0.1% Tween-80 and incubated at 37°C. After 16 h of growth at 37°C, the cells were harvested by breakage and soluble nHspX was purified by Ni²⁺ chromatography.39 Endotoxin levels were measured as described above and found to contain <10 ng EU mg⁻¹ of protein.

rHspX-pulldown protein was produced by incubation of purified, rHspX with AHspX WCL (10:1; lysate rHspX ratio) in 10 mM ammonium bicarbonate at 4°C overnight with gentle agitation. The mixture was applied to Ni²⁺ resin and rHspX-pulldown purified with any binding partners as described above.

**Vaccination procedure**
In the studies comparing rHspX and nHspX, mice were injected subcutaneously (s.c.) three times at 2-week intervals with either protein. For the dosing studies, mice were injected three times at 2-week intervals with rHspX protein at 0.1 μg, 1.0 μg or to 10 μg. As a positive control, one group of mice was vaccinated with a single dose of BCG Pasteur (1 × 10⁶ CFU) at the time of the third vaccination dose. Negative controls included one group injected s.c. with saline, and one group injected s.c. with the adjuvant formulation alone. For the BCG prime-boost study, mice were vaccinated s.c. with BCG as above, followed by one dose of recombinant or native HspX 6 weeks later. In all studies, the HspX was emulsified in an adjuvant formulation containing diocateyl ammonium bromide (250 μg per dose) and monophosphoryl lipid A (25 μg per dose) as previously described,41 and all mice were injected s.c. with 100 μl of the vaccine formulation in the scuff of the neck.

**Aerosol infection**
Mice were infected by using procedures described previously.42 Briefly, bacterial stocks were diluted in 5 ml sterile distilled water to 2 × 10⁸ CFU ml⁻¹ and placed in a nebulizer attached to an airborne infection system (Glass-Col, Terre Haute, IN, USA). At 30 days post vaccination or at 6 months post vaccination, mice were exposed to 40 min of aerosol, during which time approximately 50–100 bacteria were deposited in the lungs of each animal. Bacterial load was determined by plating whole-organ homogenates onto nutrient 7H11 agar supplemented with OADC. Colonies were enumerated after 21-day incubation at 37°C. For the BCG prime-boost study, mice were infected by the aerosol route as described above 30 days after the last vaccination.

**Spleen cell isolation**
At 30 days or 6 months after the last vaccination, mice were euthanized by CO₂ asphyxiation and the body cavity opened. The spleen was removed and passed through a cell strainer (BD Biosciences, Mountain View, CA, USA). Remaining red blood cells were then lysed with Gey’s solution (0.15 μl NH₄Cl and 10 μM KHCO₃), and the cells washed with RPMI (Invitrogen) containing 5% heat-inactivated fetal bovine serum (Atlas Biologicals, Fort Collins, CO, USA). Total viable cell numbers were determined using a Neubauer chamber (IMV International, Minneapolis, MN, USA) and 2% trypan blue solution.

**IFNγ ELISPOT**
MultiScreen 96-well high throughput screening (HTS) IP sterile plates (Millipore, Bedford, MA, USA) were coated with anti-IFNγ antibody at 4°C overnight. The plates were blocked according to the manufacturer (eBioscience, San Diego, CA, USA), and spleen cells (5 × 10⁶ cells per well) were cultured with 10 μg ml⁻¹ nHspX or rHspX protein in complete RPMI 1640 (10% fetal bovine serum, penicillin–streptomycin, non-essential amino acids,…
Flow cytometric analysis
Levels of intracellular IFN-γ, IL-2 and TNF-α produced by stimulated T lymphocytes was measured by pre-incubation of 10^5 cells with Monensin (3 μM) and anti-CD3 and anti-CD28 (both at 0.2 μg per 10^5 cells) for 4 h at 37°C, 5% CO2. The cells were then stained with fluorescent-labeled antibodies against the surface markers CD4, washed, fixed and permeabilized with PermFix/Perm Wash (BD Pharmingen, San Diego, CA, USA), and stained for intracellular IFN-γ, IL-2 and TNF-α using fluorescent-labeled antibodies and appropriate isotype controls. Cells were gated on lymphocytes by forward and side scatter according to their characteristic scatter profile. Antibodies were used at 0.2 μg per 10^5 cells, and all analyses were performed with an acquisition of at least 100,000 events on a Becton-Dickinson FACScan/Calibur (BD Biosciences). The data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Histological analysis
Whole lungs from euthanized mice (n = 3) were inflated with 10% formalin, embedded in paraffin and five, 4 μm serial sections were cut. Sections 1 and 5 were then stained with hematoxylin and eosin and examined by a veterinary pathologist with no prior knowledge of the experimental design.

Statistical analysis
Residuals from a one-way analysis of variance were tested for normality using a Wilk-Shapiro test with P-values ranging from 0.09 to 0.98 across all of the data presented in this manuscript. As these P-values are consistent with the assumption of normality (that is, P > 0.05), the Student t-test was used to determine statistical significance.

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