Characterization of Novel Multiantigenic Vaccine Candidates with Pan-HLA Coverage against Mycobacterium tuberculosis

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The low protection by the bacillus Calmette-Guérin (BCG) vaccine and existence of drug-resistant strains require better anti-Mycobacterium tuberculosis vaccines with a broad, long-lasting, antigen-specific response. Using bioinformatics tools, we identified five 19- to 40-mer signal peptide (SP) domain vaccine candidates (VCs) derived from M. tuberculosis antigens. All VCs were predicted to have promiscuous binding to major histocompatibility complex (MHC) class I and II alleles in large geographic territories worldwide. Peripheral mononuclear cells (PBMC) from healthy naïve donors and tuberculosis patients exhibited strong proliferation that correlated positively with Th1 cytokine secretion only in healthy naïve donors. Proliferation to SP VCs was superior to that to antigen-matched control peptides with similar length and various MHC class I and II binding properties. T-cell lines induced to SP VCs from healthy naïve donors had increased CD4^high/CD62L^+ activation/effector memory markers and gamma interferon (IFN-γ), but not interleukin-4 (IL-4), production in both CD4^+ and CD8^+ T-cell subpopulations. These SP VCs were selected for further characterization. In mice, each mixture manifested strong cytotoxicity against M. tuberculosis-infected macrophages, while Mix3 also manifested a VC-specific humoral immune response. Based on these results, we plan to evaluate the protection properties of these combinations as an improved tuberculosis subunit vaccine.

Mycobacterium tuberculosis, the pathogen that causes tuberculosis (TB), is resurfacing worldwide. Over 9.27 million cases of active TB were reported in 2009, up from 6.6 million in 1990, with 2 million deaths annually (1, 2). One-third of the global population is estimated to have a latent M. tuberculosis infection, of which approximately 10% will develop active disease.

The current vaccine is the live, attenuated bacterium Mycobacterium bovis bacillus Calmette-Guérin (BCG). BCG induces reasonable immunopotentiating properties (3) and prevents miliary and meningeal tuberculosis in young children, but it offers limited protection against pulmonary tuberculosis (4), development of active disease in those with latent tuberculosis or healthy carriers, and recurrent infection (5–8). Thus, BCG is not satisfactory (2, 8, 9), and more-effective vaccines are needed to block the initial infection and/or limit illness severity (10). One such strategy uses subunit vaccines (11) composed of selected proteins or peptides (epitopes), ideally with a large antigenic repertoire, presented to T lymphocytes via major histocompatibility complex (MHC) molecules (12, 13). We recently reported a strategy for selecting vaccine candidates (VCs) based on their protein domain identity (14). In this approach, signal peptide (SP) domains are selected as VCs because of their unique ability to bind multiple MHC class I and II epitopes in transporter associated with antigen presentation (TAP)-dependent or -independent manners. SPs are comprised of 15- to 40-mer-long peptides found mainly in the N termini of prokaryote and eukaryote proteins and facilitate protein trafficking between cellular compartments. Unpredictably, SPs exhibit high antigenic (sequence) variability with no particular sequence identity, while maintaining their function as chaperones (15–17).

Consequently, SP domains can be used as VCs, which allows the induction of antigen-specific responses by CD4^+ and CD8^+ T cells in a large portion of the population (14, 18). To that end, we selected 9 anti-tuberculosis SP VCs with promiscuous binding to MHC class I and II alleles from major geographic regions worldwide and with broad CD4^+ and CD8^+ T-cell response properties for potential use as antigens in a subunit vaccine. We further presented the improved MHC binding densities and immunogenicity by means of proliferation and T-cell line properties of these M. tuberculosis antigen-derived SP domains using a pool of healthy naïve donors (14).

In this study, we aimed to further characterize the immunogenicity and immunodominance properties of the best five anti-tuberculosis SP VCs both ex vivo and in vivo on a pool of naïve donors and tuberculosis patients. Our results support previous findings concerning the high immunodominance of SP-derived
VGs and suggest that these SP VCs should be further evaluated as a subunit vaccine against *M. tuberculosis*.

**MATERIALS AND METHODS**

**MHC binding predictions.** Binding predictions and scoring for HLA class I alleles (HLA-A, -B, and -C), which most frequently appear in Cauca- sian, sub-Saharan African, and Southwest Asian populations, and for DR class II were performed as previously described (14, 18, 19). Briefly, the binding strength of 9-mer to the class I alleles was defined as follows: strong, peptide score of 0.01 nM to 109 nM; medium, 10 to 100 nM; and weak, 100 nM. We categorized the binding strengths for class II as follows: strong, peptide score of >100; medium, 10 to 100; and weak, 5 to 10. Binding strength for DRB1 HLA class II binding was defined in Propred as follows: strong, top 1% of binders; medium, 1 to 2% of binders; and weak, 2% to 3% of binders. That in Immune Epitope, for HLA-DRB1-0901, was defined as follows: strong, = 50% inhibitory concentration (IC50) of 0.01 nM to 10 nM; medium, 10 to 100 nM; and weak, 100 nM.

**Peptide synthesis.** VXL is a commercial code name for all Vaxil Ltd. vaccine candidates, including control peptides. VXL SP-derived peptides were chemically synthesized by EMC Microcollections (Tubingen, Germany), and other control peptides were synthesized at GL Biochem (Shanghai, China) by fully automated solid-phase peptide synthesis using 9-fluorenlymethoxy carbonyl (Fmoc) chemistry. The purity (>90%) and identity of the peptides were determined by high-pressure liquid chromatography (HPLC)-mass spectrometry analysis.

**HLA typing.** High-resolution HLA typing was performed for selected tuberculosis patients by Maccabi Healthcare Services, Israel, as previously described (23). Briefly, DNA was extracted from peripheral blood samples using an Abbott M1000 DNA extraction workstation (Abbott Park, IL). Low-resolution tissue typing was performed using the Tepnel/Lifecodes (Stamford, CT) multiplex platform, and high-resolution typing was performed using the sequence-specific primer (SSP) method.

**Study population.** (i) Healthy naïve donors. Healthy naïve donors are uninfected individuals with no past exposure to TB and measurable immunity to BCG, as determined by a negative proliferation stimulation index (SI) (SI ≤ 2) response to tuberculosis purified protein derivative (PPD). Peripheral blood mononuclear cells (PBMC) from 6 such donors were isolated from buffy coat samples donated by the Israeli national blood bank.

(ii) TB patients. A total of 21 patients, representing diverse ethnic groups, with a history of TB were enrolled. Of the 21 patients, 15 had active disease (confirmed by positive *M. tuberculosis* culture, PCR, and chest X-ray) and 6 had latent TB infection (positive PPD by skin test but negative *M. tuberculosis* culture and normal chest X-rays). All patients were HIV negative. The study was conducted at Maccabi Healthcare Service’s Tuberculosis Center and was approved by Assuta Medical Center Institutional Ethics Committee: approval no. 2009043.

**Proliferation.** Fresh PBMC from both healthy naïve donors and TB patients were suspended at 2 × 10^6 cells/ml in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) supplemented with 5% HuAB serum, 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 µg/ml gentamicin, and 1 mM HEPES (Biological Industries, Beit Haemek, Israel). Next, 100 µl of the PBMC suspension together with 100 µl of the same medium and containing an evaluated VXL peptide at a final concentration of 10 µg/ml was cultured in triplicate in 96-well flat bottom plates for 5 to 6 days. To evaluate the PBMC maximal proliferation capacity, phytohemagglutinin (PHA) was added to the PBMC at a final concentration of 2 µg/ml. As a negative control, we used PBMC suspended with 10 µg/ml of Chrome Pure Human IgG, Fab (HuFab) (Jackson ImmunoResearch, Suffolk, United Kingdom). For proliferation assessment, 0.5 µCi of 32^P]thymidine (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) was added and incubated for an additional 18 h. Plates were harvested on UniFilter 96-well plates (PerkinElmer, Waltham, MA), and radioactivity was counted using a Matrix 96 direct beta-counter (Matrix 96 direct beta-counter; Packard, Downers Grove, IL). All cell incubations were at 37°C with 5% CO₂. The SI was calculated by dividing the number of cpm obtained in the tested VCs by the number of cpm obtained in growth medium. An SI of ≥2 was considered positive.

**Preparation of peptide-pulsed DCs.** Dendritic cells (DCs) were enriched from blood samples obtained from naive donors or *M. tuberculosis* patients as previously described (14). Briefly, PBMC were separated using ficoll tubes (UNI-SEPmaxi; Novamed, Israel) at 2,400 rpm for 30 min and cultured at a concentration of 2.5 × 10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 ml glutamine, 1 ml sodium pyruvate, 1% nonessential amino acids, 50 µg/ml gentamicin, and 1 ml HEPES (Biological Industries, Beit Haemek, Israel), termed here complete medium, for 4 h at 37°C in 150-mm by 25-mm tissue culture dishes (CellStar, Greiner, Germany). Adherent cells were collected and recultured in serum-free DCCM-1 medium (Biological Industries, Beit Haemek, Israel) supplemented with t-glutamine, human interleukin-4 (IL-4) (1,000 IU/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (80 ng/ml). Cultures at a concentration of 1 × 10^6 cells/ml were performed in 6-well tissue culture plates (Costar, Corning, Germany) for 7 days at 37°C. On day 7, floating cells were collected, washed with phosphate-buffered saline (PBS), and loaded with 50 µg/ml of the specified peptide for 18 h at 37°C. DC-loaded cells were then utilized for the different immunological assays.

**Development of VXL peptide-induced T-cell lines.** Thawed PBMC obtained from healthy naïve donors or tuberculosis patients underwent initial stimulation for 7 days with VXL SP-pulsed autologous DCs at a ratio of 20:1 in RPMI 1640 complete medium supplemented with 50 IU/ml human recombinant IL-7 (PeproTech Asia, Rehovot, Israel). PBMC underwent a second stimulation for 5 days in the same medium with adherent autologous VXL SP-pulsed macrophages. Cells were then restimulated for 48 h with fresh medium containing 1 µg/ml of VXL SP and 50 IU/ml of human recombinant IL-2 (PeproTech Asia, Rehovot, Israel).

**T-cell cytotoxicity assay against VXL peptide-loaded target cells.** A total of 1 × 10^6 autologous macrophages (Mb) (target cells) were loaded for 18 h with 50 µg/ml of various VXL peptides. Next, macrophages were radiolabeled with 14C for 14 h with 5 µCi of [14C]methionine (Amersham Biosciences, Buckinghamshire, United Kingdom) in RPMI 1640 medium without methionine (Sigma, St. Louis, MO). Targets were diluted in RPMI 1640 complete medium to 5 × 10^5 cells/ml and placed (100 µl/well) in 96-well U-plates (Cellstar, Greiner, Bio-One GmbH, Frickenhausen, Germany). Autologous VXL SP-induced T cells were diluted in RPMI 1640 complete medium, added to the target cells (100 µl/well) at 2.5 × 10^6 and 1.25 × 10^6 cell/ml (effector-to-target cell [E:T] ratios of 50:1 and 25:1, respectively), and incubated for 5 h. For the radioactive count, after centrifugation from each well, 50 µl of supernatant medium was mixed with 150 µl Microscint 40 scintillation fluid (PerkinElmer, Waltham, MA) in OptiPlate plates (Nunc, Roskilde, Denmark) and measured in a Matrix 96 direct beta-counter (Packard Instruments, Meriden, CT). Spontaneous release was determined by incubating 100 µl labeled target cells with 100 µl RPMI 1640 complete medium. Maximal release was determined by lysis of target cells in 100 µl 10% Triton X-100.

**M. tuberculosis strain.** All experiments used a single *M. tuberculosis* strain isolated from one patient with TB. The strain was verified morphologically by Gram and acid-fast Ziehl-Neelsen staining and by PCR analysis (GenoType Mycobacterium ver. 1.0; Hian Lifescience GmbH, Hehren, Germany) and maintained in Lowenstein culture medium (Hy-Laboratories, Rehovot, Israel) at 37°C.

**T-cell cytotoxicity assay against *M. tuberculosis*-infected target cells.** The macrophage target cells were infected for 18 h at a 1:10 ratio with the *M. tuberculosis* strain described above. Infected cells were collected, washed, and incubated for 48 h in RPMI 1640 complete medium.
supplemented with 100 μg/ml G418 (Sigma, Rehovot, Israel) to eliminate extracellular bacterial growth. The lowest effective G418 concentration was predetermined by a series of experiments. Next, 1 ml of infected macrophages (target cells) at 5 × 10⁴ cell/ml was dispensed into 12-well plates and cocultured for 5 h with 1 ml (5 × 10⁴/ml and 2.5 × 10⁴/ml; E:T ratios of 100:1 and 50:1, respectively) of autologous VXL peptide-induced T cells. Nonlysed target cells were separated by centrifugation (10 min at 1,200 rpm). Free bacteria were collected by additional centrifugation (6,000 rpm for 10 min), washed, resuspended in 2 ml RPMI 1640 medium, and diluted 1:1,000 in PBS. One milliliter of the final dilution was placed in Middlebrook 7H9 growing agar plates (Hy-Laboratories, Rehovot, Israel). CFU readout was performed 3 weeks later. Spontaneous release was determined by incubating 1 ml M. tuberculosis-infected macrophages with 1 ml (5 × 10⁴) naïve autologous PBMC. Maximal release was determined by lysis of 1 ml M. tuberculosis-infected macrophages in 1 ml 10% Triton X-100.

For cytotoxicity experiments in mice, we used splenocytes from immunized mice as effector cells. Ten days after the last vaccination, splenocytes were harvested and restimulated in vitro with the immunized mixture at a final concentration of 10 μg/ml for 5 days in RPMI 1640 complete medium supplemented with 10⁻³ M 2-mercaptoethanol. On the day of the experiment, effector cells were collected, separated on a Ficoll gradient, washed twice with PBS, and placed at an E:T ratio of 100:1 with primary infected macrophages (target cells).

**Phenotype analysis and intracellular staining of VXL peptide-induced T-cell lines.** VXL peptide-induced T-cell lines were suspended at a final concentration of 20 × 10⁶ cells/ml in a PBS blocking buffer containing 3% FCS and 0.1% sodium azide. A total of 1 × 10⁵ cells were transferred into a 5-ml fluorescence-activated cell sorter (FACS) tube and incubated for 30 min at room temperature (RT) in the dark with the following conjugated antibodies: anti-CD3-phycocyanin (PE), anti-CD4, anti-CD8-peridinin chlorophyll protein (PerCP)-Cy5.5, anti-CD44–allophycocyanin (APC), and anti-CD62L-fluorescein isothiocyanate (FITC) (eBiosciences, San Diego, CA). Next, cells were washed with 2 ml blocking buffer and resuspended in 0.5 ml PBS. Samples were analyzed by FACS (LSR II; BD Biosciences San Jose, CA).

For intracellular cytokine staining (ICS) analysis, T cells were restimulated for 6 h at 37°C by autologous macrophages loaded with the evaluated VXL peptides. For controls, this study used T cells restimulated by macrophages loaded with antigen-matched non-SP epitopes or with unloaded macrophages. Two hours after stimulation initiation, 1 ml of each VXL M. tuberculosis VCs separately on different plates at 5 μg/ml in carbonate buffer for incubation overnight at 4°C. Plates were then blocked with 200 μl of PBS with 0.5% gelatin and incubated for 2 h at 25°C. Next, 50 μl of each of the five VXL M. tuberculosis VCs was added to the appropriate plate and incubated for 2 h at 37°C. The plates were then developed with TMB/E solution (Chemicon, Millipore, Billerica, MA) was added at a final dilution of 1:10,000 in the blocking buffer and incubated for 1 h at 25°C. The plates were then developed with TMB/E solution (Chemicon, Millipore, Billerica, MA). At the final step, the reaction was terminated by adding 50 μl/well of 10% sulfuric acid. Results were measured at 450 nm with an Asys Expert Plus ELISA reader (Asys Hitech GmbH, Austria). In these experiments, a titer was calculated as the maximal serum dilution with an OD of ≥0.1 (which is 4-fold higher than the OD of the blocking buffer).

**Vaccination.** Six- to 8-week-old BALB/c mice were bred at the Tel Aviv University breeding facility. All experiments were conducted according to Tel Aviv University institutional rules and regulations. Six BALB/c mice were subcutaneously immunized 3 times at 7-day intervals with 100 μg/mouse/dose of the 3 peptide mixtures (Mix1 to -3) or with PBS for control group mice. For peptide mixtures, a VXL VC stock of 10 mg/ml was prepared in dimethyl sulfoxide (DMSO), and the final concentration of 100 μg/mouse was reached using PBS. The total amounts of individual VCs in Mix1, -2, and -3 were 20 μg, 25 μg, and 50 μg, respectively.

**Evaluating the humoral and cellular responses in BALB/c mice.** The following ELISA protocol was used to evaluate the humoral response in the immunized BALB/c mice. ELISA plates (F96 MaxiSorp; Nunc, Roskilde, Denmark) were activated with 0.1% glutaraldehyde in carbonate buffer (pH 9) for 1 h at room temperature, followed by coating with 50 μl of each of the five VXL M. tuberculosis VCs separately on different plates at 5 μg/ml in carbonate buffer for incubation overnight at 4°C. Plates were then blocked with 200 μl of PBS with 0.5% gelatin for 2 h at 25°C. Next, 50 μl/well of the secondary anti-mouse IgG antibody-HRP conjugate (Chemicon, Millipore, Billerica, MA) was added at a final dilution of 1:10,000 in the blocking buffer and incubated for 1 h at 25°C. The plates were then developed with TMB/E solution (Chemicon, Millipore, Billerica, MA). At the final step, the reaction was terminated by adding 50 μl/well of 10% sulfuric acid. Results were measured at 450 nm with an Asys Expert Plus ELISA reader (Asys Hitech GmbH, Austria). In these experiments, a titer was calculated as the maximal serum dilution with an optical density (OD) of ≥0.1 (which is 4-fold higher than the OD of the blocking buffer).

Target cells from BALB/c mice were enriched by injecting 1.5 ml of thioglycollate into the peritoneal cavities of the mice. After 48 h, macrophages were collected by repeated peritoneal lavage with PBS. For the assay, isolated targets were washed twice with PBS and cultured in RPMI 1640 complete medium for M. tuberculosis infection (see above). The rest of the experiment was performed similarly to the way described for the human T-cell lines.

**Statistics.** Results were analyzed using a 2-tailed Student t test or Fisher’s exact test. The minimum significance level was set at a P value of <0.05 for the two-tailed t test and at a P value of <0.01 for Fisher’s exact test.

**RESULTS**

**Selection of VXL vaccine candidates: SPs with pan-HLA binding.** We recently presented the improved MHC binding densities and proliferation properties of 9 M. tuberculosis antigen-derived SP domains using a pool of healthy naïve donors (14). These peptides were screened to bind efficiently to a defined list of abundant MHC class I and MHC class II alleles, covering major territories worldwide, as described in Materials and Methods. The five most immunogenic SP domains, VXL201, VXL203, VXL208, VXL211, and VXL212 (Table 1), underwent in-depth immunogenicity
<table>
<thead>
<tr>
<th>Identity</th>
<th>Target protein</th>
<th>Sequence</th>
<th>Position</th>
<th>Length (amino acids)</th>
<th>Binding prediction</th>
<th>MHC class I (A, B, C)</th>
<th>MHC class II (DRB1)</th>
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<tbody>
<tr>
<td>VXL201 Antigen 85B</td>
<td>MTDVSRKIRAWGRRLMIGTAAAVVLPGLVG</td>
<td>1–40</td>
<td>40</td>
<td>A2.1, A24, A68.1, B7, B35.1, B40, B51.1, Cw0702, C0401, C0602, 0301, 0401, 0701, 1101, 1302, 1501</td>
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<tr>
<td>VXL201a Antigen 85B</td>
<td>QQFIYAGSLSALLDPSQGM</td>
<td>181–199</td>
<td>19</td>
<td>A2.1, A24, B7, B51.1, Cw0702, C0401, C0602, 0301, 0401, 0701, 1101, 1302, 1501</td>
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<tr>
<td>VXL201b Antigen 85B</td>
<td>YLQVPSPSMGRDIKVQFQ</td>
<td>50–67</td>
<td>18</td>
<td>A2.1, A68.1, B35.1, B44.03, B51.1, C0702, 0301, 0701</td>
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<tr>
<td>VXL201c Antigen 85B</td>
<td>FSRPGLPVEYLQVPSPSM</td>
<td>41–58</td>
<td>18</td>
<td>A2.1, A24, B7, B35.1, B51.1, Cw0401, C0702, 0701</td>
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<tr>
<td>VXL203 Lipoprotein LpqH</td>
<td>MKRGLTVAVAGAAILVAGLSGCSS</td>
<td>1–24</td>
<td>24</td>
<td>A2.1, A24, A68.1, B35.1, B44.03, B51.1, Cw0702, C0401, C0602, 0301, 0401, 0701, 1101, 1302, 1501</td>
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<tr>
<td>VXL203a Lipoprotein LpqH</td>
<td>AATGIAAVLTDGNPPEVKSVGLGN</td>
<td>81–104</td>
<td>24</td>
<td>A24, B7, B51.1, Cw0401, C0602, 0301</td>
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<td>VXL208 ATP-dependent helicase (putative)</td>
<td>MRFAQPSALSRFSALTRDWFTSTFAAPTAAQA</td>
<td>1–32</td>
<td>32</td>
<td>A1, A2.1, A24, A68.1, B7, B8, B51.1, B35.01, B40, Cw0401, 0301, 0701, 1501</td>
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<tr>
<td>VXL208a ATP-dependent helicase (putative)</td>
<td>EVIRILRRRSLAALRAQAEPVSTAAYGRFLPA</td>
<td>1172–1203</td>
<td>32</td>
<td>A68.1, B7, B8, B35.1, B44.03, B51.1, B58.1, Cw0702, 0301, 0701, 1101, 1302, 1501</td>
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<td>VXL211 Uncharacterized protein</td>
<td>MLVLLVAVLVTAVYAFVHA</td>
<td>1–19</td>
<td>19</td>
<td>A2.1, A24, A3, A68.1, B35.1, B44.03, B51.1, Cw0702, C0602, 0301, 0401, 0701, 1101, 1302, 1501</td>
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<tr>
<td>VXL211a Uncharacterized protein</td>
<td>MSACASGVYLVDVRPKLLE</td>
<td>63–81</td>
<td>19</td>
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<tr>
<td>VXL211b Uncharacterized protein</td>
<td>AMSACASGVYLVDVRPKLL</td>
<td>62–80</td>
<td>19</td>
<td>A24, A68.1, B8, 0301, 0701, 1101, 1302</td>
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<td>VXL212 Uncharacterized protein</td>
<td>MLLRKGTVYVLVIRADLVNAMVAHA</td>
<td>1–25</td>
<td>25</td>
<td>A2.1, A24, A3, A68.1, B7, B40, B51.1, Cw0702, C0602, 0301, 0401, 0701, 1101, 1302, 1501</td>
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<td>VXL212a Uncharacterized protein</td>
<td>TEAYPSRTDVKLATEPDAHYVLVST</td>
<td>93–117</td>
<td>25</td>
<td>A2.1, A24, A3, A68.1, B7, B8, B51.1, Cw0401, 0301, 0701, 1501</td>
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<tr>
<td>VXL212b Uncharacterized protein</td>
<td>PDEACGVLAGPEGSDRPERHIPMTN</td>
<td>30–54</td>
<td>25</td>
<td>A68.1, B58.1, Cw0401, No binding</td>
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*The identity of all VXL peptides with suffix a, b, or c is from antigen-matched non-SP domains. a, strong MHC binding; b, moderate MHC binding; c, low MHC binding.*
evaluation. We initially explored the proliferation properties of the 5 VCs on PBMC isolated from 13 patients with active or latent tuberculosis (Fig. 1). These patients represent a large ethnic diversity and consequently a large HLA repertoire (Table 2). PBMC proliferation was also evaluated on tetanus toxoid (TT) and PPD to assess immune functionality in the case of TT and positive reaction in tuberculosis patients. HuFab was used in these experiments as a negative control with complete default stimulation. The proliferation response (SI) was calculated by dividing the number of cpm obtained with VC (peptide) stimulation by the number of cpm obtained with growth medium (control) and was considered positive when the result was ≥2. As a negative control, we used pure human IgG Fab fragment, which was previously reported to have no proliferation properties (25).

Positive proliferation (SI ≥ 2) in a large fraction of the patients. PPD proliferation was used based on a report that indicated a strong correlation in healthy naïve donors between proliferation to PPD and reaction to PPD in tuberculin (Mantoux) testing (24). As a negative control, we used pure human IgG Fab fragment, which was previously reported to have no proliferation properties (25).

FIG 1 VXL SP VC proliferation and cytokine secretion response in patients with M. tuberculosis. (A) For proliferation, PBMC derived from 13 M. tuberculosis patients were stimulated for 5 to 6 days with 10 μg/ml of the five VXL SP VCs. PBMC were also evaluated on tetanus toxoid (TT) and PPD to assess immune functionality in the case of TT and positive reaction in tuberculosis patients. HuFab was used in these experiments as a negative control with complete default stimulation. The proliferation response (SI) was calculated by dividing the number of cpm obtained with VC (peptide) stimulation by the number of cpm obtained with growth medium (control) and was considered positive when the result was ≥2. (B) For cytokine secretion, PBMC derived from 6 of these M. tuberculosis patients were stimulated for 48 h by the same VCs. Secretion of the IFN-γ, IL-2, and IL-4 cytokines was evaluated by ELISA and correlated to the proliferation response (SI) manifested by the same patients. The proliferation of all of VCs except VXL208 was significantly higher (P ≤ 0.03 by t test and/or P ≤ 0.0012 by Fisher’s exact test) than that of human Fab. Results show averages from 8 different experiments. SI, stimulation index.
was manifested mainly by VXL211 and to some extent by VXL201, VXL203, and VXL212 (Fig. 1A). The proliferation of all VCs other than VXL208 was significantly higher (by t test [P < 0.03] and/or Fisher’s exact test [P < 0.0012]) than that of human Fab. However, we found no correlation between the positive (SI ≥ 2) proliferation obtained in these patients and the poor or no secretion of the Th1 and Th2 cytokines, namely, gamma interferon (IFN-γ), IL-2, and IL-4 (Fig. 1B) analyzed for patients 2, 3, 10, 14, 15 and 16. These results could be related to inhibition of immunological function of peripheral lymphocytes by M. tuberculosis, manifested by an altered cytokine secretion profile as previously shown (12, 26).

VXL SP VCs induced stronger proliferation in PBMC than antigen-matched non-SP epitopes. To explore the immunogenic properties of the five SP VCs further, their proliferation properties were compared to those of antigen-matched non-SP peptides (Table 1) using PBMCs from 6 healthy naïve donors and M. tuberculosis patients (no. 14, 16, and 18) with active tuberculosis and one (no. 20) with latent disease, who responded positively (SI > 2) to at least 2 of the 5 peptides. Proliferation in healthy naïve donors (Fig. 2A) was more robust with each of the 5 SP VCs tested than with the antigen-matched non-SP peptides (t test, P < 0.01), except for VXL201. In these donors, SP-derived VCs attained average SI scores of 4 to 7, while all antigen-matched VCs, excluding VXL201a, displayed a negative score (SI ≤ 2) (Fig. 1A). The ability of SP domains to induce a proliferation response in unprimed PBMC from healthy naïve donors is related not to in vitro immunization but rather to their unique sequence, as previously demonstrated (14, 18). Comparable results (t test, P < 0.05) were observed for 4 of 5 VCs, excluding VXL212, with PBMC obtained from patients with latent and also, importantly, active disease having heterogeneous ethnic backgrounds and HLA repertoires (Table 2; Fig. 1B).

VXL SP VCs induce mixed CD4+ and CD8+ T-cell populations to express effector memory markers and produce IFN-γ. The goal for any M. tuberculosis vaccine is to enrich CD4+ and CD8+ T-cell populations with effector and memory profiles and robust activity. Therefore, we analyzed the phenotype and function of the T-cell lines generated from healthy naïve donors to selected SP VCs. VXL-generated T-cell lines comprised mainly CD4+ cells (ranging from 40 to 50%) but also CD8+ T cells (ranging from 32 to 35%) (data not presented). Figure 3A shows a significant elevation in two subpopulations after stimulation with VXL203 and VXL211. The first is CD4+ and CD8+ CD44high activated effector T cells (up to 1.5-fold in CD8+ T cells), and the second is CD4+ and CD8+ CD62Lhigh effector memory cells (up to 5- and 6-fold, respectively, for CD4+ and CD8+ T cells). In these experiments, VXL203- and VXL211-stimulated T-cell populations also presented elevated CD45RO levels (data not shown). We further analyzed the levels of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 in the same VXL203- and VXL211-stimulated T-cell populations (Table 2; Fig. 1B).

**TABLE 2** Characterization of the tuberculosis patients included in the study

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Date of diagnosis (mo/yr)</th>
<th>Gender*</th>
<th>Ethnic group</th>
<th>Disease stage</th>
<th>PPD test (mm)</th>
<th>Culture test</th>
<th>Multidrug resistanceb</th>
<th>HLA typing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>08/09</td>
<td>M</td>
<td>Ethiopian</td>
<td>Latent</td>
<td>15</td>
<td>Negative</td>
<td>M. tuberculosis</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>08/09</td>
<td>M</td>
<td>Ethiopian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>02/09</td>
<td>F</td>
<td>Ethiopian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10/08</td>
<td>M</td>
<td>Russian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>02/09</td>
<td>M</td>
<td>Ethiopian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>01/09</td>
<td>M</td>
<td>Ethiopian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>07/09</td>
<td>M</td>
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<td>Latent</td>
<td>20</td>
<td>Negative</td>
<td>M. tuberculosis</td>
<td>N</td>
</tr>
<tr>
<td>8</td>
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<td>F</td>
<td>Ethiopian</td>
<td>Active</td>
<td>22</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>9</td>
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<td>M</td>
<td>Russian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>Y</td>
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</tr>
<tr>
<td>10</td>
<td>06/09</td>
<td>M</td>
<td>Native Israeli</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
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<tr>
<td>11</td>
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<td>M</td>
<td>Russian</td>
<td>Active</td>
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<tr>
<td>12</td>
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<tr>
<td>13</td>
<td>08/09</td>
<td>F</td>
<td>Arabic</td>
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<td>M. tuberculosis</td>
<td>N</td>
</tr>
<tr>
<td>14</td>
<td>09/09</td>
<td>M</td>
<td>Russian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>11/09</td>
<td>F</td>
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<td>Latent</td>
<td>21</td>
<td>Negative</td>
<td>M. tuberculosis</td>
<td>N</td>
</tr>
<tr>
<td>16</td>
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<td>F</td>
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<td>Active</td>
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<td></td>
</tr>
<tr>
<td>17</td>
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<td>M</td>
<td>Native Israeli</td>
<td>Latent</td>
<td>42</td>
<td>Negative</td>
<td>M. tuberculosis</td>
<td>N</td>
</tr>
<tr>
<td>18</td>
<td>10/09</td>
<td>M</td>
<td>Russian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>09/10</td>
<td>M</td>
<td>Russian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>03/10</td>
<td>F</td>
<td>Native Israeli</td>
<td>Latent</td>
<td>25</td>
<td>Negative</td>
<td>M. tuberculosis</td>
<td>N</td>
</tr>
<tr>
<td>21</td>
<td>03/10</td>
<td>M</td>
<td>Ethiopian</td>
<td>Active</td>
<td>Not done</td>
<td>M. tuberculosis</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

* M, male; F, female.

**a** N, no; Y, yes.

Additional information:
- Table 2 provides a detailed summary of the tuberculosis patients included in the study.
- Figure 1A depicts the proliferation profile of the five SP VCs compared to the antigen-matched non-SP peptides.
- Figure 2A shows the proliferation response of PBMCs from healthy naïve donors to SP VCs.
- Figure 3A illustrates the phenotypic and functional analysis of the T-cell lines generated.
- Table 2 lists the characteristics of the tuberculosis patients, including date of diagnosis, gender, ethnic group, disease stage, PPD test, culture test, multidrug resistance, HLA typing, and other relevant details.

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matched VXL203a and VXL211a peptide-loaded macrophages (Fig. 3B, middle panels) showed no (0%) stimulation by either CD4\(^+\) or CD8\(^+\) cells. In addition, stimulation of healthy naïve PBMC by VXL203 and VXL211 VCs (Fig. 3B, lower panels) also showed no (0%) stimulation. These results emphasize the robustness and specificity of the SP VCs. In addition, as there was no IL-4 production, we suggest that cytokine production in these donors was mainly Th1 rather than Th2 mediated.

VXL SP VC-induced T-cell lines exhibit specific lysis of VXL SP VC-loaded autologous target cells. Next, we analyzed the cytotoxic properties of the T-cell lines generated against the 5 SPs. Autologous macrophages loaded with the same VCs were used as targets in these assays. Figure 4 represents the average cytotoxic activity in three different experiments using 5 healthy naïve donors. A strong, specific lysis ranging from 90% to 100% at an E:T ratio of 50:1 was manifested by all 5 VC-induced T cells (\(t\) test, \(P < 0.01\)) compared to their antigen-matched non-SP peptide-loaded target, which manifested lower specific lysis in the range of 5% to 30%. These results support the recognition of peptide-MHC complexes on the target cells by VC-induced T cells. These results also

FIG 2 VXL SP VC proliferation properties compared with those of antigen-matched non-SP peptides. PBMC derived from 6 healthy naïve donors (A) and 4 patients with latent TB infection or active tuberculosis (B) were stimulated for 6 days with 10 \(\mu\)g/ml VXL201, VXL203, VXL208, VXL211, VXL212, and non-SP antigen-matched peptides (Table 1). Positive controls PHA and TT and negative control HuFab were also used (data not shown). The proliferation response (SI) was calculated by dividing the number of cpm obtained with VC (peptide) stimulation by the number of cpm obtained with growth medium (control) and was considered positive when the result was \(\geq 2\). In healthy naïve donors, the proliferation of all of VCs except VXL201 was significantly higher (\(t\) test, \(P < 0.01\)) than that of antigen-matched non-SP peptides. In tuberculosis patients, the proliferation of all VCs except VXL212 was significantly higher (\(t\) test, \(P < 0.05\)) than that of antigen-matched non-SP peptides. Results represent one of two similar experiments. SI, stimulation index.
confirm cross-presentation of the VCs on the loaded autologous macrophages. Interestingly, T-cell lines induced to VXL201, VXL203, and VXL211 VCs also caused 50% to 90% specific lysis of PPD-loaded macrophages. This may indicate the presence of these epitopes in the PPD’s antigenic repertoire.

VXL SP VC-induced T-cell lines specifically lyse *M. tuberculosis*-infected autologous target cells while secreting IFN-γ. We concluded this set of experiments by evaluating the ability of the T-cell lines induced against the 5 VCs from 5 healthy naïve donors and 4 patients with tuberculosis to lyse autologous macrophages.
infected with live \textit{M. tuberculosis} (Fig. 5). Patients were selected for this assay based on PBMC availability and properties of proliferation to the VCs, with the rationale of having a T-cell line against each of the 5 VCs. Results from healthy naïve donors (Fig. 5A) showed highly specific lysis by T cells, potentially a mixture of both CD4\textsuperscript{+} and CD8\textsuperscript{+} cells (as shown in Fig. 3), induced to VXL201 (82\%) or VXL203 (60\%) and moderate lysis by T cells induced to VXL208 (25\%) and VXL211 (32\%). In these experiments, VXL212 did not manifest any lysis of infected target cells. The profile of lysis by effector T cells induced to the same VCs from patients 17 to 20 (Table 2; Fig. 5B) was weaker but showed a trend similar to that observed in healthy naïve donors. In particular, anti-VXL201 and VXL203 T-cell lines were the most potent effectors, exhibiting 60 to 67\% specific lysis, while anti-VXL211, VXL212 (28\% to 22\%), and VXL208 (11\%) exhibited moderate to low lysis levels. Importantly, these results confirm the processing and presentation of epitopes from these VCs of host \textit{M. tuberculosis}-infected macrophages. HLA typing of these patients (Table 2) demonstrated a heterogeneous HLA repertoire for both class I and II and supported the promiscuous MHC binding/T-cell activation properties of these VCs, as predicted \textit{in silico}. Although SP-specific tetramer analysis was not part of our evaluation, this broad T-cell activation could potentially also cover rare alleles such as A2902, A3001, A3201, B1517, B4002, B5701, DR B0404, DR B0301, DR B1305, and DR B0405, which were initially not included in our \textit{in silico} prediction but are part of the HLA repertoire in the four evaluated patients and were later confirmed \textit{in silico} to have binding restrictions for each of five VXL peptides.

Significant levels of IFN-\(\gamma\) secretion were measured during the lysis process in healthy naïve donor samples (3,600 to 15,100 pg/ml) and also in tuberculosis patient samples (2,600 to 13,800 pg/ml), which was unexpected in light of the poor cytokine secretion results manifested by PBMCs from TB patients (Fig. 1). These were detected by the different T-cell lines induced to each of the 5 VCs and correlated with cytotoxic potency. This finding indicates a potential role for these SP VCs in improving immune function for \textit{M. tuberculosis} patients. Importantly, cytokine secretion correlated with the lytic properties of the same T-cell lines. Anti-VXL201, anti-VXL203, and anti-VXL211 T cells exhibited the strongest lytic properties and the highest IFN-\(\gamma\) secretion levels. These results corresponded with the high IFN-\(\gamma\) production levels shown in intracellular staining (Fig. 3B).

**FIG 4** Cytotoxicity analysis of VC-loaded autologous targets by T-cell lines generated from healthy naïve donors with VXL SP VCs. T-cell lines were generated as described in Materials and Methods and evaluated for their cytotoxicity properties with autologous macrophages loaded with the same VCs as targets. The lysis to each of the 5 VC-loaded autologous targets was statistically significant (\(t\) test, \(P < 0.01\)) compared to that to its antigen-matched non-SP peptide-loaded target. Results represent an average cytotoxic activity at an E:T ratio of 50:1 in three experiments using 5 different healthy donors.

**VXL SP VC combinations show synergic proliferation of PBMC and improved immunogenic properties \textit{in vivo}**. We then
evaluated the immunogenic properties of selected combinations of the five VCs. Five mixtures, termed Mix1 to -5, were prepared as follows: Mix1 contained all 5 VCs, Mix2 contained all the VCs excluding VXL208, Mix3 contained VXL201 and VXL203, Mix4 contained VXL211 and VXL212, and Mix5 contained VXL201, VXL203, and VXL211. Initially, we evaluated the proliferation properties of the mixtures directly via PBMC obtained from 6 different donors (see Materials and Methods). As in previous experiments, we observed diverse yet positive proliferation (SI > 2) among the different donors (Fig. 6A). Nonetheless, proliferation to Mix1, -2, -3, and to some extent to Mix5 (Fig. 6B), showed significant ($P < 0.03$) additive and (in selected donors) synergistic properties, with average SIs of 9.71 and 17, respectively, for Mix 5 and Mix 2, versus the SIs of the individual VCs. Apparently, we found a significant ($P < 0.02$) antagonistic effect on the proliferation between VXL211 and VXL212 in Mix4 (SI < 2) versus the proliferation of the individual VCs.

Based on the results of this analysis, we next tested the cellular (Fig. 6C) and humoral (Fig. 6D) immunodominant properties of the three best combinations, Mix1, Mix2, and Mix3 in BALB/c syngeneic mice. Figure 6C shows that splenocytes from BALB/c mice immunized with Mix1, -2, and -3 effectively lysed *M. tuberculosis*-infected targets, which were thioglycolate-induced peritoneal macrophages (see Materials and Methods). This lysis was significantly greater ($P < 0.01$ by $t$ test) than the lysis of the control PBS-injected mice. These results reconfirm our data with the VC-induced T-cell lines (Fig. 5) regarding the expression of SP-derived epitopes on *M. tuberculosis*-infected targets. Moreover, these results stress the immunogenic properties of the three mixtures in inducing a strong and specific cellular response following a short regimen of active vaccination. In parallel, sera from the immunized mice were evaluated for the presence of anti-VXL antibodies. A titer of up to 1:3,200 was detected against VXL201 and VXL203, primarily in Mix3.

**FIG 5** Analysis of cytotoxicity and cytokine production to live *M. tuberculosis*-infected autologous targets by T-cell lines generated from healthy naive donors and tuberculosis patients with VXL SP VCs. T-cell lines were generated as described in Materials and Methods from naive healthy donors (A) and tuberculosis patients (B) and evaluated for their cytotoxicity properties and cytokine secretion properties on live *M. tuberculosis*-infected autologous macrophages. Results represent average cytotoxicity activity at an E:T ratio of 100:1 in three experiments using 5 different healthy naive donors and 4 *M. tuberculosis* patients.
DISCUSSION

This study was designed to identify VCs in key antigens of *M. tuberculosis* that could be used as multiepitopes in a subunit vaccine with a wide antigenic repertoire and MHC coverage against tuberculosis infection. A straightforward method for achieving this goal is to use defined protein domains with an inherent broad immunogenicity, such as SPs (14). We previously demonstrated that SP domains have several advantages as VCs (14, 18, 27): (i) antigen-specific promiscuous binding to MHC class I and II alleles, as SP binding to multiple MHC alleles induces stronger CD4+ and CD8+ T-cell responses in most of the population regardless of its MHC repertoire; (ii) TAP-dependent and independent presentation, as SP domains have the unique ability to bypass TAP deficiencies induced by intracellular pathogens such as *M. tuberculosis* (15, 28); and (iii) less complex vaccines, as unlike most peptide vaccines, SP domains have hydrophobic/lipophilic sequences with properties that enable prompt delivery across the cell membrane, as well as improved immunogenicity, which reduce the dependency on external adjuvants (14, 18, 29, 30). Previous reports focused on the immunogenicity of SP-derived epitopes rather than on that of the full domain. In addition, they did not specifically relate to the knowledge around SP domain biology, in particular, its antigen specificity and broad pan-MHC immunological properties. Jiang et al. showed that vaccination of mice with an 18-mer SP derived from Ag2/PRA induced protective immunity against coccidioidomycosis. The protective response was superior to that of the mature protein without the SP and was highly specific, as frameshift mutation in the Ag2/PRA SP sequence abolishes the specific activity (27). McMurry et al. identified an *M. tuberculosis*-originated, SP-derived sequence that induced IFN-γ secretion in a large percentage of PBMC taken from *M. tuberculosis*-immune subjects (31).

In the current study, we selected 5 SP VC domains from *M. tuberculosis* antigens. Each VC predicted promiscuous binding to at least 50% of both human class I A, B, and C alleles and class II DRB1 alleles worldwide and no identity with another human sequence, as verified by BLAST analysis (www.ncbi.nlm.nih.gov/blast/). For biovalidation, we used immunological responses of

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**FIG 6 Immunogenic properties of VXL VC mixtures on PBMC and in mice.** (A and B) PBMC derived from 6 healthy naïve donors were stimulated for 6 days with 10 μg/ml of individual VCs (A) or mixtures 1 to 5 (B). Mix1 contained all 5 VCs, Mix2 contained all the VCs excluding VXL208, Mix3 contained VXL201 and VXL203, Mix4 contained VXL211 and VXL212, and Mix5 contained VXL201, VXL203, and VXL211. Positive control PHA and negative control HuFab (data not shown) were also used. Mix1, -2, and -3 were then used in vaccination and immunogenicity studies. (C and D) Cellular (C) and humoral (D) responses were evaluated. The proliferation response (SI) was calculated by dividing the number of cpm obtained with VC (peptide) stimulation by the number of cpm obtained with growth medium (control) and was considered positive when the result was $>2$. The proliferation of Mix1, -2, -3, and -5 on PBMC was significantly higher ($t$ test, $P < 0.01$) than that of the individual VCs. Mix1 and -2 manifested strong cytotoxicity; in addition, Mix3 induced a humoral response. The results represent one of two similar experiments (on both PBMC and mice). SI, stimulation index.
heterogeneous groups of PBMC and induced T-cell lines from healthy naïve donors and TB patients. Since both preventive and therapeutic vaccines against *M. tuberculosis* are needed, we evaluated the immune responses in patients with active TB and latent TB infection from diverse ethnic groups (Table 2) and those in healthy naïve donors to better identify the best immunodominant VCs. Next, the immunogenicity of selected combinations of these VCs was further evaluated on heterogeneous groups of PBMC and in mice.

Results with these SP VCs showed positive proliferative effects (SI ≥ 2) when used directly on PBMCs from healthy donors and from tuberculosis patients (Table 2). This phenomenon is unique to SP domains (14, 18) and is associated less with most peptides, which are considered poor immunogens that require indirect presentation by DC.

Although the current study did not include a large group of subjects, the highly heterogeneous MHC profile within our patient population originating from diverse ethnic groups, including Ethiopians, persons from the former Soviet Union, and native Israelis (Table 2), compensates for the group size. Our observations support this claim, as despite the high HLA diversity, proliferation results with the 5 SP VCs on PBMC were mostly positive (SI > 2) (Fig. 1) and stronger than that with any other antigen-matched, non-SP multipptide sequence predicted in silico to have low to high MHC binding density (Table 1; Fig. 2). These results suggest superior immunogenicity of SP domains.

The strong proliferation of the SP domains positively correlated with high Th1 cytokine secretion only in healthy naïve donors. The poor cytokine secretion in TB patients (Fig. 1) agrees with previous reports (12, 26) and presumably indicates poor immune status and inhibited immunological function of peripheral lymphocytes. The challenge, therefore, is to induce a specific, potent response even in immune-suppressed patients with TB.

CD4\(^+\) and CD8\(^+\) T-cell lines produced from healthy donors and patients with TB against the 5 VCs were evaluated in detail. This is important because the role of CD8\(^+\) T-cell induction in *M. tuberculosis* is known (32) but is less well documented than that of CD4\(^+\) T cells (33–35). Results with T-cell lines from healthy naïve donors indicate that the VCs induced robust, antigen-specific immune activation of both T-cell subpopulations (Fig. 3A). These T cells express the CD4\(^+\)high activated effector and CD62L\(^+\) effector memory markers, suggesting that stimulation with the SP VCs led to differentiation of nonactivated PBMC to functional subsets of T cells with potential memory properties. The same T-cell lines demonstrated strong, antigen-specific IFN-γ (Th1), but not IL-4 (Th2) cytokine production (Fig. 3B). This is important because IFN-γ is associated with potent anti-*M. tuberculosis* immunity, while IL-4 is associated with more severe disease (36) and correlates with earlier disease onset in *M. tuberculosis*-exposed individuals. Increased IFN-γ/IL-4 ratios in *M. tuberculosis* patients also decrease the likelihood of relapse (12). In summary, these results emphasize the ability of the evaluated VCs to prime functional, activated CD4\(^+\) and CD8\(^+\) T-cell populations, which are necessary for generating effective anti-*M. tuberculosis* immunity.

In additional experiments, T-cell lines, containing both CD4\(^+\) and CD8\(^+\), induced from healthy naïve donors and TB patients to the 5 isolated VCs also exhibited robust pan-HLA lysis of peptide-loaded and bacterium-infected targets (Fig. 4 and 5), which was associated with strong IFN-γ secretion. These results confirmed the processing and cross-presentation of the SP VCs on target cells and on DCs for the induction of potent SP-specific T-cell lines. Moreover, they also support previous reports suggesting that the mechanism of virulent *M. tuberculosis* allows transport of antigens to generate MHC class I-restricted T cells (37). The abilities of T-cell lines isolated from healthy naïve donors and, more importantly, from TB patients to induce effective lysis and high Th1 cytokine secretion (Fig. 5) are important indicators of the quality of the evaluated VCs.

The purpose of a TB vaccine is to manifest efficacy in humans. However, since the SP VC peptides were initially designed to bind human MHC alleles, the use of syngeneic mice with a limited MHC repertoire represents the key advantages of these multiepitopes less well. This is a special challenge for SP domains, which are known to have significantly lower MHC binding in murine versus human proteins (14). Therefore, the use of mice in this study was mainly intended to prove the immunogenicity of the SP VC in vivo, while dose and regimen calibrations were a minor issue based on our past experience with other SP domains. Moreover, predicated on the above realities, it is important to stress that these results have limited applicability to the final dose and regimen in humans.

Despite these limitations, in vivo results from BALB/c mice for three mixtures containing 2 to 5 of the VXL SP domains and designed to mimic subunit vaccines were encouraging. The mixtures, in particular Mix3, were able to generate antigen-specific humoral and cellular responses even without a dedicated adjuvant. These results might reflect the total amount of peptides in the different mixtures used for vaccination. While Mix3 contained 50 μg of VXL201 and VXL203, Mix1 contained only 20 μg of each of these peptides. Nevertheless, the ability to induce both cellular and antibody responses with peptides is not obvious, especially because there was no carrier or adjuvant in the peptide vaccination mixtures. We previously observed a similar phenotype in mice with the MUC1 vaccine ImMucin (18), which is potentially related to the lipophilic sequences of SP (29, 30). Although the combined T-cell and B-cell response induced by Mix3 is novel, additional studies are needed to further explore the potential anti-*M. tuberculosis* role of the generated antibodies.

The importance of bioinformatics in evaluating proteomes of pathogenic organisms is underscored by the approach presented here. While previous publications reported shortcomings of predicted peptide binding to MHC alleles, especially for HLA class II, our current results demonstrated the opposite. We could specifically compare the class II binding prediction score of the evaluated SP domains with the function of T cells generated to these specific VCs and originating from TB patients who were HLA typed for class I and class II (DRB1). Using this methodology, we found a positive trend between strong MHC class I and class II binding to key alleles and function of both CD4 and CD8 T cells from patients with the same set of alleles (Fig. 3 to 5). A recent report by Widenmeyer et al. reached similar conclusions with a survivin peptide that has promiscuous MHC class II (DRB1) binding and potent (CD4\(^+\)) T-cell responses in the majority of vaccinated cancer patients (38).

Whereas a functional immunodominant role was previously assigned to three of the five proteins (39–42), the remaining two are hypothetical bacterial proteins with no known function. Although the exact identities, structures, and functions of the pro-
teins from which the VCs were derived are yet unknown, this does not impair their ability to serve as effective VCs.

We plan further experimentation to assess the immunogenicity and anti-M. tuberculosis properties of the VXL VC mixtures developed, both alone and in combination with BCG.

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All other authors declare no conflicts of interest.

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