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ML1419c Peptide Immunization Induces *Mycobacterium leprae*-Specific HLA-A*0201–Restricted CTL In Vivo with Potential To Kill Live Mycobacteria

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MHC class I-restricted CD8$^+$ T cells play an important role in protective immunity against mycobacteria. Previously, we showed that p113-121, derived from *Mycobacterium leprae* protein ML1419c, induced significant IFN-γ production by CD8$^+$ T cells in 90% of paucibacillary leprosy patients and in 80% of multibacillary patients' contacts, demonstrating induction of *M. leprae*-specific CD8$^+$ T cell immunity. In this work, we studied the in vivo role and functional profile of ML1419c p113-121–induced T cells in HLA-A*0201 transgenic mice. Immunization with 9mer or 30mer covering the p113-121 sequence combined with TLR9 agonist CpG induced HLA-A*0201–restricted, *M. leprae*-specific CD8$^+$ T cells as visualized by p113-121/HLA-A*0201 tetramers. Most CD8$^+$ T cells produced IFN-γ, but distinct IFN-γ$^+$/TNF-α$^+$ populations were detected simultaneously with significant secretion of CXCL10/IFN-γ–induced protein 10, CXCL9/MIG, and VEGF. Strikingly, peptide immunization also induced high ML1419c-specific IgG levels, strongly suggesting that peptide-specific CD8$^+$ T cells provide help to B cells in vivo, as CD4$^+$ T cells were undetectable. An additional important characteristic of p113-121–specific CD8$^+$ T cells was their capacity for in vivo killing of p113-121–labeled, HLA-A*0201$^+$ splenocytes. The cytotoxic function of p113-121/HLA-A*0201–specific CD8$^+$ T cells extended into direct killing of splenocytes infected with live *Mycobacterium smegmatis* expressing ML1419c: both 9mer and 30mer induced CD8$^+$ T cells that reduced the number of ML1419c-expressing mycobacteria by 95%, whereas no reduction occurred using wild-type *M. smegmatis*. These data, combined with previous observations in Brazilian cohorts, show that ML1419c p113-121 induces potent CD8$^+$ T cells that provide protective immunity against *M. leprae* and B cell help for induction of specific IgG, suggesting its potential use in diagnostics and as a subunit (vaccine) for *M. leprae* infection. *The Journal of Immunology*, 2011, 187: 1393–1402.

Host defense activity against mycobacteria is chiefly dependent on cell-mediated immunity in which the adaptive immune response plays a crucial role in inhibiting mycobacterial multiplication. It has long been established that CD4$^+$ T cells are key mediators of immunity to mycobacteria, notably in the acute phase of infection (1), but it has taken longer to acknowledge the importance of CD8$^+$ T cells (2). Moreover, the role of CD8$^+$ T cells, at least in *Mycobacterium tuberculosis* infection, seems to be more focused on control of latent infection (3, 4) and can be mediated by production of Th1 cytokines like IFN-γ that activate microbicidal effector functions of infected macrophages, as well as by the release of cytotoxic granules containing perforin, granzyme, and granulysin, leading to the killing of infected phagocytes and intracellular mycobacteria (5).

Mycobacterium leprae, the causative agent of leprosy, has a predilection for nerve cells and skin leading to severe nerve damage and subsequent disabilities. Clinical leprosy presents as a spectrum in which interindividual variability in resistance correlates with the host's ability to mount effective cell-mediated immunity to the pathogen (6). This is clear from the characteristic immunological and clinical leprosy spectrum, ranging from strong cellular immunity in tuberculoid/borderline tuberculoid (TT/BT) patients with localized disease to predominantly humoral responses and lack of T cell immunity in lepromatous (LL) leprosy (7). The strong *M. leprae*-specific Th1 cell responses (CD4$^+$ and CD8$^+$) present in TT/BT leprosy patients, characterized by production of substantial levels of IFN-γ, are believed to be responsible for bacterial control. Similarly, in animal studies of *M. leprae* infection, IFN-γ–producing T cells have been reported to control bacterial growth (8). These differences in outcome of infection in leprosy are most likely caused by different host defense mechanisms (9–11), and a recent genome-wide association study showed that susceptibility to leprosy was associated with polymorphisms in seven genes in the innate NOD2-signaling pathway, in addition to HLA (12).

Despite the efforts and successes of the World Health Organization during the past 20 years to decrease markedly the number of registered leprosy cases worldwide, the decline in new cases is

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Abbreviations used in this article: BT, borderline tuberculoid; HLA-A2tg, HLA-A*0201 transgenic; IP-10, IFN-γ–induced protein 10; LL, lepromatous; β_2m, β_2-microglobulin; PGL-I, phenolglycolipid; RT, room temperature; TB, tuberculosis; TT, tuberculoid.

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stagnant demonstrating that transmission of *M. leprae* is persistent and not affected sufficiently by current control measures (13–15). There are no tools available to identify subclinical *M. leprae* infection: although the level of anti-*M. leprae*–specific phenolglycolipid (PGL-I) Abs in serum reflects the bacterial load in individuals exposed to *M. leprae*, it does not represent a reliable marker for subclinical *M. leprae* infection progressing to active disease (16).

Deciphering the sequences of various mycobacterial genomes, including those of two *M. leprae* strains (17), has provided the necessary data for selecting *M. leprae*-specific Ags as tools to analyze *M. leprae*-specific immunity; for example, induction of in vitro IFN-γ production (18–21). Using algorithms for binding to HLA class I molecules, an *M. leprae*-specific nonamer p113-121, derived from the regulatory protein ML1419c, was selected from *M. leprae* unique candidate proteins (19, 21). After in vitro stimulation of PBMC with this peptide, IFN-γ production was induced in $CD8^+$ T cells derived from BT leprosy patients and contacts of multibacillary patients, providing higher sensitivity than PGL-I–based tests to detect *M. leprae* infection in these individuals (21). However, the molecular basis of this epitope's HLA-restriction remains unknown. Moreover, the function of these $CD8^+$ T cells, in particular their potential inhibitory activity on mycobacterial replication, remains equally unidentified.

As mentioned, HLA class I-restricted $CD8^+$ T cells play a role in immunity against leprosy and tuberculosis (4), but evidence showing that $CD8^+$ T cells participate in protective immunity to *M. leprae* infection in humans is lacking (5, 22). Immunohistological analysis of lesions has shown that the $CD8^+$ T cell frequency and function depends on the clinical phenotype, as in lesions of LL patients higher numbers of $CD8^+$ T cells are found than those in TT lesions (23) although the ratios are again different in peripheral blood.

HLA-A*0201 is one of the most prevalent class I alleles, with a frequency of >30% in most populations. Because the amino acid sequence of ML1419c p113-121 contains amino acids that fit the HLA-A*0201–peptide binding motif (24), we argued that this allele very likely represents the restriction element via which this peptide is in vivo presented to $CD8^+$ T cells. To address the in vivo function of ML1419c p113-121 and determine whether the *M. leprae*-specific $CD8^+$ T cells induced by this epitope have a protective or pathogenic effect, we used HLA-A*0201 transgenic (HLA-A2tg) mice. This mouse model has proved to be an appropriate tool for the identification of human HLA-A*0201–restricted T cell epitopes (25, 26). By immunizing HLA-A2tg mice with synthetic ML1419c peptides, we demonstrate induction of cytotoxic $CD8^+$ T cells specific for ML1419c p113-121 that lead to killing of live mycobacteria and induction of specific IgG Abs against ML1419c protein.

Materials and Methods

Synthetic peptides

ML1419c p113-121 (9mer; RLDGTTLEV), ML1419c p108-122 (15mer; EAVLLRLDGTTLEVE), and the synthetic long peptide ML1419c p100-129 (30mer; VGDASQPS EAVLLRLDGTTLEVEAVSVLTV) were purchased from Peptide 2.0 (Chantilly, VA). Homogeneity and purity were confirmed by analytical HPLC and by mass spectrometry. Purity of all peptides was ≥80%. All impurities consist of shorter versions of the peptides caused by <100% coupling efficiency in each round of synthesis.

*HLA-A*0201–peptide binding*

Peptide binding to HLA-A*0201 was performed as described previously (25). Briefly, recombinant HLA-A*0201 (previously determined to yield 20–40% binding) was incubated in 96-well serocluster plates (Costar; Corning) at 20°C for 48 h with 0.5 μl β_2-microglobulin (β_2m; 15 pmol) and 1 μl (100 fmol) fluorescent-labeled peptide (HBV core 47–56 with a cysteine substitution at position 52) in 92.5 μl assay buffer (100 mM Na-phosphate, 75 mM NaCl, 1 mM CHAPS, pH 7), 2 μl protease inhibitor mixture (1 μM chymostatin, 5 μM leupeptin, 10 μM pepstatin A, 1 mM EDTA, 200 μM Pefabloc; Sigma, St. Louis, MO), and 2 μl of test peptide at different concentrations to establish a dose-response curve. HLA–peptide complexes were separated from free peptide by gel filtration on a Synchropak GPC 100 column (250 mm × 4.6 mm; Synchrom, Lafayette, IN) using assay buffer containing 5% CH_3CN. Fluorescent emission was measured at 528 nm on a Jasco FP-920 fluorescence detector (B&L Systems, Maarssen, The Netherlands). The percentage of labeled peptide bound was calculated as the amount of fluorescence bound to MHC divided by total fluorescence. The concentration of test peptide yielding 50% inhibition (IC_{50}) was deduced from the dose-response curve.

Mice

HLA-A2tg mice B6.Cg-Tg (HLA-A/H2-D, Enge/J stock no. 004191; The Jackson Laboratory, Bar Harbor, ME) were bred under specific pathogen-free conditions at the Leiden University Medical Center animal facility. These mice express an interspecies hybrid class I MHC gene, AAD, which contains the α1 and α2 domains of the human HLA-A2.1 gene and the α3 transmembrane and cytoplasmic domains of the mouse H-$2D^d$ gene, under the direction of the human HLA-A2.1 promoter (27). Immunodetection of the HLA-A2.1 recombinant transgene established that expression was at equivalent levels to endogenous mouse class I molecules. The mouse α3 domain expression enhances the immune response in this system. Surface expression of the HLA-A*0201 molecule was confirmed by FACS analysis for each mouse.

Immunizations

Because immunization with peptide alone did not cause appreciable responses, mixtures of CpG adjuvant with Ag are routinely used. Mice (four to five animals per group) were injected twice, with a 2-wk interval, s.c. in the flanks with 50 μg CpG (ODN1826 5′-TCC ATG ACG TTC CTG ACG TT-3′; InvivoGen, San Diego, CA) in 200 μl PBS and either 50 μg (40 nmol) ML1419c p113-121 (nonamer) or 140 μg (40 nmol) ML1419c p100-129 (30mer). Splenocytes were harvested 7–10 d after final injections. Since ODNs containing unmethylated CpG motifs can activate immune cells to produce cytokines (28), we also routinely immunize with CpG alone as a (negative) control to assess the Ag specificity of immunization.

In vitro cultures

Splenocytes were isolated from individual animals by homogenizing spleens through a plastic cell strainer (BD Biosciences), and splenocytes (3×10^6 cells/ml) were resuspended in IMDM (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen), 100 U/100 μg/ml penicillin/streptomycin solution (Invitrogen), 8% heat-inactivated FCS, and 5×10^{-5} M 2-mercaptoethanol (Sigma). Cell suspensions (100 μl) were added to 96-well round-bottom microtiter plates (Costar; Corning). Cells were incubated in quadruplicates with 100 μl of medium, peptide (1 or 10 μg/ml), or *M. leprae* whole-cell sonicate (1 or 10 μg/ml). The mitogen Con A (2 μg/ml; Sigma) was used in all experiments as a positive control for cell viability. After 6 d, supernatants were taken from each well and quadruplicates pooled and frozen at −20°C until performance of ELISA.

M. leprae *whole-cell sonicate*

Irradiated armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to >95% breakage. This material was provided through the National Institutes of Health/National Institute of Allergy and Infectious Diseases "Leprosy Research Support" Contract N01 AI-25469 from Colorado State University (these reagents are now available through the Biodefense and Emerging Infections Research Resources Repository at http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx).

IFN-γ ELISA

Detection of IFN-γ in culture supernatants of in vitro-cultured splenocytes was performed by ELISA (BD Biosciences) according to the manufacturer's instructions. OD values were converted into concentrations using Microplate Manager software, version 5.2.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands). The cutoff value to define positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 20 pg/ml. Values for unstimulated whole-blood cultures were typically <30 pg/ml.

Intracellular cytokine staining

For polychromatic flow cytometry, splenocytes (3×10^6 cells/ml) were cultured in vitro with peptide (5 μg/ml). After 7 d, cells were incubated with medium or fresh peptide (5 μg/ml). After 1 h, brefeldin A (5 μg/ml; Sigma) was added. After 5 h, cells were permeabilized and fixed using Cytofix/Cytoperm (BD Biosciences) and Perm/Wash (BD Biosciences) according to the manufacturer's instructions and stained using PE-conjugated anti-CD8β_2 (BD Pharmingen), PECy5-conjugated anti-CD4 (BD Pharmingen), ebioV405-conjugated anti-CD19 (eBioscience), Vivid (Invitrogen), allophycocyanin-conjugated anti–IL-2 (BD Pharmingen), Alexa Fluor 700-conjugated anti–IFN-γ (BD Pharmingen), and PeCy7-conjugated anti–TNF (BD Pharmingen).

Multiplex determination of cytokines and chemokines

According to the manufacturer's guidelines, 16 inflammatory and immunomodulatory cytokines or chemokines (MIG, VEGF, IFN-γ–induced protein 10 [IP-10], IFN-γ, GM-CSF, IL-4, IL-6, MIP-1β, IL-10, IL-12p70, IL-17, IL-1α, IL-1β, IL-2, TNF, MCP-1) were measured in unstimulated, Ag-stimulated, or mitogen-stimulated samples by Milliplex Multi-Analyte Profiling (Millipore, Billerica, MA): 96-well microtiter filter plates were prewetted with washing buffer (200 μl/well; Millipore), sealed, and shaken at room temperature (RT). After 10 min, washing buffer was removed by vacuum, and subsequently assay buffer (12.5 μl), test sample (12.5 μl), and mixed cytokine beads (12.5 μl) were added to each well. Fourfold dilutions of standards were used for each analyte starting from 10,000 pg/ml. Plates were sealed and incubated at RT on a microtiter plate shaker. After 2 h, fluids were removed, and plates were washed twice with washing buffer (200 μl/well; Millipore). To each well, detection Ab was added (12.5 μl; Millipore), and plates were incubated at RT on a plate shaker at 300 rpm. After 1 h, PE-labeled streptavidin (12.5 μl; Millipore) was added to each well and incubated at RT. After 30 min, fluids were removed and plates washed twice with washing buffer (200 μl/well; Millipore). To each well, sheath fluid was added (80 μl; Millipore) and mixed well for 5 min on a plate shaker at 300 rpm after which plates were placed in the Bio-Plex System (Bio-Rad Laboratories). From each well, a minimum of 50 analyte-specific beads were analyzed for fluorescence with the Bio-Plex Manager Software 4.0 (Bio-Rad Laboratories). A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. Analyte concentrations outside the upper or lower limits of quantification were assigned the values of the limits of quantification of the cytokine or chemokine.

*HLA-A*0201/ML1419c p113-121 tetramer production and staining*

Tetrameric complexes were prepared essentially as described (1). Briefly, recombinant HLA-A*0201/K^d and human β_2m were produced in *Escherichia coli* as inclusion bodies. Prefolded human β_2m and HLA-A-*0201/K^d solubilized in urea were added with synthetic peptide (ML1419c p113-121) into a refolding buffer consisting of 100 mM Tris (pH 8), 400 mM arginine, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione. Refolded complexes were biotinylated by incubation for 90 min at 30°C with BirA enzyme (Avidity, Denver, CO), and the biotinylated complex was purified by gel filtration on a Superdex 75 column (Amersham Pharmacia Biotech). Tetrameric HLA–peptide complexes were produced by the stepwise addition of streptavidin-conjugated allophycocyanin (Sigma) to achieve a 1:6 molar ratio (streptavidin–allophycocyanin/biotinylated HLA class I).

Splenocytes were stained in PBS with 0.1% BSA using allophycocyanin-conjugated HLA-A*0201/ML1419c p113-121 tetramer (HLA-A2/p113 TM; 50 μl; 1:50), PE-conjugated anti-CD8β_2 (Ly-3.2 clone RM4-5; 50 μl; 1:100; BD Pharmingen), and propidium iodide (50 μl; 1:2000; Sigma).

Determination of anti-ML1419c Abs

Levels of Ab directed against ML1419c in serum from immunized mice were determined by ELISA. Briefly, plates were coated overnight at 4°C with recombinant ML1419c Ag (5 μg/ml) or PBS (0.4% BSA) as a negative control. Plates were blocked for 2 h using PBS containing 1% BSA and 1% Tween 20. Different sample dilutions (100 μl/well) were added to wells and incubated at 37°C for 2 h. Plates were washed three times using PBS containing 0.05% Tween 20 and 100 μl/well HRP-labeled, rabbit anti-mouse total IgG (Dako, Glostrup, Denmark). After 2 h at 37°C, plates were washed three times using PBS containing 0.05% Tween 20, and 100 μl/well TMB substrate (Sigma) was added for 15 min at RT. The reaction was stopped by addition of H_2SO_4 (1 M; 100 μl/well). OD values at 450 nm were determined using BioRad Microplate reader 680 (Bio-Rad Laboratories). Mean Ab concentration was calculated from the linear part of the titration curve.

In vivo cytotoxicity assay

Erythrocytes in splenocytes suspension were lysed with ammonium chloride treatment, and the single-cell suspension was split into two equal fractions. Cells were differentially labeled at 37°C for 10 min with CFSE (Invitrogen, Carlsbad, CA) to 5 μM (target = $CFSE^{high}$) or 0.02 μM CFSE (control = $CFSE^{low}$) concentration in PBS with 0.1% BSA. The reaction was stopped by addition of FCS (Invitrogen) to a final 10% v/v. The target population was pulsed for 2 h with 5 μg/ml ML1419c nonamer, and the control population remained unpulsed. Cells were washed four times with PBS before the two populations were mixed in 1:1 ratio, and a total of 15×10^6 cells was injected intravenously in the tail. After 2 d, spleens were removed and splenocytes analyzed for specific killing by FACS cytometry. The ratio of $CFSE^{low}/CFSE^{high}$ cells was determined by flow cytometry. Specific killing of ML1419c p113-121–pulsed $CFSE^{high}$ target cells was calculated as follows: $[1 - (CFSE^{high}/CFSE^{low})] \times 100\%$.

Mycobacterium smegmatis *strains*

M. smegmatis strains were produced including empty vector control (pVV16) or *M. smegmatis* expressing ML1419c (pVV:ML1419). Western blot analysis using mouse Abs directed against ML1419c was used to check the expression level of ML1419c in the latter two *M. smegmatis* strains. To compare viability of *M. smegmatis* strains, three clones per strain were grown in Luria–Bertani broth containing 0.05% Tween 80 (Sigma), kanamycin (25 μg/ml; Sigma), and hygromycin (25 μg/ml; Invitrogen). The OD (at 600 nm) was checked every 2 h and indicated similar growth rates for all three strains. The pVV16, *E. coli–Mycobacterium* shuttle vector was generously provided by Dr. V. Vissa (Department of Microbiology, Immunology and Pathology, Colorado State University).

Killing of recombinant M. smegmatis *by splenocytes of immunized mice*

M. smegmatis strains were grown in Middlebrook 7H9 medium supplemented with 10% ADC (BD Biosciences) until log phase. Splenocytes (10^7/well) derived from immunized HLA-A2tg mice were plated in 48-well cell cluster plates (Costar; Corning) at 37°C together in IMDM supplemented with 8% FCS and 2 mM glutamine (Life Technologies, Paisley, U.K.) together with 1.5×10^7 CFU *M. smegmatis* pVV:ML1419 or *M. smegmatis* pVV16, and plates were centrifuged for 3 min at 1000 rpm. Based on our findings that ~30% of the splenocytes (= 3×10^6/well) are macrophages and because macrophages are specifically infected by the added mycobacteria (1.5×10^7/well), the multiplicity of infection used in these experiments was 5. The number of CFU used for infection was calculated using OD (600 nm) and standard growth curves, and the inocula were confirmed by growth on Middlebrook 7H10 agar medium supplemented with 10% OADC (BD Biosciences). After 1-h incubation, splenocytes were washed three times with PBS. To prevent extracellular growth of *M. smegmatis*, cells were incubated at 37°C with 50 μg/ml gentamicin (Sigma). After 1 h, splenocytes were washed three times with and incubated for 24 h at 37°C in the presence of 5 μg/ml gentamicin in IMDM supplemented with FCS and glutamine. Splenocytes were lysed with 0.1% Triton X-100 (Sigma) for 5 min and plated on Middlebrook 7H10 agar plates. CFU was determined by counting after 3 d.

Results

Immunization of HLA-A2tg mice with ML1419c peptides induces high levels of IFN-γ–producing, ML1419c-specific $CD8^+$ T cells

In view of its unique T cell recognition pattern in PBMC of leprosy patients and their contacts (21), we decided to study the role of ML1419c p113-121–reactive T cells in more detail and analyze whether this nonamer can induce protective T cell responses in vivo. For this purpose, we used the HLA-A2tg mouse model that has been shown to be suitable for identification of human HLA-A*0201–restricted T cell epitopes (25, 26). First, after immunization of mice with ML1419c p113-121 (9mer) or ML1419c p100-129 (30mer), IFN-γ secretion induced by in vitro stimulation of splenocytes with ML1419c peptide or *M. leprae* whole-cell sonicate was analyzed by ELISA (Fig. 1*A*). Naive mice and mice immunized with CpG alone showed no IFN-γ secretion in response to *M. leprae* Ags. In contrast, mice immunized with either ML1419c p113-121 or p100-129 both induced high levels

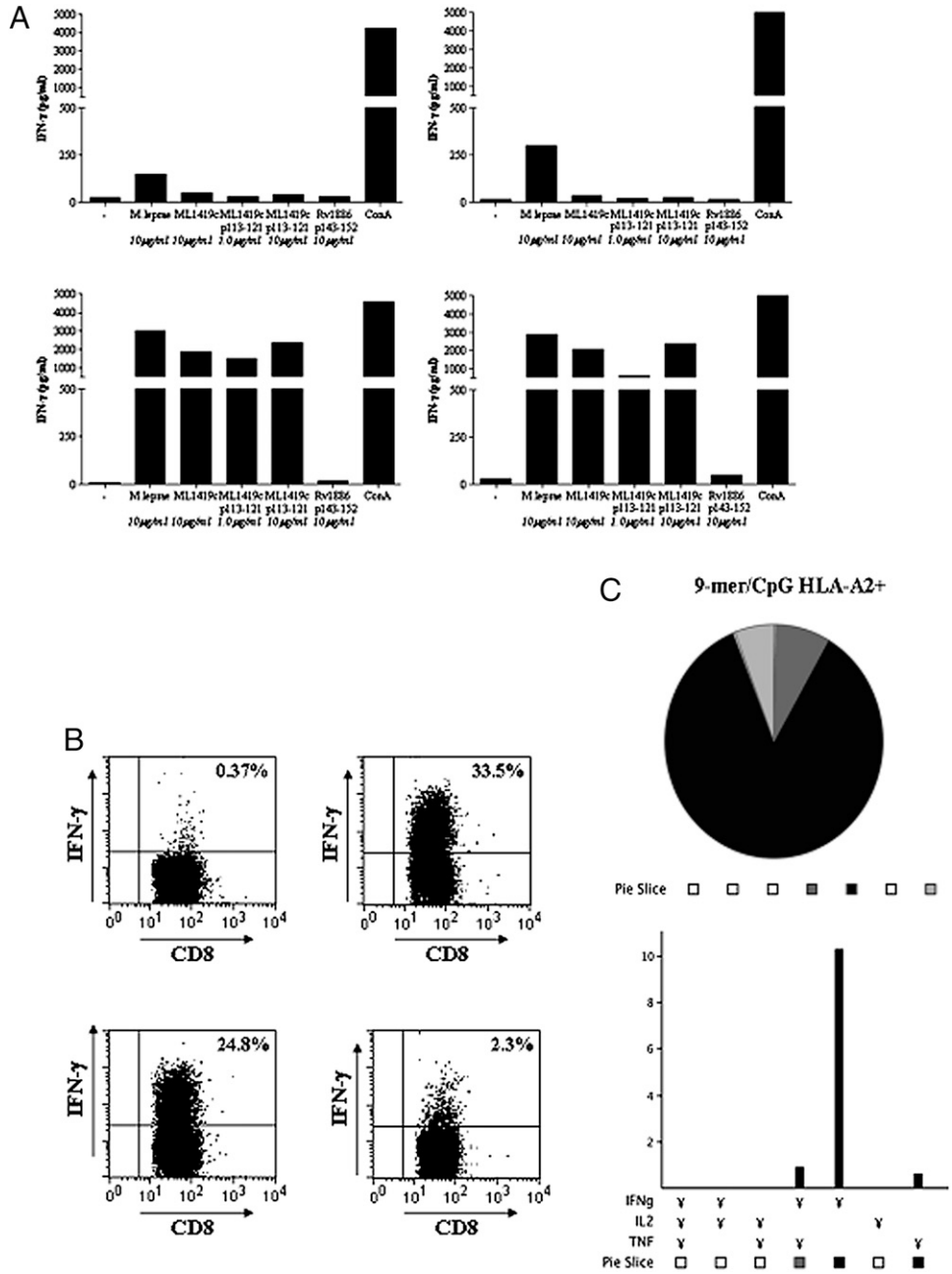

FIGURE 1. *A*, IFN-γ secretion. IFN-γ secretion was analyzed after 5 d in vitro stimulation of splenocytes with ML1419c p113-121, *M. leprae* sonicate, or mitogen (Con A). HLA-A2tg mice were unimmunized (*upper left panel*) or immunized with CpG alone (*upper right panel*), ML1419c p113-121 (9mer/CpG; *lower left panel*), or ML1419c p100-129 (30mer/CpG; *lower right panel*). The HLA-A*0201–restricted epitope Rv1886 (*M. tuberculosis* Ag85B) p143-152 (25) was used as a control for ML1419c specificity. All test groups included five mice. All mice were separately analyzed. Results are shown for one animal and are representative for each test group. *B*, Intracellular IFN-γ production by $CD8^+$ T cells after ML1419c p113-121 immunization of HLA-A2tg mice. Mice were immunized twice with CpG alone (*upper left panel*), ML1419c p113-121 (9mer/CpG; *upper right panel*), p108-122 (15mer/CpG; *lower left panel*), or p100-129 (30mer/CpG; *lower right panel*). Splenocytes were stimulated in vitro with the same peptides used for immunization. After 7 d, cells were incubated with medium or fresh peptide for 1 h before addition of brefeldin A and analysis for intracellular IFN-γ production. *C*, Frequency of polyfunctional $CD8^+$ T cells. Percentage of $CD8^+$ T cells in HLA-A2tg mice, producing combinations of IFN-γ, TNF-α, or IL-2 after in vitro stimulation with peptide (see *B*). Mice were immunized with ML1419c p113-121 (9mer/CpG). The total number of $CD8^+$ T cells analyzed in immunized mice was 56,000. Slices in the pie chart represent the proportion of single-, double-, or triple-positive $CD8^+$ T cells for each Ag. Only $CD8^+$ populations of $>5 \times 10^4$ events were analyzed. In naive mice, the number of $CD8^+$ T cells producing cytokines was $<5 \times 10^4$ cells.

of IFN-γ to the ML1419c 9mer. Because the HLA-A*0201–restricted epitope Rv1886 (*M. tuberculosis* Ag85B) p143-152 (25) has a high binding affinity for HLA-A*0201 (Table I), it was used as a control for ML1419c specificity. Even though Rv1886 p143-152 binds with higher affinity to HLA-A*0201 than that of ML1419c p113-121 (IC_{50}: 0.01 μM versus 0.035 μM, respectively), no IFN-γ was produced in response to this *M. tuberculosis* epitope, demonstrating the specificity for ML1419c in HLA-A2tg mice immunized with ML1419c peptides (Fig. 1*A*).

To define the phenotype of ML1419c-responsive T cells more precisely, splenocytes of HLA-A2tg mice immunized with ML1419c p113-121 (9mer), p108-122 (15mer), or p100-129 (30mer) were analyzed directly ex vivo or stimulated in vitro for 1 wk with the same ML1419c peptides they had been immunized with, after

Table I. ML1419c 9mer, 15mer, and 30mer peptides

Peptides	Amino Acid Sequences[a]	HLA-A*0201 Binding Affinity (IC_{50}), μM[b]	In Vivo CTL Induction
ML1419c p113–121	**RLDGTTLEV**	0.035	++
ML1419c p108–122	EAVLL**RLDGTTLEV**E	>50	+
ML1419c p100-129	VGDASQPS EAVLL**RLDGTTLEV**EAVSVLTV	>50	+
Rv1886 p143-152	FIYAGSLSAL	0.01	−

[a]The minimal peptide epitope is indicated in bold.
[b]Peptide binding affinity (IC_{50}) was defined as high affinity (<1 μM), intermediate affinity (1–10 μM), weak affinity (10–100 μM), or nonbinding (>100 μM), according to Ref. 46.
++, specific lysis >85% in 5/5 mice; +, specific lysis 50–85%; −, no specific lysis detected.

which intracellular IFN-γ production was assessed by FACS analysis. As shown in Fig. 1*B*, $CD8^+$ T cells were responsible for the ML1419c-specific IFN-γ production after in vitro stimulation with both 9mer and 15mer peptides. Directly ex vivo intracellular staining of splenocytes of ML1419c peptide-immunized HLA-A2tg mice resulted in specific $CD8^+$ T cells as well, but percentages of $CD8^+$ IFN-γ^+ T cells were slightly lower, ranging from 4.3 to 5.6% (data not shown). Thus, direct ex vivo analysis of splenocytes of HLA-A2tg mice immunized with ML1419c peptide showed the presence of $CD8^+$ T cells specific for ML1419c 9mer (19.5%, Fig. 2), part of which (4.3–5.6%) also produced IFN-γ^+ ex vivo (data not shown), and which was further expanded by in vitro ML1419c peptide stimulation (Fig. 1*B*). This in vitro expansion was specific as in vitro stimulation with ML1419c peptides of splenocytes of unimmunized mice did not induce $CD8^+$ IFN-γ^+ T cells (Fig. 1*B*).

Intracellular IFN-γ production in response to the 30mer was much less, probably because presentation in the context of HLA class I requires processing of the 30mer, whereas the 15mer can induce IFN-γ by $CD8^+$ T cells as well (21). The 1-h incubation time with freshly added peptide followed by 5 h in combination with brefeldin A (Fig. 1*B*) may have been too brief for the 30mer to be processed. This was in agreement with our finding that immunization with 30mer followed by in vitro stimulation with the 9mer induced 29% IFN-γ–producing $CD8^+$ T cells showing that 30mer induced ML1419c-specific cellular responses (data not shown).

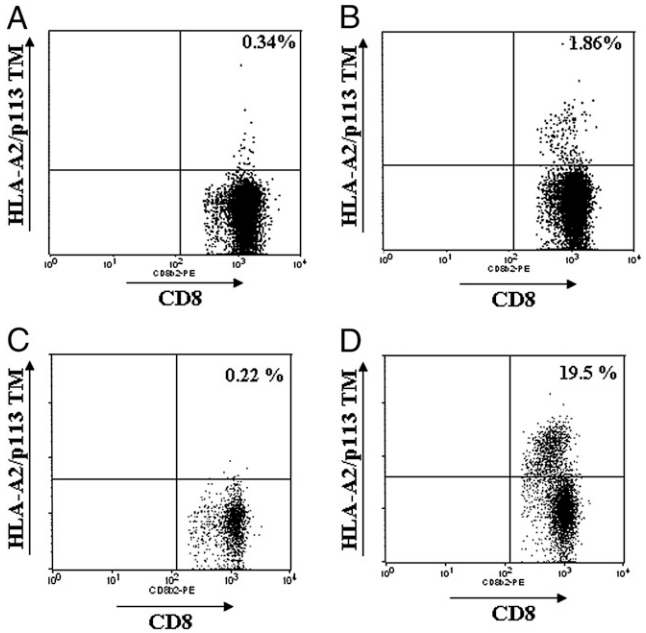

FIGURE 2. Tetramer staining. Splenocytes of unimmunized (*A*, *C*) or ML1419c p113-121–immunized (*B*, *D*) HLA-A2tg mice were stained with PE-conjugated anti-CD8 and allophycocyanin-conjugated HLA-A*0201/ML1419c p113-121 tetramer (HLA-A2/p113 TM) directly ex vivo (*A*, *B*) or after 1 wk in vitro restimulation with ML1419c p113-121 (*C*, *D*).

To estimate the frequency of polyfunctional $CD8^+$ T cells, intracellular TNF-α and IL-2 production was assessed simultaneously with IFN-γ (Fig. 1*C*) by polychromatic flow cytometry. After 9mer immunization, the majority (85%) of these $CD8^+$ T cells produced IFN-γ, but distinct IFN-γ^+/TNF-α^+/$CD8^+$ and TNF^+/$CD8^+$ populations were observed as well. Similar results were observed using 15mer immunization, in which case we found 78% of the $CD8^+$ T cells to be single positive for IFN-γ (data not shown).

Finally, in the intracellular staining analyses, no IFN-γ production by $CD4^+$ T cells was observed after in vitro ML1419c peptide restimulation (data not shown). Mice with a genetic background identical to the HLA-A2tg mice but lacking the human HLA-A2 molecule did not, however, show ML1419c-specific IFN-γ–producing $CD8^+$ (or $CD4^+$) T cells. Both these findings strongly support the HLA class I restriction of ML1419c p113-121–specific T cells.

Multiplex determination of cytokines and chemokines in response to ML1419c stimulation

Immunological correlates of protection in leprosy are still lacking: although Ag-specific IFN-γ production is often used as a biomarker for *M. leprae* infection (18), it is possible that additional cytokines might allow more specific or qualitatively different detection of immune responses against *M. leprae* peptides. To characterize further the cellular immune response directed against ML1419c, 15 additional cytokines and chemokines were tested in multiplex assays on supernatants of splenocytes of ML1419c p113-121–immunized HLA-A2tg mice after in vitro 6-d stimulation with ML1419c p113-121 (Table II). As expected based on the ELISA data (Fig. 1*A*), immunization with ML1419c p113-121, but not with CpG only (data not shown), induced IFN-γ production in response to this peptide but not to Rv1886 p143-152. Similar responses were observed for two proteins that can be induced by IFN-γ: the 10-kDa protein IP-10/CXCL10 and the T cell chemoattractant MIG/CXCL9. Production of MIP-1β, which is produced by macrophages after stimulation with bacterial endotoxins as well as by regulatory $CD8^+$ T cells in humans (29), was observed to a higher extent in ML1419c p113-121–immunized mice, but production in supernatants from medium-stimulated cultures of peptide-immunized mice was already substantial indicating only partial *M. leprae* specificity in the assay. Immunization with ML1419c p113-121 specifically induced VEGF secretion, as significant production was only observed in splenocytes in response to ML1419c p113-121 and not to Rv1886 p143-152 and, additionally, as immunization with ML1419c p113-121, and not CpG alone (data not shown), induced VEGF. Similarly, for TNF-α and IL-6, secretion was only observed in response to the *M. leprae* peptide, but the amounts of the cytokines measured in the supernatants were only marginal.

Finally, levels of IL-2, IL-4, IL-5, IL-10, IL-12p70, and IL-17 in samples stimulated with ML1419c p113-121 were below detection

Table II. Multiplex analysis of ML1419c peptide-immunized HLA-A2tg

		In Vitro Stimuli			
Analyte (pg/ml)	Medium	ML1419c p113-121 (1 μg/ml)	ML1419c p113-121 (10 μg/ml)	Rv1886 p143-152 (10 μg/ml)	Con A
IFN-γ	0	390	287	0	5112
IP-10	20	289	147	23	251
MIG	29	370	236	36	533
MIP-1β	272	917	731	248	787
TNF-α	1.0	21	9.3	2.9	33
VEGF	2.8	48	24	1.2	83
IL-6	3.8	41	26	4.4	299

In response to in vitro stimulation of splenocytes with ML1419c p113-121 for 5 d, production was assessed for 16 analytes. Shown here are IFN-γ, IP-10, MIG, MIP-1β, TNF-α, VEGF, and IL-6. HLA-A2tg mice tested after immunization with ML1419c p113-121 or CpG alone (data not shown). Levels of IL-2, IL-4, IL-5, IL-10, IL-12p70, and IL-17 in samples stimulated with ML1419c p113-121 were below detection threshold; for IL-1α, IL-1β, and GM-CSF, all stimuli induced secretion levels equal to those of unstimulated samples. Both groups included five mice. All mice were separately analyzed. Results are shown for one animal and are representative for each test group.

threshold, whereas for IL-1α, IL-1β, and GM-CSF, all stimuli induced secretion levels in splenocytes that were equal to those in unstimulated samples.

*Frequency of ML1419c p113-121–specific, HLA-A*0201–restricted CD8$^+$ T cells*

Using allophycocyanin-conjugated tetramers composed of HLA-A*0201 and ML1419c p113-121 (HLA-A2/p113 TM), the frequency of CD8$^+$ T cells after immunization of HLA-A2tg mice with ML1419c p113-121 was addressed. Tetramer staining of splenocytes was determined directly ex vivo (Fig. 2*A*, 2*B*) or after 7 d in vitro incubation with 5 μg/ml peptide (Fig. 2*C*, 2*D*).

The percentage of TM$^+$/CD8$^+$ splenocytes induced by peptide immunization was 1.86% (CpG only: 0.34%), which could be increased further to 19.5% by in vitro stimulation with ML1419c peptide. This increase was specific for ML1419c as no increase in frequency of TM$^+$/CD8$^+$ T cells was observed after in vitro peptide restimulation of splenocytes derived from HLA-A2tg mice immunized with CpG only (0.22%).

Immunization with ML1419c nonamer induces Ab specific for ML1419c protein

Contrasting with the high cell-mediated immune response against *M. leprae* in TT leprosy is the strong *M. leprae*-specific humoral response in LL leprosy. This response is primarily directed to PGL-I, but also *M. leprae* protein Ags can be recognized by sera from LL patients (30, 31). In view of this, Ab levels against the ML1419c protein were analyzed after immunization with its HLA-A*0201–restricted nonamer (Fig. 3). Immunization with ML1419c p113-121 induced high Ab titers not only to the nonamer itself but also to the 30mer and even to the whole recombinant ML1419c protein. Mock-immunized mice, in contrast, did not show any Ab reactivity, indicating that the ML1419c p113-121 is capable of inducing not only a cellular but also a humoral immune response. Furthermore, the Ab levels present after this peptide immunization indicate that CD8$^+$ T cells are capable of providing T cell help to induce the production of Ab by plasma cells, as no CD4 T cells could be detected after peptide immunization.

Immunization with ML1419c peptides in HLA-A2tg mice induces in vivo CTL activity

Besides producing IFN-γ, CD8$^+$ T cells also contribute to protection by exerting cytolytic functions. Therefore, we determined whether ML1419c p113-121 could induce HLA-A*0201–restricted CTL using in vivo cytotoxicity assays (32). For this purpose, mice were immunized with CpG alone or with ML1419c 9mer and 30mer peptides combined with CpG. As shown in Fig. 4*B*, ML1419c p113-121 immunization in HLA-A2tg mice induced high levels of in vivo cytotoxic activity specific for the ML1419c nonamer ranging from 88 to 94%. Likewise, immunization with the 30mer ML1419c p100-129 induced efficient specific lysis in a similar range (83–95%) (Fig. 4*C*). As expected, no lysis was observed in CpG-immunized mice.

Killing of M. smegmatis *expressing ML1419c by splenocytes of ML1419c p113-121–immunized mice*

Finally, to assess the ability of ML1419c-specific, HLA-A*0201–restricted T cells to kill live mycobacteria, *M. smegmatis* was transfected with ML1419c (pVV:ML1419). The two strains, *M. smegmatis* pVV:ML1419 and *M. smegmatis* transduced with the

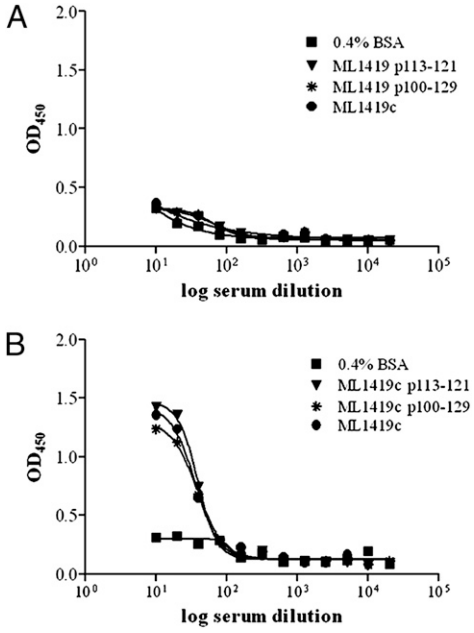

FIGURE 3. Quantification of serum Abs to ML1419c. After immunization of HLA-A2tg mice with CpG alone (*A*) or with ML1419c p113-121/CpG (*B*), Ab titers (OD_{450}) against ML1419c p113-121 (▼), ML1419c p100-129 (*), or ML1419c protein (●) were determined by ELISA. As a control, affinity for BSA (0.4% in PBS) alone (■) is shown. Serum dilutions are shown on the *x*-axis. All test groups included five mice. All mice were separately analyzed. Results are shown for one animal and are representative for each test group.

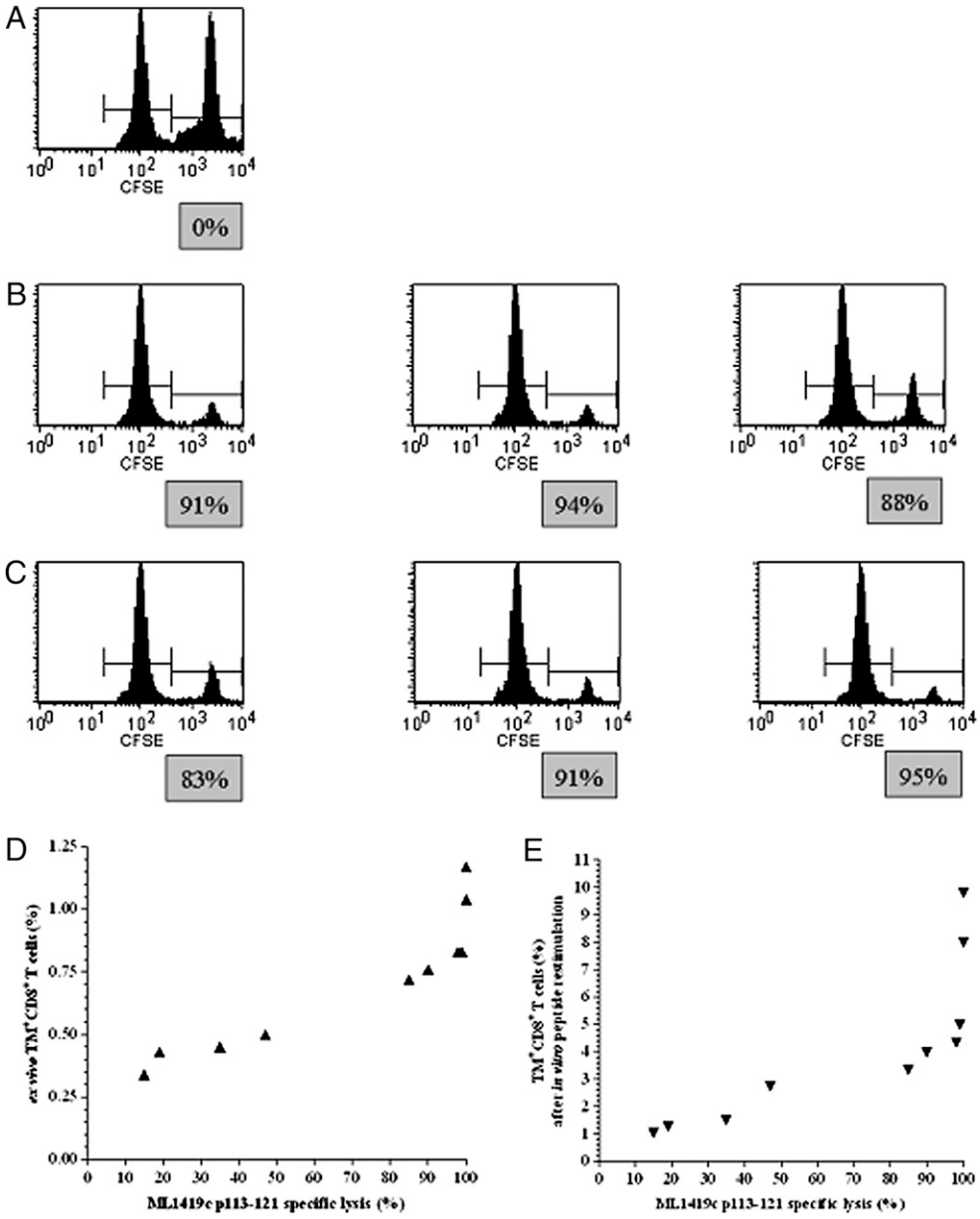

FIGURE 4. In vivo cytotoxicity. CTL response in HLA-A2tg mice against ML1419c p113-121 as detected by lysis of ML1419c p113-121–pulsed, $CFSE^{high}$-labeled syngeneic target cells using flow cytometry. The *y*-axis indicates the number of cells and the *x*-axis the CFSE intensity. The figure shows one representative CpG-immunized animal (*A*; $n = 5$) and three representative animals from the groups immunized with ML1419c p113-121/CpG (*B*; $n = 5$) and ML1419c p100-129/CpG (*C*; $n = 5$). Results shown are representative of at least four separate experiments. Correlation of ML1419c p113-121–specific in vivo lysis in various experiments with the percentage of HLA-A2/p113 TM^+CD8^+ T cells directly ex vivo (*D*) or after in vitro peptide restimulation (*E*).

empty vector (pVV16), were used to infect splenocytes derived from CpG-immunized, ML1419c p113-121–immunized, or ML1419c p100-129–immunized HLA-A2tg mice, and after 3 d, CFU of each strain was determined (Fig. 5).

Splenocytes of mice that had been immunized with either 9mer or 30mer ML1419c peptide in CpG respectively caused 95% (1,850,000 to 420,000 CFU) and 62% (1,850,000 to 1,140,000 CFU) reduction in CFU of *M. smegmatis* pVV:ML1419, which was not observed for mice immunized with CpG only (Fig. 5*A*). In contrast, no difference in CFU of wild-type *M. smegmatis* was observed when this strain was cultured in the presence of splenocytes from ML1419c peptide-immunized mice compared with that after culture in the presence of splenocytes from CpG-immunized mice (Fig. 5*B*).

These data show that immunization with ML1419c 9mer or 30mer induced a strong $CD8^+$ T cell response with Th1, cytolytic, as well as B cell help-functional activity, specific for the *M. leprae* peptide Ag ML1419c p113-121 as well as whole protein, and which, importantly, are able to inhibit mycobacterial growth. Thus, ML1419c-specific $CD8^+$ T cells (as present in BT/TT patients and contacts) have all characteristics of a protective host response against *M. leprae.*

Discussion

Identification of *M. leprae* Ags that induce protective $CD4^+$ and $CD8^+$ Th1 immune responses is important to the development of both diagnostic tools and new leprosy vaccines. Previously, we demonstrated that the *M. leprae* unique nonamer ML1419c p113-121 induced ex vivo IFN-γ production by $CD8^+$ T cells of BT leprosy patients and healthy household contacts of leprosy patients allowing more sensitive detection of *M. leprae*-specific immunity in

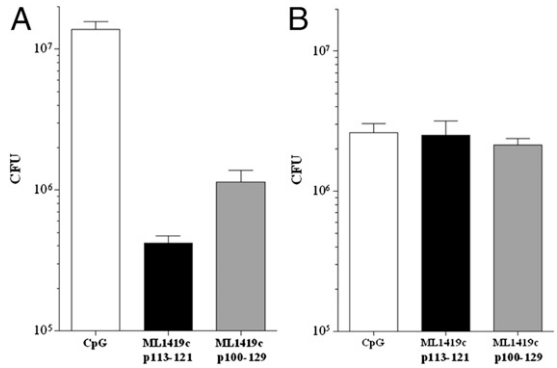

FIGURE 5. Determination of CFU of recombinant *M. smegmatis* expressing ML1419c Ag. CFU was determined for *M. smegmatis* expressing ML1419c (pVV:ML1419) (*A*) or *M. smegmatis* with empty vector (pVV16) (*B*) after incubation with splenocytes derived from HLA-A2tg mice immunized with CpG (white bar), ML1419c p113-121/CpG (black bar), or ML1419c p100-129/CpG (gray bar).

these individuals than by PGL-I–based tests (21). However, because the in vivo function and genetic restriction of these responding $CD8^+$ T cells and their possible link to protection (e.g., their ability to inhibit growth of *M. leprae*) was unknown, the characteristics of these cells in vivo were analyzed in detail in the current study.

In the absence of a relevant experimental infection model for leprosy that permits analysis of HLA-restricted T cell responses directed against *M. leprae* peptides, and because of the impossibility to grow the causative agent in vitro, we used an HLA-A2tg mouse strain that enables the modeling and identification of human T cell immune responses presented in the context of HLA-A*0201. These mice express chimeric HLA-A*0201/H2-D^d MHC class I molecules that, compared with unmodified HLA-A*0201, mediate efficient positive selection of mouse T cells to provide a more complete T cell repertoire capable of recognizing peptides presented by HLA-A*0201 class I molecules.

Immunization of peptides with adjuvant has been shown to induce stronger responses and better protection than immunization of the whole protein alone (33). Thus, we administered both the HLA-A*0201–restricted, ML1419c minimal peptide epitope and a synthetic long peptide (30mer) containing the 9mer in combination with CpG. The advantage of using synthetic long peptide for immunization is that these peptides are targeted to and processed by professional APCs, namely dendritic cells, resulting in efficient $CD4^+$ and $CD8^+$ T cell responses (34). Minimal HLA class I binding epitopes, in contrast, can in addition bind to nonprofessional APCs bearing the risk of tolerance induction.

Immunization of HLA-A2tg mice with ML1419c peptides led to production of IFN-γ by HLA-A2–restricted $CD8^+$ T cells that were specific for whole *M. leprae*, ML1419c protein, and ML1419c peptides, whereas other HLA-A2–restricted mycobacterial peptides such as the *M. tuberculosis* Ag85B epitope Rv1886 p143-152 (25) did not induce any IFN-γ production against ML1419c (Fig. 1). As expected, splenocytes derived from C57BL/6 mice immunized with ML1419c p113-121 that did not express the chimeric HLA-A*0201/H2-D^d MHC class I molecule did not produce IFN-γ in response to similar in vitro stimulation with this peptide (data not shown), further indicating that the response to ML1419c peptides is restricted by HLA-A*0201 and not by murine MHC.

After ML1419c p113-121 immunization, the majority (85%) of the ML1419c-specific $CD8^+$ T cells produced only IFN-γ although distinct, yet less significant, IFN-γ^+TNF-α^+CD8^+ and TNF^+CD8^+ T cell populations were observed as well. Research on antiviral immunity has shown that the presence of polyfunctional (IFN-γ^+IL-2^+TNF-α^+) profiles of virus-specific T cell responses correlated with disease activity (35), but recent studies on *M. tuberculosis*-infected cohorts demonstrated a substantial increase of TNF-α single-positive *M. tuberculosis*-specific $CD4^+$ T cells in active disease (36). In contrast, vaccine-induced protection against *Leishmania major* infection in mice has been associated with polyfunctional (IFN-γ^+IL-2^+TNF-α^+) $CD4^+$ T cells (37). However, polyfunctional $CD4^+$ T cells were specifically detected in patients with active *M. tuberculosis* infection (38). Although the exact contribution of the IFN-γ produced by ML1419c-specific $CD8^+$ T cells to protection against mycobacterial infection is not exactly clear, as specific Abs and CTL activity were induced simultaneously, our finding that ML1419c p113-121/CpG adjuvant immunization induces a protective immune response dominated by IFN-γ single-positive $CD8^+$ cells with CTL activity against *Mycobacterium*-infected cells suggests that these cells may well have a protective role. Moreover, it has been shown recently that the frequency and cytokine profile of *M. tuberculosis*-specific T cells did not correlate with protection against tuberculosis (TB) (39). Therefore, critical components of immunity against mycobacteria, such as IFN-γ production by $CD4^+$ T cells, may not necessarily translate into immune correlates of protection against mycobacterial disease by itself, and other functions (cytotoxicity or help for Ab production) may be required as well.

Besides IFN-γ, specific production was detected for IP-10, MIG, VEGF, and to a lesser extent for TNF-α, which were also specific for ML1419c p113-121 (Table II), indicating the proinflammatory nature of the response against ML1419c. VEGF has recently been found to have potential to differentiate between *M. tuberculosis* infection states, as levels of VEGF in combination with epidermal growth factor, TGF-α, and sCD40L levels were higher in TB patients (40). MIP (CXCL9) and IP-10 (CXCL10) are potent chemoattractants for monocytes; both are induced by IFN-γ and have potential as biomarkers for TB as well (41, 42). Multiplex biomarker signatures will probably be more informative as candidate signatures of vaccine-induced immunological protection.

The striking observation that the 9mer ML1419c p113-121 unexpectedly appeared to induce efficient IgG Abs at high titers in sera of immunized mice indicated the multifunctionality of this *M. leprae* epitope: the IgG Abs specifically recognized both the nonamer peptide, the 30mer peptide, and the whole ML1419c protein. Classically, B cells and Abs are thought to offer no significant contribution toward protection against *M. tuberculosis* or other mycobacterial pathogens. However, emerging experimental evidence suggest that B cells play a role in many intracellular infections, probably by interacting with T cells, and thereby contributing to long-lived protection in vaccination settings (43). Our findings also suggest that B cells may play a more important role in antimycobacterial immunity than hitherto appreciated (44).

Thus, ML1419c p113-121 immunization induces specific $CD8^+$ T cells capable of providing B cell help for production of IgG. It is generally thought that only $CD4^+$ T cells provide help for B cells, and this unusual phenomenon has only been observed rarely (45). In contrast to the previously reported $CD8^+$ helper T cell clones that provided B cell help by secreting IL-4 (10), no IL-4 production by the ML1419c-specific $CD8^+$ T cells was detected in our study. Expression of CD40L in ML1419c-specific $CD8^+$ T cells was slightly increased compared with that in CpG-immunized mice (data not shown) indicating that CD40–CD40L interaction may activate B cells to produce Abs. Thus, these data provide a novel function of $CD8^+$ T cells by which they participate in antimycobacterial immunity.

p113-121–immunized or 30mer-immunized HLA-A2tg mice showed specific in vivo killing of p113-121–labeled, HLA-

A*0201$^+$ splenocytes, whereas no such lysis was observed in unimmunized mice or after immunization with an irrelevant HLA-*0201–binding peptide. Lysis was even detected in immunized mice after 9 mo (data not shown). Importantly, p113-121–specific HLA-A*0201$^+$ CD8$^+$ T cells directly and strongly inhibited mycobacterial growth using recombinant *M. smegmatis* expressing ML1419c. Immunization with both 9mer and 30mer peptides inhibited growth of *M. smegmatis* strains expressing ML1419c, whereas no such inhibition was observed using wild-type mycobacteria without the *M. leprae* protein. Analysis of leprosy lesions has revealed that CD8$^+$CD28$^+$ (T cytotoxic) cells are more prevalent in TT than in LL lesions (23), which is in line with our findings in Brazilian BT patients (21). Thus, it is possible that the CD8$^+$ T cells induced by ML1419c vaccination are able to kill *M. leprae*-infected cells in vivo and contribute to reducing the mycobacterial load in infected individuals. Analyzing the killing of *M. smegmatis* expressing *M. leprae* Ags like ML1419c may be a useful correlate of the efficacy of vaccines against *M. leprae.*

The data described in this study show that immunization with 9mer or 30mer peptides from *M. leprae*-specific ML1419c induced HLA-A*0201–restricted, multifunctional CD8$^+$ T cells that produce various Th1 and proinflammatory cytokines, have a strong cytolytic capability that is specific for the *M. leprae* Ag ML1419c, mediate B cell help for specific Ab production, and induce mycobacterial killing. The novel characteristics of these peptide-specific CD8$^+$ T cells may be exploited for the development of diagnostic tools as well as subunit vaccines to augment protection against leprosy.

In summary, these data show that immunization with 9mer or 30mer peptides from the *M. leprae*-specific protein ML1419c induces a strong CD8$^+$ T cell response with Th1, cytolytic, as well as B cell help-functional activity. These responses are directed to the *M. leprae* ML1419c peptide, ML1419c protein, and whole *M. leprae* and, importantly, are able to inhibit mycobacterial growth. Thus, ML1419c-specific CD8$^+$ T cells possess all key functions characteristic of a protective host response against *M. leprae.*

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Disclosures

The authors have no financial conflicts of interest.

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