Discovery of dormancy associated antigens of Mycobacterium tuberculosis : novel targets for the development of post-exposure or therapeutic tuberculosis vaccines

Lin, M.Y.

Chapter 5

T-cell recognition of the HspX protein of *Mycobacterium tuberculosis* correlates with latent *M. tuberculosis* infection but not with *M. bovis* BCG vaccination

Annemieke Geluk, May Young Lin, Krista E. van Meijgaarden, Eliane M. S. Leyten, Kees L. M. C. Franken, Tom H. M. Ottenhoff, and Michèl R. Klein

Department of Immunohematology & Blood Transfusion and Department of Infectious Diseases, Leiden University Medical Centre, the Netherlands

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Abstract

During stationary growth or in vitro conditions mimicking relevant aspects of latency such as hypoxia, the HspX protein (Rv2031c) is specifically upregulated by *M. tuberculosis*. In this study we compared T cell responses against HspX and the secreted *M. tuberculosis* protein Ag85B (Rv1886c), in tuberculosis (TB) patients, tuberculin skin test positive individuals (TST+), BCG-vaccinated individuals and healthy negative controls. IFN-γ responses against HspX were significantly higher in *M. tuberculosis* exposed individuals than in *M. tuberculosis* unexposed BCG vaccinees. In contrast, no such differences were found with respect to T cell responses against Ag85B. Therefore, BCG-based vaccines containing relevant fragments of HspX, may induce improved responses against this TB latency antigen. To identify relevant MHC class I- and class II-restricted HspX-specific T cell epitopes, we immunized HLA-A2/K^b and HLA-DR3.Ab^0 transgenic (tg) mice with HspX. Two new T cell epitopes were identified, p91-105 and p31-50, restricted via HLA-A*0201 and HLA-DRB1*0301, respectively. These epitopes were recognized by human T cells as well, underlining the relevance of HspX T cell recognition both in vivo and in vitro. In line with the data in humans, BCG immunization of both tg strains did not lead to T cell responses against HspX-derived epitopes, whereas nonlatency antigens were efficiently recognized. These data support the notion that BCG vaccination per se does not induce T cell responses against the latency antigen, HspX. Thus, we suggest that subunit vaccines incorporating HspX and/or other latency antigens, as well as recombinant BCG strains expressing latency antigens need to be considered as new vaccines against TB.

Introduction

Tuberculosis (TB) is one of the leading causes of mortality (two million annually) from an infectious disease, particularly in the developing world (36). It is estimated that 2 billion people are latently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the etiologic agent of TB which causes active disease in 5-10% of infected individuals. Most individuals initially control the infection by mounting cell-mediated immunity. However, residual mycobacteria remain viable for many years in healthy immunocompetent hosts in the absence of disease (4) and the majority of contagious TB disease cases will arise from this enormous source of latent TB infection.

The currently available vaccine against TB, *M. bovis* BCG, is largely ineffective at protecting against pulmonary disease in adults (16, 23). The precise nature of the T cell response needed for protection against adult pulmonary TB is incompletely defined, as is the cause for the lack of protection by BCG vaccination (3).

The 16kDa heat shock protein HspX (Rv2031c) is required for mycobacterial persistence within the macrophage and is a dominant protein produced during static growth or under oxygen deprivation (37). Under these conditions, it can account for up to 25% of the total bacillary protein expression. It is proposed that HspX plays an active role in slowing the growth of *M. tuberculosis* in vivo immediately after infection, as *M. tuberculosis* mutants lacking the hspX gene exhibited increased growth both in mice and in macrophages (24). In addition to the presence of specific humoral responses against HspX in sera of cavitary TB patients (29), T cell responses to HspX were also found to be associated with latent *M. tuberculosis* infection (13, 14) pointing to the importance of HspX as an antigenic target of immune responses during latent TB infection.

Since new vaccines containing relevant fragments of HspX, may induce improved responses against this TB latency antigen, we have generated and characterized HspX-specific, human $CD8^+$ and $CD4^+$ T cells, restricted by common HLA class I and class II alleles. In addition, PBMC from *M. tuberculosis* infected individuals (active as well as latent infection) and BCG vaccinated individuals with and without exposure to *M. tuberculosis* were examined for their in vitro response to HspX. Finally, the effect of BCG- or HspX immunization on induced immunity against HspX was analyzed in HLA-$A2/K^b$ and HLA-$DR3.Ab^0$ transgenic (tg) mice.

Materials and Methods

Antigens

BCG (*M. bovis* bacillus Calmette Guérin, Danish 1331) was purchased from the Statens Serum Institute (Copenhagen, Denmark), killed *M. tuberculosis* H37Rv sonicate was obtained from Dr. D. van Soolingen (RIVM, The Netherlands). The antigen 85B gene (Rv1886c) and the hspX gene (Rv2031c) of *M. tuberculosis* were amplified by PCR and cloned by Gateway Technology (Invitrogen, San Diego, CA) in a bacterial expression vector containing an N-terminal histidine tag. The proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified, as described (17).

Study subjects

The study included 63 individuals, amongst whom 17 TB patients, 18 tuberculin skin test (TST) positive individuals, 17 BCG vaccinated individuals and 11 non-BCG vaccinated, TST negative, healthy Dutch controls. All individuals gave informed consent before blood sampling. The study protocol was approved by the institutional review board of the Leiden University Medical Centre.

ELISPOT assay for single-cell IFN-γ release

Venous blood was obtained from study participants in heparinized tubes and PBMC isolated by Ficoll density centrifugation. PBMC ($1x10^6$) were pulsed (16h) in 48-well plates with antigen (10 μg/ml) in IMDM (Life Technologies) with 10% pooled human serum. PVDF-backed 96-well plates (MAIPS45, Millipore, Bedford, MA) were pre-coated with 5 μg/ml anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden), washed 6x with IMDM and blocked (2 h) with IMDM containing 10% FCS. Antigen-pulsed PBMC ($2.5x10^5$ cells/well; 150 μl) were incubated in the PVDF-backed 96-well plates at 37°C, 5% CO_2. After 16 h plates were washed (PBS 0.05% Tween-20), incubated with 100 μl biotinylated anti-IFN-γ mAb (0.3 μg/ml) for 3 h at RT, washed, incubated with streptavidin-alkaline phosphatase conjugate (1:1000; Mabtech) for 2 h, washed and incubated with 100 μl NBT/BCIP substrate (Sigma, St. Louis, MO). The reaction was stopped by addition of water. Plates were dried and analyzed using a Zeiss Axioplan 2 microscope and KS ELISPOT software (CarlZeiss Vision, Hallbergmoos, Germany). A positive response to antigen was taken as twice the background.

Synthetic peptides

15-mer and 20-mer peptides were synthesized by simultaneous multiple peptide synthesis as described (30). Homogeneity was confirmed by analytical HPLC and by mass spectrometry. Fluorescence labeled peptides were synthesized as described previously (19).

HLA-DR/ peptide binding assay

As a source of HLA-DR molecules EBV-transformed B lymphoblastoid cell lines (EBV-BLCL) homozygous for HLA-DR were used. HLA-DR molecules were purified by affinity chromatography and peptide binding to purified HLA-DR3 molecules (60-600 nM) was determined as described (21). As standard fluorescent peptides influenza hemagglutinin (HA) p308-319 (PKYVKQNTLKLAT) was used for HLA-DR1 and HLA-DR2, and hsp65 p3-

13 (KTIAYDEEARR) for HLA-DR3. Peptide binding affinity was defined as high (IC_{50} < 1μM), intermediate (1μM - 10 μM), weak (10 μM - 100 μM) or absent (> 100 μM) (21).

Generation and epitope mapping of human, HspX-reactive $CD8^+$ T cell lines
PBMC ($1x10^5$cells/ well) derived from an HLA-A*0201$^+$ donor were stimulated with peptide pools containing four 15-mer HspX peptides overlapping 10 AA (10 μg/ ml of each peptide) for 7 days in IMDM (10% HS) in the presence of r-IL7 (5 ng/ ml, Biocarta) and r-IL15 (5 ng/ ml, Biocarta) in 96 wells round bottom plates. After 2 days r-IL2 (25 U/ ml, Cetus) was added to the cultures.

Intracellular IFN-γ staining
Seven days later, cells were collected and co-cultured for 6 h with the HLA-A*0201-positive EBV-BLCL JY, that had been pulsed overnight with single HspX peptides in IMDM (10% FCS) and washed twice with IMDM (10% FCS). During the last 2 h of co-culture, Brefeldin A was added (10 μg/ ml, Sigma). Cell-surface staining was performed using CD19-FITC, CD4-PerCP and CD8-APC (all Becton Dickinson, CA) after which the cells were permeabilized with Perm buffer (Pharmingen, CA) and stained with anti-IFN-γ-PE (Becton Dickinson, CA). Stimulation with phorbol myristate acetate-ionomycine was used as a positive control and unstained cells as a negative control.

Generation and epitope mapping of human, HspX-reactive $CD4^+$ T cell lines
PBMC (1.5×10^6 cells/ well) of healthy human donors were stimulated with HspX (10 μg/ ml) in IMDM (10% human serum) in 24-well plates. After 6 days r-IL-2 (25 U/ ml (Chiron, CA) was added and cells were additionally cultured for 10 to 15 days, frozen and stored in liquid nitrogen until further use. Human $CD4^+$ T cell lines (1.5×10^5 cells/ well) and HLA-DR matched irradiated PBMC (5×10^5 cels/ well) were incubated with either HspX (10, 1.0, 0.1 or 0.01 μg/ ml) or HspX-derived peptides (10, 1.0, 0.1 or 0.01 μg/ ml) in flat-bottom 96-well plates. After 72 h, 0.5 μCi of [^{3}H]thymidine was added to each well. After 18 h, cells were collected on glass fiber filter strips and the radioactivity incorporated into the DNA was determined by liquid scintillation counting. Results are the mean of triplicate cultures. SEM were < 20%.

HLA transgenic (tg) mice
HLA-DRB1*0301/DRA tg, murine class II deficient (HLA-DR3.Ab^0) mice were generated as described (18). During breeding, PBMCs were typed for expression and segregation of the transgene by flow cytometry and PCR (27). HLA-A2/K^b mice expressed, in addition to the murine class I alleles H2-K^b and H2-D^b, a chimeric HLA-A*0201/K^b gene encoding H2-K^b α3 domain and the HLA-A*0201 α1 and α2 domains (20). HLA-A2/K^b mice were bred under specific pathogen-free conditions at TNO-PG (Leiden, The Netherlands). Surface expression of HLA-A*0201/K^b molecule was confirmed by flow cytometry.

Immunizations
Emulsions comprising equal volumes of HspX recombinant protein in PBS and incomplete Freund's adjuvant (IFA; DIFCO, Detroit, MI) were prepared and administered as subcutaneous (s.c.) injections into the flanks (in total 10 μg protein per mouse, n = 4).

Live *M. bovis* BCG (Montreal strain) was diluted with PBS. Each mouse was s.c. injected with 50 μl around each hind leg (in total $1x10^6$ CFU/ mouse). Seven days post-injection, spleens were removed and cell suspensions prepared for in vitro culture. For DNA immunizations mice were injected intramuscularly 3 times (at 3-wk intervals) in both quadriceps (2x 50 μl) with HspX plasmid (1 mg/ml) or control DNA (empty vector) in PBS. Splenocytes were harvested three weeks after the last DNA injection.

In vitro culture

Splenocytes from each mouse (1.5×10^5 cells/ well) were stimulated in triplicate cultures with antigen in round bottom 96-well plates in 200 μl RPMI 1640 (Life Technologies) supplemented with 2mM L-glutamine (Life Technologies), 100 U/100 μg/ml penicillin/ streptomycin solution (Life Technologies) and 10 % heat-inactivated FCS (Life Technologies). HspX and *M. tuberculosis* sonicate were tested at 5 μg/ ml, peptides at 25 μg/ ml. After 6 days, 0.5 μCi of [^{3}H]thymidine was added to each well. After 18 h cells were collected on glass fiber filter strips and the radioactivity incorporated into the DNA was determined by liquid scintillation counting. Results are the mean of triplicate cultures. SEM were <20 %.

ELISA murine IFN-γ

Splenocytes were resuspended in RPMI-1640 medium (Life Technologies, Rockville, MD, USA) supplemented with 10% heat-inactivated FCS (INTEGRA Biosciences AG, Switzerland) were added to 96-well U-bottom plates (Corning B.V. Life Sciences, The Netherlands) and stimulated in triplicates with antigens. After 72 hours, culture supernatants were taken and evaluated for their IFN-γ content using murine IFN-γ CytoSet™ ELISA kit (Biosource, Camatillo, CA, USA). The assay was performed according to the manufacturer guidelines.

Cytotoxicity assays

The human EBV-BLCL JY (HLA-A*0201, -B7, -Cw7) was incubated at 37° C for 1 hour with 0.1 mCi $Na_2{}^{51}CrO_4$ (Amersham, UK), washed and plated with pooled splenocytes from immunized mice (n = 4), in triplicates in 96-well round-bottomed plates (2500 cells/ well) together with medium, peptide (2 μg) or 5 % Triton-X100 (20). After 6 h supernatants were harvested and % specific lysis was calculated as: [(release – spontaneous release) / (maximum release – spontaneous release)] x 100%.

Statistical analysis

Differences in IFN-γ responses to HspX between different test groups were analyzed with the two-tailed Mann-Whitney U test for non-parametric distribution using Graph Pad Prism (version 4). *P*-values were corrected for multiple comparisons. The statistical significance level used was $P<0.05$.

Results

T cell responses against HspX do not correspond to BCG vaccination per se

Individuals with different stages of *M. tuberculosis* infection were previously reported to respond differentially to HspX (14). Since the effect on T-cell responses in BCG vaccination in areas where TB is and not endemic may vary (5), we analyzed HspX responses in IFN-γ ELISPOT assays using PBMC from BCG-vaccinated individuals and from non-BCG vaccinated, healthy controls in addition to TB patients and TST+ individuals (Figure 1).

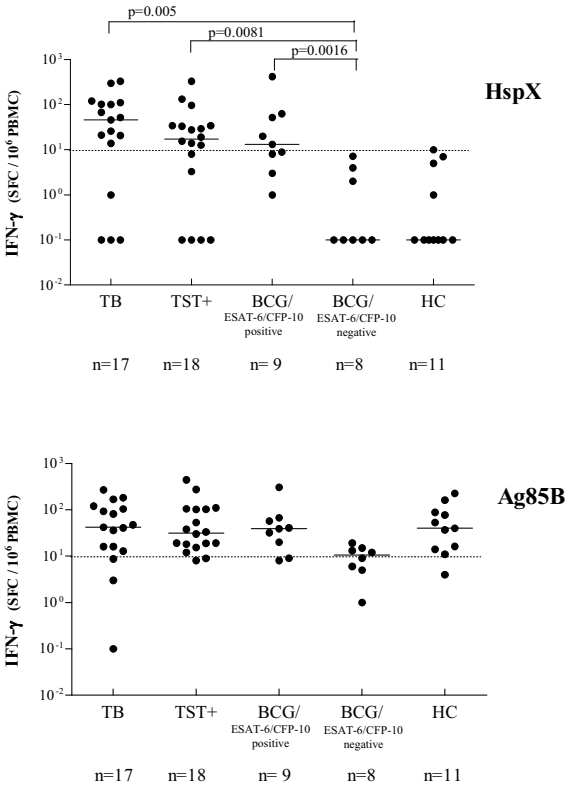

Figure 1. *IFN-γ production against HspX and Ag85B of M. tuberculosis by PBMC of 17 TB patients (TB), 18 tuberculin skin test positive (TST+) individuals, 17 BCG vaccinated individuals (nine in vitro positive for ESAT-6 and/or CFP-10 and eight in vitro negative for ESAT-6 and/or CFP-10), and 11 non-BCG vaccinated, healthy controls (HC). Spot forming cells (SFC) per 10⁶ PBMC corrected for medium values are given on the y axis. Median values are indicated by horizontal lines. P values are calculated by the non-parametric Mann Whitney U test. T cell responses were considered positive if the medium-corrected number of SFC was > 10/10⁶ cells.*

Based on their in vitro IFN-γ response to the *M. tuberculosis*-specific proteins ESAT-6 and/ or CFP-10 (32), we divided BCG vaccines into *M. tuberculosis*-unexposed individuals (<10 spot-forming cells [SFC]/10^6 PBMC; n=8) and individuals with likely exposure to *M. tuberculosis* (> 10 SFC/ 10^6 PBMC; n = 9; ranging from 16 to 116 SFC/10^6 PBMC).

Table 1. HspX/Ag85B ratios

Test group	Median HspX/ Ag85B value
TB patients	0.55
TST^+	0.33
BCG, ESAT-6/CFP-10 positive[a]	0.35
BCG, ESAT-6/CFP-10 negative[b]	0.060
Controls, *M. tuberculosis* unexposed	0.0062

[a] *In vitro responsive to ESAT-6 and/or CFP-10*
[b] *In vitro unresponsive to ESAT-6 and/or CFP-10*

HspX induced high numbers of IFN-γ producing T cells in three of the five groups (TB, TST^+ and BCG vaccinees responding to ESAT-6 and/or CFP-10). These T cell responses differed significantly ($P \leq 0.0081$) from BCG-vaccinated individuals without a response to ESAT-6 and/or CFP-10 and from healthy controls. As a control, the number of IFN-γ producing T cells induced by the secreted Ag85B was analysed in the same individuals. Interestingly, no significant differences between the five groups were detected in the number of SFC induced by Ag85B. This indicates that the lack of IFN-γ production in response to HspX in BCG vaccinees and controls is not caused by the absence of anti-mycobacterial responses. The median values of the HspX/Ag85B response ratio were 0.060 and 0.0062 in BCG-vaccinated, *M. tuberculosis*-unexposed individuals and the healthy control group, respectively, whereas *M. tuberculosis* infected or exposed groups had 10-fold higher HspX/Ag85B response ratios ranging between 0.55 and 0.33 (Table 1).

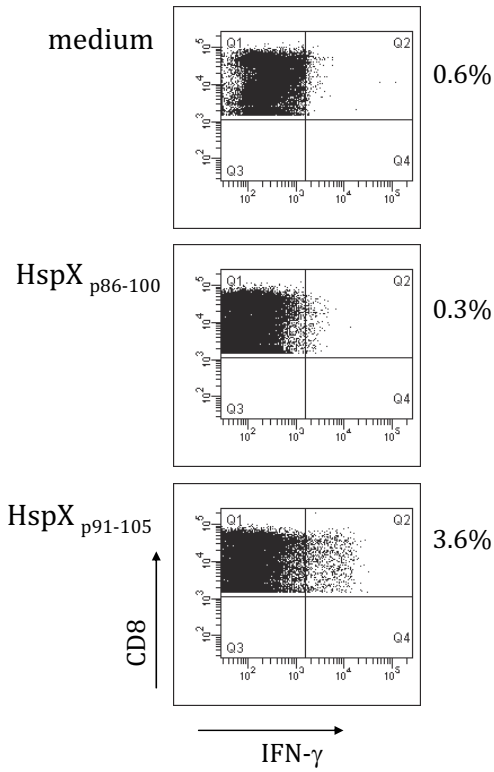

Figure 2. *Intracellular IFN-γ staining of HLA-A*0201+, CD8+ human T cell lines generated by stimulation of PBMC with seven peptide pools, each containing four HspX 15-mers. T cell lines were co-cultured for 6 h with the HLA-A*0201-positive EBV-BLCL JY, that had been pulsed overnight with one single HspX peptide (indicated on the left). Cells were incubated with PE-labeled anti-IFN-γ and APC-labeled anti-CD8 MAb. For the analysis, only lymphocytes were used with the exclusion of CD19-positive cells to avoid contamination of the JY cells.*

HLA-A*0201-restricted T cell epitopes

Since mounting evidence from human studies and murine models of TB points toward a role of $CD8^+$ T cells in controlling (latent) *M. tuberculosis* infection (22, 28, 31), we set out to identify HLA-class I-restricted T cell responses to the HspX *M. tuberculosis* latency antigen. PBMC derived from an HspX responding HLA-A*0201+ donor were stimulated with seven peptide pools, each containing four HspX-derived 15-mer peptides with an overlap of 10 amino acids. After 7 days, cells were

collected and co-cultured for 6 h with HLA-A*0201-positive EBV-BLCL, that had been pulsed overnight with single HspX peptides, and induction of intracellular IFN-γ was assessed by FACS analysis (Figure 2). Intracellular IFN-γ producing $CD8^+$ T cells were detected in the T cell line generated with pool 5. In this pool only one peptide, HspX p91-105, induced significant levels of intracellular IFN-γ production (Figure 2).

Murine HLA-A*0201-restricted epitopes

HLA-A2/K^b mice represent a suitable model for the induction and detection of high affinity HLA-A*0201-restricted $CD8^+$ CTL responses in vivo(11, 20). Thus, these mice were immunized three times with plasmid DNA encoding *hspX* or empty vector DNA. Three weeks after the last DNA injection, splenocytes were incubated with peptide pulsed, ^{51}Cr-labeled HLA-A*0201-positive JY cells (which express HLA-A*0201 but not H2-K^b or H2-D^b) in order to analyze their cytolytic potential. JY cells pulsed with HspX p91-105 were lysed by splenocytes of immunized mice (Figure 3). Splenocytes derived from mice immunized similarly with empty vector DNA did not show any lysis (data not shown). These data show that the same HLA-A*0201-restricted cytotoxic T lymphocyte (CTL) epitope is identified in HLA-A*0201 humans and in HLA-A2/K^b.

The relatively low level of lysis by the CTL raised in HLA-A2/K^b mice (maximal 25 %) could be due to the presence of the chimeric HLA-A*0201/K^b gene in the tg mice. This expresses the H2-K^b α3- and the HLA-A*0201 α1 and α2 molecules allowing efficient CD8 interaction of murine $CD8^+$ T cells and thus interacts less efficiently with the human HLA-A*0201 molecule expressed on the JY target cells.

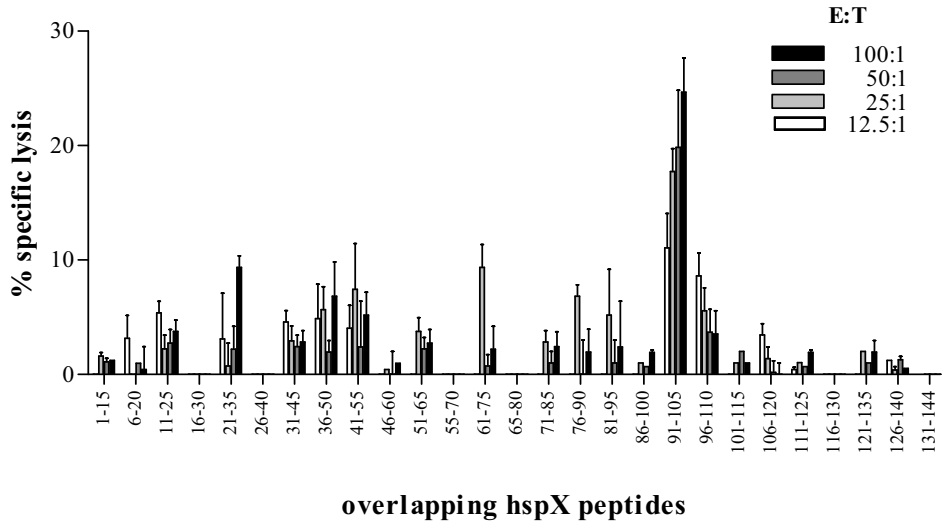

Figure 3. *Cytotoxic activity of splenocytes derived from HLA-A2/K^b mice, immunized with HspX plasmid DNA. M. tuberculosis-derived peptides used to pulse HLA-A*0201$^+$ target cells (JY) are indicated on the x axis. The percentage specific lysis is indicated on the yaxis. E:T, effector to target ratio.*

HLA-DR binding of HspX-derived peptides and HLA-DR-restricted T cell epitopes

Previously, several studies have shown that different regions of the *M. tuberculosis* HspX latency antigen can provoke human CD4+ T cell responses. Especially the immunodominant region p91-105 is permissively recognized, since peptide epitopes were defined in the context of HLA-DRB1*0101, -DRB1*1101, -DRB1*1301, -DRB1*1501, and DRB1*0401 (1, 2). Other peptide sequences recognized by human T cells are: p21-40 (HLA-DRB1*0401) (1), p21-29 and p120-128 (both HLA-A*0201) (10).

To study CD4+ T cell responses to HspX, we first assessed HLA-binding affinity of HspX-derived peptides. Ten amino acids overlapping 20-mer peptides, covering the entire sequence of the HspX protein of *M. tuberculosis* were used to analyze peptide binding to purified HLA molecules (Table 2). HLA-DR1 and HLA-DR2 showed similar binding affinities for the HspX peptides, particularly p11-30 and p91-105 bound with high affinity to these alleles. HLA-DR3, on the other hand, bound especially well to p31-50, which was confirmed by the presence of the HLA-DR3 peptide binding motif (21) with L (Leucine) at position n, E (Glutamate) at position n+2 and K (Lysine) at position n+4 (Table 2).

HspX 20-mers were used for epitope mapping in three human, HspX-specific T cell lines from individuals homozygous for HLA-DRB1*0101, -*01501 or -*0301 (Figure 4). The HLA-DRB1*0101+ T cell line recognized p91-105 (Figure 4A), which also binds with high affinity to both HLA-DR1 and HLA-DR2. Another peptide that bound with high affinity to these alleles, p21-40 (Table 2), induced high proliferative responses, whereas p71-90, which bound with lower affinity, induced moderate responses in the HLA-DRB1*01501+ T cell line (Figure 4B). The HLA-DR3-restricted epitope identified here, p31-50 (Figure 4C), corresponded well with the high binding affinity for HLA-DR3 as described for other HLA-DR3-restricted, mycobacterial peptide epitopes (18). T cell responses were HLA-DR-restricted as MAbs directed against HLA-DR (B8.11.2) decreased proliferation of all three T cell lines (data not shown).

Table 2. HLA-DR binding of overlapping HspX peptides[a]

Amino acids	Sequence	IC50 (μM)		
		HLA-:DR*0101	DR*1501	DR*0301
1-20	MATTLPVQRHPRSLFPEFSE	15	22	>100
11-30	PRSLFPEFSELFAAFPSFAG	< 0.7	< 0.7	42
21-40	LFAAFPSFAGLRPTFDTRLM	5	**0.9**	68
31-50	LRPTFDTRLMR**L**ED**EM**K**E**GR	24	35	**0.5**
41-60	RLEDEMKEGRYEVRAELPGV	8	13	41
51-70	YEVRAELPGVDPDKDVDIMV	12	17	35
61-80	DPDKDVDIMVRDGQLTIKAE	5	3	4
71-90	RDGQLTIKAERTEQKDFDGR	14	9	>100
81-100	RTEQKDFDGRSEFAYGSFVR	6	1	>100
91-110	SEFAYGSFVRTVSLPVGADE	**< 0.7**	**< 0.7**	45
101-120	TVSLPVGADEDDIKATYDKG	>100	>100	>100
111-130	DDIKATYDKGILTVSVAVSE	>100	70	>100
121-140	ILTVSVAVSEGKPTEKHIQI	>100	>100	>100
125-144	SVAVSEGKPTEKHIQIRSTN	>100	>100	>100

[a] *T cell epitopes and associated values are depicted in boldface.*

Murine HLA-DR3 restricted epitopes

To address the in vivo immunogenicity and epitope specificity of the HspX protein, we have used HLA-DR3.Ab^0 tg mice. After immunization of the mice with HspX protein in IFA, splenocytes were restimulated in vitro with the overlapping HspX 20-mer peptides. Since the HLA-DR3.Ab^0 mice are devoid of any murine class II molecules expressed at the cell surface, all CD4$^+$ T cells are restricted by HLA-DR3. Figure 4D shows that murine HLA-DR3-restricted T cells recognize the same immunodominant HspX peptide (p31-50) as human HLA-DR3-restricted T cells, indicating that p31-50 is an in vivo processed and presented HLA-DR3-restricted T cell epitope.

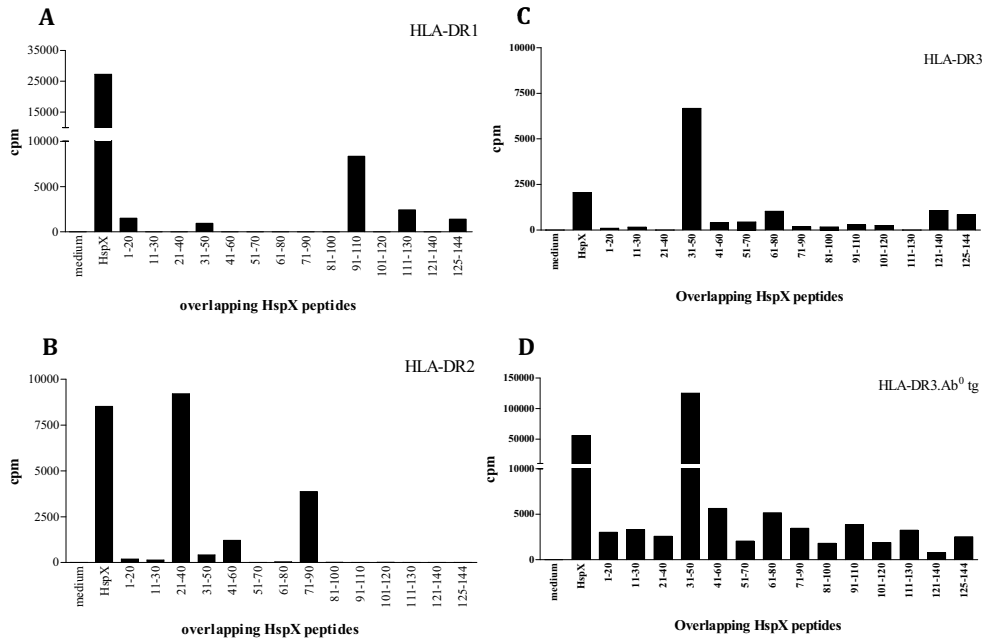

Figure 4. *T cell proliferation of HLA-DR1$^+$ (**A**), -DR2$^+$ (**B**) or -DR3$^+$ (**C**) human T cell lines responsive to the HspX protein, or of splenocytes from HLA-DR3.Ab^0 tg mice immunized with recombinant protein of HspX (**D**). T cell proliferation (in counts per minute [cpm]) corrected for medium values is shown on the y axis. The SEM were < 12%. The amino acid numbering of the overlapping HspX peptides from Table 2 is given on the x axis.*

BCG immunization of HLA-A2/K^b and HLA-DR3.Ab^0 does not induce T cell responses against HspX

Since BCG vaccinated, *M. tuberculosis* unexposed individuals in a cross-sectional comparison did not show T cell responses directed against HspX (Figure 1 and (33), we decided to evaluate this in HLA-tg mice. Splenocytes were harvested ten days post BCG-vaccination, and analyzed for their ability to lyse the human target cell JY, which expresses HLA-A*0201. Target cells were pulsed with one of seven known HLA-A*0201-epitopes (10 μg/ml per peptide): hsp65 p369-377 (11), Ag85B p143-152 and

Ag85B p199-207(20), HspX p91-105 (the present study), HspX p21-29 and HspX p120-128 (10) or the HLA-A2-binding influenza A matrix peptide: Flu p58-66 (34).
The hsp65 and Ag85 epitopes were strongly recognized by CTL in a dose-dependent fashion (Figure 5A). However, none of the target cells pulsed with HspX peptides were lysed. No lysis was observed for control cells: splenocytes pulsed with HLA-A*0201-binding influenza virus p58-66 (Figure 5A) or splenocytes derived from unimmunized A2/K^b mice (data not shown). Similarly, we investigated the T cell responses in HLA-DR3.Ab^0 tg mice after immunization with BCG. Splenocytes of BCG-immunized mice were restimulated in vitro with the HspX protein, its HLA-DR3-restricted epitope p31-50, *M. tuberculosis* hsp65, its HLA-DR3-restricted epitope p3-13, Ag85B or its HLA-DR3-restricted epitope p51-70 (Figure 5B). Again, no IFN-γ responses were detected in response to HspX or its HLA-DR3-restricted epitope. In contrast, both other proteins and their HLA-DR3-restricted epitopes induced significant levels of IFN-γ. No T cell responses could be detected against any of the antigen in mock-immunized HLA-DR3.Ab^0 mice.

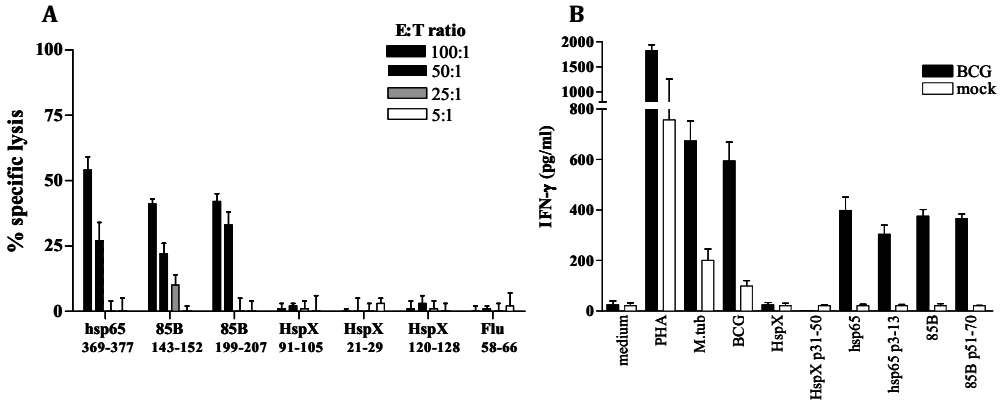

Figure 5. (A) *Cytotoxic activity of splenocytes derived from HLA-A2/K^b mice, immunized with BCG. Ten days after immunization, splenocytes were harvested and analyzed for their ability to lyse human HLA-A*0201-positive target cells (JY). HLA-A*0201-restricted peptides used to pulse target cells are indicated on the x axis. The HLA-A*0201-binding peptide of influenza A matrix (F58-66) was used as a control. E:T, effector to target ratio. The sequences of peptides used in the CTL assay were as follows: hsp65p369-377: KLAGGVAVI, Ag85B p143-152: FIYAGSLSAL, Ag85B p199-207: KLVANNTRL, HspX p91-105: SEFAYGSFVRTVSLP, HspX p21-29: LFAAFPSFA, HspX p120-128: GILTVSVAV and influenza A matrix p58-66: GILGFVFTL. (**B).** IFN-γ production of splenocytes of HLA-DR3.Ab^0 mice immunized with BCG in PBS (BCG) or with PBS only (mock). The antigens used for in vitro challenge are given on the x axis.*

Discussion

Since BCG vaccination does not protect against reactivation of TB, mycobacterial antigens that induce differential T cell responses in individuals latently infected with *M. tuberculosis* compared to unexposed BCG-vaccinated individuals may help to identify protective antigens and immune responses against TB. Such antigens may be applied in development of post-exposure vaccines as well as for new specific diagnostic tools.
By means of whole genome DNA microarray expression profiling and proteomic analysis 48 so-called latency antigens have been identified (35). In a previous study, our group analyzed the immunogenicity of 25 of these latency antigens and observed strong IFN-γ responses particularly in latently infected individuals (25).
In the present study we have focussed on the latency antigen HspX, as it is required for bacterial growth within the macrophage and is predominantly present during stationary growth of *M. tuberculosis* (37). Moreover, strong T cell responses to HspX in African populations were observed which were mostly restricted to latently infected individuals (14). In addition, T cells from TB patients recognizing the HspX protein showed a switch from Th0 towards Th1 after chemotherapy, indicating their potential to induce protective T cell responses (9, 15). These characteristics of HspX make it an interesting target for post-exposure TB vaccination as well as for the possible diagnosis of preclinical infection.
We here describe HspX-specific T cell responses in *M. tuberculosis* (likely)-exposed individuals (TB patients, TST^+ asymptomatic individuals, BCG vaccinees with positive ESAT-6 and/ or CFP-10 T cell responses) in comparison to *M. tuberculosis* unexposed individuals (BCG vaccinees and healthy controls lacking a T cell response to ESAT-6 and/ or CFP-10). Our results show that most (24 of 34, [71%]), *M. tuberculosis* infected or –exposed individuals responded well in the ELISPOT assay to HspX whereas sporadic and significantly lower responses were observed in *M. tuberculosis* unexposed individuals including BCG vaccinated individuals without any known exposure to *M. tuberculosis.* This suggests that BCG vaccination alone does not induce T cell responses against the HspX antigen. Similar findings come from a study in The Gambia where neonatal BCG immunization did not lead to IFN-γ responses to HspX or CFP-10, whereas these proteins were well-recognized in *M. tuberculosis*-exposed household contacts and health care workers (33).
Several studies have indicated that BCG can express the HspX homologue during oxygen depletion (6, 12, 26); however, our data suggest that vaccination with BCG in humans does not induce immune responses against HspX. We hypothesize that the expression of HspX by BCG in vivo after vaccination is limited and insufficient to induce an immune response.
To confirm this assumption in vivo, we applied BCG immunization to an HLA-class I and HLA-class II tg mouse models -HLA-A2/K^b and HLA-DR3.Ab^0, respectively- which provide powerful models to help characterizing in vivo T cell responses against mycobacterial antigens in the context of HLA polymorphism (18, 20). Furthermore, the HLA-A*0201 and HLA-DRB1*0301 alleles are major HLA alleles as frequencies are 42% and 24%, respectively in Caucasian, African/ Afro-Caribbean, and Oriental populations (8).

Splenocytes derived from BCG-immunized HLA-A*0201.K^b mice failed to lyse target cells pulsed with HLA-A*0201-restricted HspX peptides, whereas target cells pulsed with HLA-A*0201-restricted epitopes derived from hsp65 or Ag85B of *M. tuberculosis* were lysed up to 54% (Figure 5A). Similarly splenocytes from BCG-immunized DR3.Ab^0 mice were challenged in vitro with several mycobacterial antigen and their HLA-DR3-restricted peptides. Whereas immunization of the HLA-DR3.Ab^0 mice with the HspX protein induced responses against HspX p31-50 and the HspX protein (Figure 4D), such responses were not detected after BCG immunization of HLA-DR3.Ab^0 mice (Figure 5B). In contrast, both the cytosolic hsp65 and the secreted Ag85B of *M. tuberculosis* and their respective HLA-DR3-restricted epitopes, p3-13 and p51-70, induced significant levels of IFN-γ in these mice, a finding consistent with our previous findings (18). These data indicate that BCG immunization does not provoke T cell responses against the HspX latency antigen, while it is possible to induce $CD4^+$- and $CD8^+$ T cell responses to HspX using protein- or DNA vaccination. Thus, these data suggest that expression of HspX by BCG after in vivo vaccination is probably low compared to the expression of its homologue in *M. tuberculosis* under latent conditions and thus will not lead to a significant immune response directed against HspX.

This study shows that BCG vaccination alone does not induce T cell responses against the HspX antigen but that HspX is an immunogenic antigen that harbors several T cell epitopes. Thus, we anticipate that improved (BCG) vaccines, expressing relevant fragments of *M. tuberculosis* latency antigens (7, 25), may have potential as vaccines against latent TB.

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