# Identification of Epitopes of *Mycobacterium tuberculosis* 16-kDa Protein Recognized by Human Leukocyte Antigen–A*0201 $CD8^+$ T Lymphocytes

**Nadia Caccamo,[1] Salvatore Milano,[1] Caterina Di Sano,[1,2] Diego Cigna,[2] Juraj Ivanyi,[3] Alan M. Krensky,[4] Francesco Dieli,[1] and Alfredo Salerno[1,2]**

*[1]Department of Biopathology, University of Palermo, and [2]Institute of Advanced Diagnostic Methodologies, National Research Council, Palermo, Italy; [3]Department of Oral Medicine and Pathology, King's College London Medical and Dental School at Guy's Hospital, London, United Kingdom; [4]Department of Pediatrics, Stanford University School of Medicine, Stanford, California*

**$CD8^+$ T cells could make an important contribution to protection against tuberculosis (TB), but the antigenic determinants recognized in the context of major histocompatibility complex class I molecules remain ill defined. Our aim was to identify nonamer peptides derived from the *acr*/16-kDa antigen. Two immunogenic peptides (p21–29 and p120–128) were identified by their ability to elicit cytotoxic $CD8^+$ T cells from juvenile patients recovering from TB. Epitope-specific recognition was demonstrated by the lysis of both *Mycobacterium tuberculosis*–infected and peptide-pulsed macrophages, the release of cytotoxic granules, and interferon-$\gamma$ and tumor necrosis factor–$\alpha$ production. $CD8^+$ T cell responses to p21–29 and p120–128 were detected ex vivo in freshly isolated peripheral blood mononuclear cells from patients with TB but not in those from healthy control subjects. Our data suggest that these antigenic peptides can play a critical role in effective immunity against mycobacterial infection and TB.**

Tuberculosis (TB) is an infectious disease that affects millions of people worldwide. *Mycobacterium tuberculosis,* the primary etiologic agent of TB, causes ~8 million new cases each year, and the disease is responsible for at least 3 million deaths annually [1]. Many parameters—notably socioeconomic factors, coinfection with human immunodeficiency virus, the increasing incidence of antibiotic resistance, and genetic predisposition of the host—influence susceptibility to disease.

Several studies have demonstrated that cellular immunity mediated by interferon (IFN)–$\gamma$–secreting $CD4^+$ T cells plays a critical role in the protective immune response against *M. tuberculosis.* However, evidence exists that major histocompatibility complex (MHC) class I–restricted $CD8^+$ cytotoxic T lymphocytes (CTL) are involved in the immune response against *M. tuberculosis* [2–5]. These cells are able to secrete IFN-$\gamma$ and tumor necrosis factor (TNF)–$\alpha$ on recognition of mycobacterial antigens. Moreover, $CD8^+$ T cells can kill infected cells via a granule-dependent mechanism that involves perforin and granulysin, which also have direct antimicrobial activity [6].

Relatively few epitopes in mycobacterial antigens have so far been identified for human $CD8^+$ T cells. In the present study, we selected nonamer peptides derived from the 16-kDa protein of *M. tuberculosis,* on the basis of the MHC class I HLA-A*0201–binding motif. HLA-A*0201 frequency is 29% in the white population. The 16-kDa antigen was selected because this protein is predominantly expressed by *M. tuberculosis* during stationary growth or when the organism is subjected to oxygen deprivation, and it can account for as much as 25% of total bacillary protein expression under these circumstances. Because containment within the granuloma may induce similar conditions, the 16-kDa protein may be an important antigenic target during bacillary latency [7]. In addition, we have found that postchemotherapy changes in T cell proliferative and IFN-$\gamma$ responses in patients with TB are particularly striking in $CD4^+$ T cells that recognize the 16-kDa protein [8].

The present article reports the identification of two 16-kDa peptide epitopes recognized by $CD8^+$ T cells from individuals with HLA-A*0201. These $CD8^+$ T cells produce IFN-$\gamma$ and TNF-$\alpha$, express perforin and granulysin, and perform cytolytic functions directed against *M. tuberculosis*–infected macrophages.

## Subjects, Materials, and Methods

*Human subjects.* Peripheral blood was obtained from 8 children with HLA-A*0201 who had tuberculous infection (5 boys and 3 girls; age range, 6–12 years) from the Children's Hospital G. Di Cristina, Palermo, Italy. The subjects had pulmonary TB, as established by the presence of clinical symptoms of TB, positive results of tuberculin (purified protein derivative; PPD) skin testing, and signs visible on chest radiography. All patients included in this study had a positive PPD skin test. None of the children had been vaccinated in infancy with bacille Calmette-Guérin (BCG), had evidence of human immunodeficiency virus infection, or were being treated with steroid or antituberculosis drugs at the time of sam-

Received 21 March 2002; revised 22 May 2002; electronically published 13 September 2002.

Financial support: Italian National Research Council (to F.D.); Ministry for Education and Scientific and Technologic Research (60% to A.S. and F.D.).

Reprints or correspondence: Prof. Alfredo Salerno, Dept. of Biopathology, University of Palermo, Corso Tukory 211, 90134 Palermo, Italy (asalerno @unipa.it).

**The Journal of Infectious Diseases 2002; 186:991–8**

0022-1899/2002/18607-0014$15.00

pling. PPD skin tests were considered to be positive when the induration diameter was >5 mm at 72 h after injection of 1 U of PPD (Statens Seruminstitut).

Subjects underwent serologic HLA typing. Presence of the HLA subtype A*0201 was confirmed by a polymerase chain reaction amplification technique that used sequence-specific oligonucleotide primers.

*Synthetic peptides and cell lines.* A total of 5 nonamer peptides derived from the sequence of the 16-kDa protein of *M. tuberculosis* and containing HLA-A*0201–binding motifs [9] were prepared using solid-phase/Fmoc chemistry, as described in detail elsewhere [8]. The peptides were of 90% purity, and their homogeneity was confirmed by analytical reverse-phase high-performance liquid chromatography, mass spectroscopy, and amino acid composition analysis. The sequences of the peptides were SLFPEFSEL (p13–21), LFAAFPSFA (p21–29), EMKEGRYEV (p45–53), GAVEDDIKA (p107–115), and GILTVSVAV (p120–128).

Epstein-Barr virus–transformed homozygous lymphoblastoid B (EBV-B) cell lines were obtained from the Istituto Tumori Genova, Genoa, Italy; the HLA types of these cells are shown in table 1. The HLA-A2–positive, premyelomonocytic cell line THP-1 was also obtained from the Istituto Tumori Genova.

*Isolation of $CD8^+$ T cells.* PBMC were isolated from heparinized blood by centrifugation on Ficoll-Paque (Pharmacia). Cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated pooled human $AB^+$ serum, 2 m*M* L-glutamine, 20 m*M* HEPES, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, $5 \times 10^{-5}$ *M* 2-mercaptoethanol, and 150 IU/mL recombinant interleukin (IL)–2, hereafter referred to as "IL-2–supplemented culture medium" (IL-2/CM).

$CD8^+$ T lymphocytes were sorted by immunomagnetic beads (Myltenyi Biotec) using an anti-CD8 monoclonal antibody (MAb; clone MEM31), and $CD8^+$ T cells were then expanded in vitro in IL-2/CM supplemented with purified phytohemagglutinin (leucoagglutinin, 0.5 $\mu$g/mL; Sigma) and irradiated (30 Gy from a cesium source) allogeneic feeder cells (peripheral blood lymphocytes and EBV-B cells at a ratio of 10:1) as described above. Cells were maintained for 3–4 weeks without restimulation before functional analysis was done. The purity of the $CD8^+$ T cells was assessed by 2-color flow cytometry. Phycoerythrin-conjugated anti-CD3 antibody was used with fluorescein isothiocyanate (FITC)–conjugated anti-CD8 (all from Becton Dickinson). The cells were analyzed by FACScan flow cytometer (Becton Dickinson). This T cell expansion procedure, in which phytohemagglutinin and allogeneic stimulation are used, induces expansion of virtually all $CD8^+$ T cells and does not introduce any bias in the T cell repertoire [10].

*Generation of CTL lines from healthy HLA-A*0201–positive donors by in vitro immunization.* The method used for in vitro CTL induction followed the protocol of Plebanski et al. [11], which provides, after 4 cycles of stimulation, >85% peptide-specific CTL. In brief, peripheral blood mononuclear cells (PBMC) from HLA-A*0201–positive donors were pulsed with 50 $\mu$g/mL peptides at $3 \times 10^7$ cells in RPMI medium at 37°C for 90 min. These cells were washed and cultured at $3 \times 10^6$ cells/well in complete medium. Every 3 days, the culture was refreshed by complete medium plus human recombinant IL-2 (40 U/mL). Cultures were restimulated weekly with peptide-pulsed irradiated autologous cells as feeders, as described elsewhere [10]. After 4–5 cycles of restimulation, $CD4^+$ T cells were depleted by immunomagnetic sorting, and the remaining enriched $CD8^+$ T cells (80%–90%) were restimulated weekly as described earlier.

**Table 1.** Levels of TNF-$\alpha$ production by $CD8^+$ T cell lines in response to stimulation with peptides p21–29 and p120–128 in the presence of EBV-B cell lines of the indicated HLA-A type.

| EBV-B line (HLA-A type), peptide | TNF-$\alpha$ level, pg/mL |
|---|---|
| KAS011 (*0101) | |
| None | 70 |
| p21–29 | 50 |
| p120–128 | 65 |
| BTB (*0201) | |
| None | 35 |
| p21–29 | 780 |
| p120–128 | 950 |
| WT100BIS (*1101) | |
| None | 25 |
| p21–29 | 50 |
| p120–128 | 40 |
| WT51 (*2301) | |
| None | 20 |
| p21–29 | 30 |
| p120–128 | 25 |
| LKT3 KT3 (*2402) | |
| None | 55 |
| p21–29 | 45 |
| p120–128 | 65 |
| DO208915W (*2501) | |
| None | 30 |
| p21–29 | 30 |
| p120–128 | 30 |
| PITOUT (*2902) | |
| None | 60 |
| p21–29 | 45 |
| p120–128 | 70 |
| SPACH SPL (*3101) | |
| None | 40 |
| p21–29 | 55 |
| p120–128 | 20 |
| HOR (*3303) | |
| None | 20 |
| p21–29 | 40 |
| p120–128 | 15 |

NOTE. EBV-B, Epstein-Barr virus–transformed homozygous lymphoblastoid B; TNF, tumor necrosis factor.

*In vitro stimulation of peptide-specific $CD8^+$ T cells.* $CD8^+$ T cells ($5 \times 10^4$) were incubated with autologous or allogeneic cells in the presence of synthetic peptides at different concentrations. After 6 h, the supernatants were collected and stored at −70°C until testing. IFN-$\gamma$ and TNF-$\alpha$ levels were assessed by a 2-MAb sandwich ELISA kit (Euroclone).

*Intracellular fluorescence-activated cell cytometry (FACS) analysis.* $CD8^+$ T cells or fresh PBMC were stimulated with peptides, as described earlier, in the presence of monensin for 6 h at 37°C in 5% $CO_2$. The cells were harvested, washed, and stained with quantum red (QR)–conjugated anti-CD8 MAb (Sigma) in incubation buffer (PBS–1% fetal calf serum [FCS]–0.1% sodium azide) for 30 min at 4°C. The cells were washed twice in PBS–1% FCS and fixed with PBS–4% paraformaldehyde overnight at 4°C. Fix-

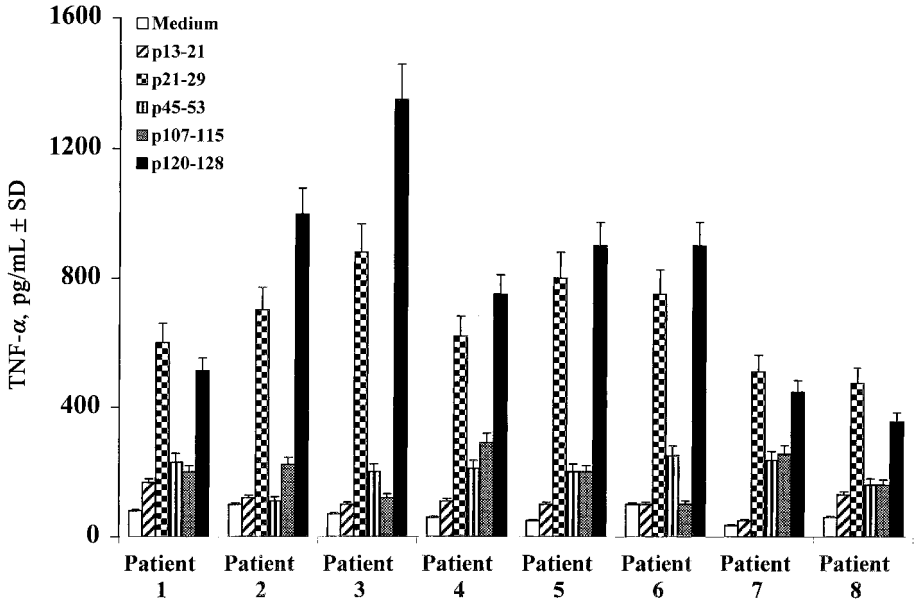


**Figure 1.** Identification of immunogenic peptides from the 16-kDa protein recognized by $CD8^+$ T cells from patients with tuberculosis. Polyclonal $CD8^+$ T cell lines from HLA-A*0201–positive patients were stimulated with the indicated peptides in the presence of autologous peripheral blood mononuclear cells. Supernatants were collected, and tumor necrosis factor (TNF)–$\alpha$ production was measured.

ation was followed by permeabilization with PBS–1% FCS–0.3% saponin–0.1% sodium azide for 15 min at 4°C. Staining of intracellular cytokine was performed by incubation of fixed permeabilized cells with FITC-labeled anti–IFN-$\gamma$ antibody. Viable lymphocytes were gated by forward and side scatter, and analysis was performed on 100,000 acquired events for each sample.

For detection of granulysin and perforin, $CD8^+$ T cells were stimulated with peptides and autologous cells for 3 h. The cells were washed and fixed with PBS–4% paraformaldehyde for 30 min at 4°C. After 2 washes with permeabilization buffer (PBS–1% FCS–0.3% saponin–0.1% sodium azide), the cells were incubated with rabbit anti-perforin MAb ($\delta$G9 [Alexis]; final concentration, 2 $\mu$g/mL), mouse anti-granulysin MAb (DH-4; final concentration, 2 $\mu$g/mL), or an isotype-matched control MAb of irrelevant specificity for 1 h on ice in PBS–1% FCS. After 2 washes in PBS–1% FCS, cells were stained with QR-conjugated anti–human CD8, phycoerythrin-conjugated anti–rabbit IgG, and FITC-conjugated anti–mouse IgG. All the antibodies were purchased from Sigma. After 2 more washes in PBS–1% FCS, the cells were analyzed by FACS [12].

*Cytotoxicity assay.* THP-1 target cells ($4 \times 10^3$ cells/well) were incubated with phorbol 12-myristate 13-acetate (Sigma) at a final concentration of 10 ng/mL in 96-well round-bottom plates for 24 h at 37°C in the presence of 5% $CO_2$. Nonadherent cells were removed, and the macrophages were infected with an avirulent *M. tuberculosis* strain, H37Ra, or BCG (ratio of colony-forming units to macrophages, 10:1) for 24 h or pulsed with peptides for 6 h at 37°C in 5% $CO_2$. The macrophages were washed with RPMI 1640 medium, and CTL effector cells were added at different ratio and incubated for 5 h at 37°C in 5% $CO_2$. Cytotoxicity was analyzed using a nonradioactive colorimetric cytotoxicity assay (CytoTox 96; Promega).

## Results

*Identification of HLA-A*0201 peptides from* M. tuberculosis *16-kDa protein.* Because HLA-A*0201 is one of the most prominent MHC class I HLA alleles, we selected potential candidate peptides within the sequences of the 16-kDa protein for the presence of allele-specific peptide motifs for the HLA-A*0201 molecules. This analysis was carried out using the SYFPEITHI database for MHC ligand and peptide motifs [9]. From this analysis, a total of 50 sequences of 9 aa, with HLA-A*0201–binding scores ranging from 5 to 27, were found to contain the binding motifs for the HLA-A*0201 molecule. Five peptides were synthesized that corresponded to the candidate sequences and had a high probability, on the basis of score (<15), of being presented by the HLA-A*0201 molecule. These peptides were used in subsequent studies.

We analyzed the response to 16-kDa–derived peptides of $CD8^+$ T cells from 8 HLA-A*0201–positive patients with TB. For each patient, $CD8^+$ T cell lines were generated at the time of diagnosis, and the peptide recognition pattern was analyzed by measuring the amounts of TNF-$\alpha$ and IFN-$\gamma$ produced after stimulation with peptides and autologous PBMC. Figure 1 shows that responses to peptides p21–29 and p120–128 were detected in $CD8^+$ T cells from 8 of 8 patients with TB, whereas very low TNF-$\alpha$ production was observed after stimulation with peptides p13–21, p45–53, and p107–115. Similar data were obtained for IFN-$\gamma$ production (data not shown). The $CD8^+$ T cell response to peptides p21–29 and p120–128 was both

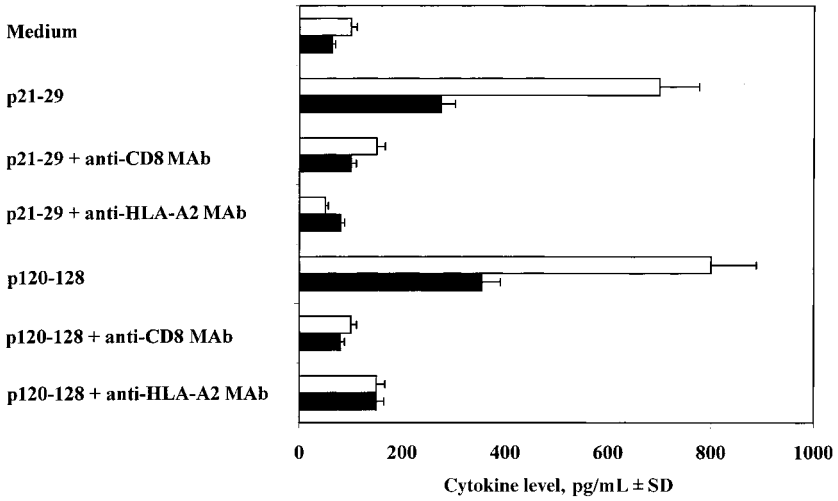


**Figure 2.** Production of tumor necrosis factor (TNF)–$\alpha$ and interferon (IFN)–$\gamma$ in response to stimulation with mycobacterial 16-kDa peptides. Polyclonal $CD8^+$ T cell lines were incubated with peptides p21–29 or p120–128 and autologous peripheral blood mononuclear cells in the presence of monoclonal antibodies (MAbs) to CD8 or HLA-A2. Supernatants were collected, and TNF-$\alpha$ *(black bars)* and IFN-$\gamma$ *(white bars)* levels were measured. Recognition of p21–29 and p120–128 peptides by $CD8^+$ T cell lines is HLA-A*0201 restricted.

CD8- and HLA-A*0201–mediated, as demonstrated by inhibition of TNF-$\alpha$ and IFN-$\gamma$ production in the presence of MAbs to CD8 and HLA-A2 (figure 2). Moreover, TNF-$\alpha$ production was only detected when $CD8^+$ T cells were stimulated with peptides and HLA-A*0201–positive EBV-B cell lines; it was not seen in the presence of 8 HLA-A*0201–negative cells and relevant peptides or EBV-B cells alone (i.e., without the addition of peptides to cultures; table 1). In addition, we consistently failed to detect any TNF-$\alpha$ and IFN-$\gamma$ production by polyclonal $CD8^+$ T cell lines from 5 healthy (negative results of PPD testing), HLA-A*0201–positive donors after stimulation with either p21–29 or p120–128 peptides and EBV-B cell lines (data not shown).

*Cytotoxic activity of mycobacterial 16-kDa peptide–specific $CD8^+$ T cells.* To assess the role of $CD8^+$ T cells as CTL, the HLA-A*0201–positive THP-1 cell line was pulsed with p21–29 or p120–128 peptides or infected with the avirulent *M. tuberculosis* strain H37Ra or BCG and used as a target for $CD8^+$ T cells. Results with 3 representative $CD8^+$ T cell lines from 3 different patients are shown in figure 3; similar results were obtained with $CD8^+$ T cell lines from all subjects with TB.

Significant lysis of THP-1 cells pulsed with p21–29 or p120–

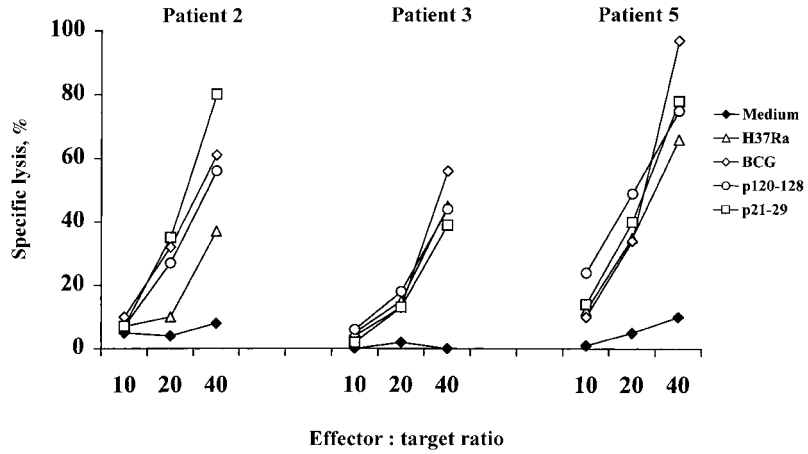


**Figure 3.** Cytotoxic activity of human HLA-A*0201 T cells. Polyclonal $CD8^+$ T cell lines from 3 different patients were cocultured with THP-1 cells pulsed with p21–29 or p120–128 peptides or infected with bacille Calmette-Guérin (BCG) or the avirulent *Mycobacterium tuberculosis* strain H37Ra, at the indicated ratio of effector to target cells. The percentage of specific lysis was calculated as described in Subjects, Materials, and Methods.

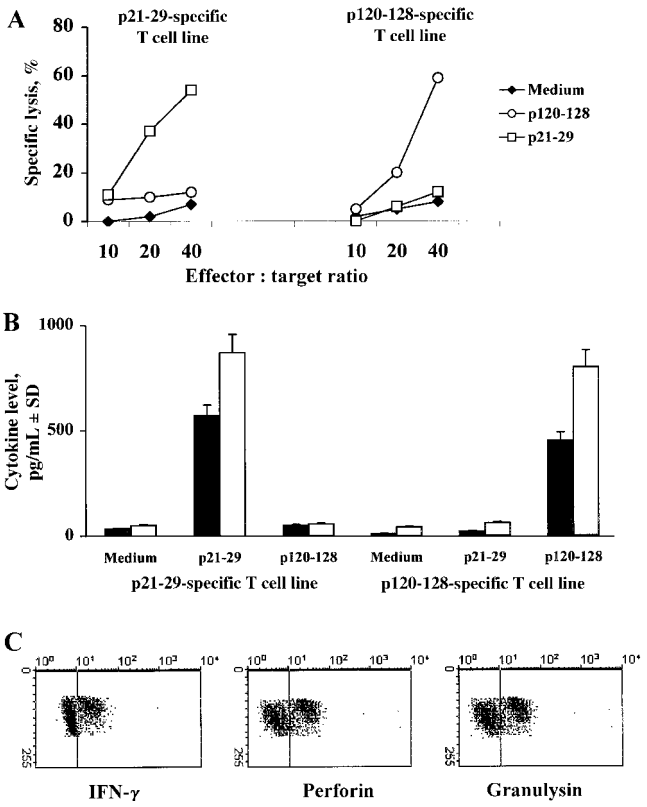


**Figure 4.** Functional activity of peptide-specific CD8+ T cell lines. *A,* Cytotoxic activity of p21–29- and p120–128-specific CD8+ T cell lines toward THP-1 cells pulsed with peptides. *B,* Production of tumor necrosis factor–α *(black bars)* and interferon (IFN)–γ *(white bars)* by p21–29- and p120–128-specific CD8+ T cells after stimulation with peptide-pulsed THP-1 cells. *C,* Intracellular staining of a p21–29-specific T cell line for IFN-γ, perforin, and granulysin.

128 peptides was observed. Similarly, specific lysis was seen in THP-1 target cells infected with BCG or H37Ra (figure 3), whereas CD8+ T cells failed to lyse either unpulsed or uninfected THP-1 cells. These results provide evidence that CD8+ T cells are able to recognize peptides from mycobacterial antigens presented by monocyte-derived macrophages and to mediate cytolytic activity.

*Characterization of CD8+ CTL specific for mycobacterial 16-kDa peptides.* To verify that CD8+ T cell responses against p21–29 or p120–128 epitopes can also be induced in healthy subjects, CD8+ T cell lines were obtained in vitro: PBMC from uninfected HLA-A*0201–positive donors were cultured and stimulated with autologous PBMC pulsed with either p21–29 or p120–128 peptides for 4–5 cycles, as described in Subjects, Materials, and Methods. Both T cell lines were able to kill, in an antigen-specific fashion, peptide-pulsed THP-1 cells (figure 4*A*). On stimulation with a specific peptide, CD8+ T cell lines produced TNF-α and IFN-γ (figure 4*B*). CD8+ CTL specific for p21–29 and p120–128 peptides were further analyzed for the presence of intracellular IFN-γ, perforin, and granulysin. As shown in figure 4*C,* after stimulation with peptide p21–29, >40% of CD8+ T cells contained IFN-γ and stained positive for perforin and granulysin. These results indicate that CD8+ CTL lines have cytolytic potential in addition to their cytokine-producing function.

*Endogenous generation of p21–29 and p120–128 epitopes during mycobacterial infection.* To demonstrate that p21–29 and p120–128 are endogenously generated epitopes, we infected THP-1 cells with BCG and measured recognition by peptide-specific CD8+ T cell lines. Two T cell lines specific for either p21–29 or p120–128 peptides promptly secreted IFN-γ and TNF-α in response to BCG-infected cells (figure 5*A*), and the amount of IFN-γ induced by BCG-infected targets was similar to that induced by activation with the corresponding peptide (see, for comparison, figures 4*B* and 5*A*).

The ability of p21–29- and p120–128-specific CD8+ T cell lines to lyse infected target cells was also investigated. Figure 5*B* shows that CD8+ T cell lines specific for p21–29 and p120–128 peptides efficiently kill BCG-infected THP-1 target cells but have no cytotoxic activity toward uninfected targets. These results indicate that CD8+ T cells recognizing peptides from the 16-kDa protein can efficiently recognize and lyse *M. tuberculosis*–infected target cells.

*Analysis of IFN-γ release by freshly isolated CD8+ T cells in response to mycobacterial 16-kDa peptides.* To answer the question of whether CD8+ T cell responses are normally elicited during the course of *M. tuberculosis* infection in vivo and are not the results of artifacts arising from polyclonal or peptide-specific expansion during in vitro cultures, peptides p21–29 and p120–128 were used to assess the level of IFN-γ release in CD8+ T cells, using intracellular FACS analysis in fresh PBMC from healthy HLA-A*0201–positive donors or HLA-A*0201–positive subjects with TB.

As shown in figure 6, we were able to detect a statistically significantly higher response in all 8 patients with TB than in healthy control subjects. Moreover, the frequency of IFN-γ–producing CD8+ T cells in response to p120–128 was higher than the frequency of IFN-γ–producing CD8+ T cells in response to p21–29. These results, obtained using intracellular FACS analysis, clearly demonstrate that the observed CD8+ T cell response to 16-kDa–derived peptides is elicited during the course of *M. tuberculosis* infection in vivo.

## Discussion

Cellular immunity, which involves the interaction between activated infected macrophages and antigen-specific CD4+ T cells, is known to be the major protective response of the human body against infection. This model of defense has long been established as essential for mounting a strong protective immune response against infection with *M. tuberculosis* [1]. However, it has been suggested, on the basis of observations of experimental TB in mice, that MHC class I–mediated CD8+

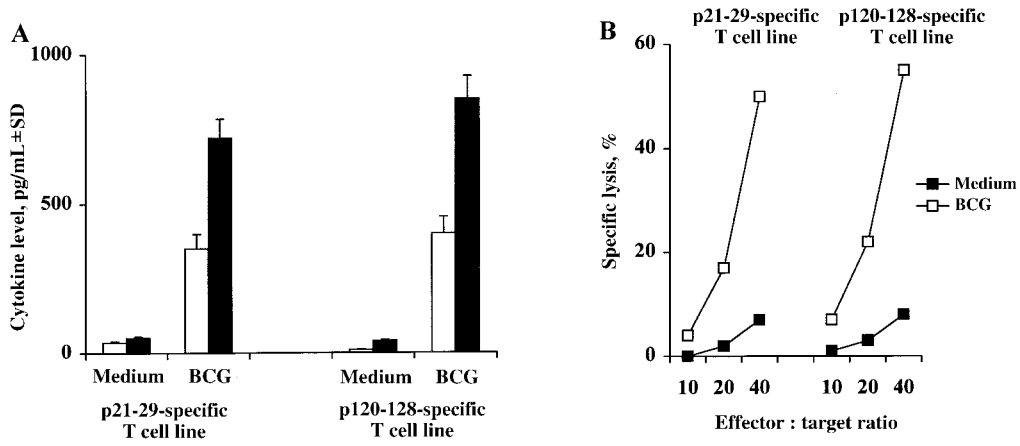


**Figure 5.** Recognition of bacille Calmette-Guérin (BCG)–infected macrophages by peptide-specific $CD8^+$ T cells. $CD8^+$ T cell lines specific for p21–29 and p120–128 were coincubated with BCG-infected THP-1 cells. *A,* Supernatants were collected and assayed for tumor necrosis factor–$\alpha$ *(black bars)* and interferon-$\gamma$ *(white bars). B,* Specific lysis of BCG-infected THP-1 cells is shown.

cytotoxic T cells have a protective role, because mice lacking functional $CD8^+$ T cells were more susceptible to mycobacterial infection [13–15].

Human studies have provided evidence that MHC class I–restricted $CD8^+$ T cells recognize *M. tuberculosis*–infected cells and can release IFN-$\gamma$, lyse infected target cells, and kill intracellular bacteria [16–18]. For these reasons, there has been an intense effort to define *M. tuberculosis* epitopes that can be presented by MHC class I molecules to $CD8^+$ T cells [19–21]. In the present study, we analyzed the 16-kDa protein of *M. tuberculosis* to identify HLA-A*0201–binding peptides with the capability of activating $CD8^+$ T cells. We identified 2 new epitopes (p21–29 and p120–128) from the sequence of the 16-kDa protein that are recognized by $CD8^+$ T cells from HLA-A*0201–positive patients with TB.

$CD8^+$ T cell responses against these 2 epitopes can also be induced in healthy (uninfected) HLA-A*0201–positive subjects by prolonged stimulation with peptides in vitro, which indicates that both p21–29 and p120–128 can sensitize naive $CD8^+$ T cells and, as a consequence, enable the generation of peptide-specific CTL lines in vitro. Most notably, these peptide-reactive $CD8^+$ T cells killed not only peptide-pulsed target cells but also *M. tuberculosis*– or BCG-infected HLA-A*0201–positive human macrophages [22]. This suggests that p21–29 and p120–128 epitopes gain entry into mycobacteria-infected host cell cytoplasm, allowing processing and presentation via the MHC class

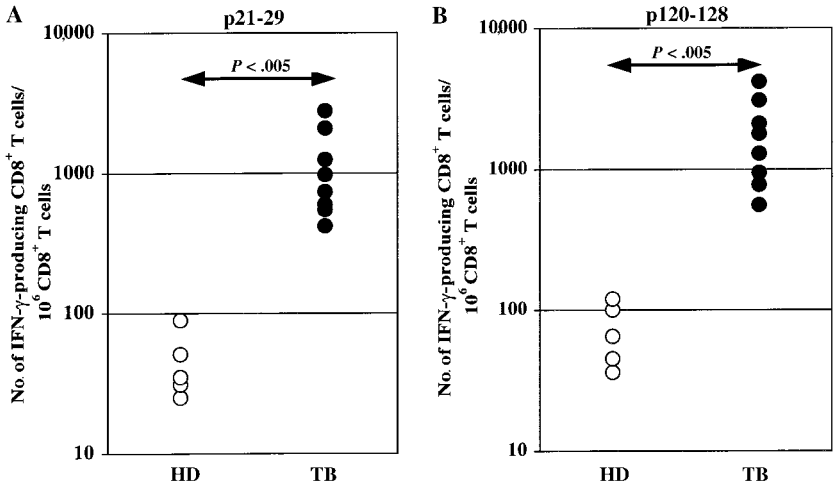


**Figure 6.** Interferon (IFN)–$\gamma$–producing $CD8^+$ T cells in fresh peripheral blood mononuclear cells stimulated with p21–29 and p120–128 peptides. The frequency of $CD8^+$ T cells producing IFN-$\gamma$ in response to in vitro stimulation with peptides p21–29 and p120–128 was determined by combined intracellular and membrane fluorescence-activated cell cytometric staining, as described in Subjects, Materials, and Methods. Results show the absolute no. of IFN-$\gamma$–producing $CD8^+$ T cells/$10^6$ $CD8^+$ T cells in individual healthy donors (HD) and patients with tuberculosis (TB).

I pathway. Of interest, immunization of mice with a peptide spanning aa 24–31 of the 16-kDa protein (thus partially overlapping p21–29) induces peptide-specific CD8$^+$ T cells with cytotoxic activity toward peptide-pulsed targets [23].

Finally, using an intracellular assay for assessing IFN-$\gamma$ production to measure peptide-specific CD8$^+$ T cell frequencies, we demonstrated that CD8$^+$ T cell responses to p21–29 and p120–128 are detected ex vivo in freshly isolated PBMC from patients with TB but not in those from healthy control subjects, which indicates that the CD8$^+$ T cell response to 16-kDa–derived peptides is elicited during the course of *M. tuberculosis* infection in vivo [24].

There are at least 3 possible pathways by which these CD8$^+$ T cells can play a critical role in the immune response against *M. tuberculosis* infection: the release of IFN-$\gamma$, lysis of the infected target, and direct antimicrobial activity [14, 19]. In our study, *M. tuberculosis* peptide–reactive CD8$^+$ T cell lines release IFN-$\gamma$ and TNF-$\alpha$ on recognition of peptide-pulsed cells, providing a mechanism by which this T cell subset might contribute to immunity against *M. tuberculosis* infection. In fact, IFN-$\gamma$ and TNF-$\alpha$ synergize for the induction of inducible nitric oxide synthase and production of nitric oxide from macrophages, which, in turn, exerts cytocidal effects on intracellular bacteria such as *M. tuberculosis.*

The second mechanism by which MHC class I–restricted T cells can contribute to host defense against *M. tuberculosis* infection is their ability to lyse infected target cells [22, 23, 25, 26]. CD8$^+$ T cells specific for the p21–29 and p120–128 peptides of the 16-kDa protein lysed TB-infected targets of the monocyte/macrophage lineage. The continued lysis of infected cells could lead to release of bacteria from this safe intracellular harbor so that they can be taken up at a low multiplicity by freshly activated macrophages and destroyed [27].

Ultimately, CD8$^+$ T cells can mediate antimicrobial activity by the release of perforin and granulysin from cytotoxic granules that can directly kill the intracellular microbial pathogen [5]. In our study, the presence of both perforin and granulysin in these CD8$^+$ T cells was demonstrated by intracellular staining. Granulysin, which is homologous to the saposin-like protein family of lipid-binding proteins, exerts its cytolytic function and antibacterial activity via interaction with lipids that induce lesions on the surface of *M. tuberculosis* with a direct action on the mycobacterial glycolipid envelope [28, 29]. Perforin colocalizes in the cytotoxic granules of CD8$^+$ T cells and is released, together with granulysin, when antigen activation occurs. Perforin forms pores in the membranes of infected cells and shows no antibacterial activity when isolated, whereas the combination of both molecules resulted in a dramatic decrease in the viability of intracellular mycobacteria [5].

In conclusion, we have demonstrated that human MHC class I–restricted CD8$^+$ T cells specific for epitopes of the 16-kDa antigen from *M. tuberculosis* play a critical role in the immunologic response against mycobacterial infections. The presence of peptide-specific CD8$^+$ T cells in patients with TB suggests that these epitopes are presented during the natural course of infection [19]. Our data have demonstrated that these cells can also be induced in healthy HLA-A*0201–positive subjects, which suggests that these epitopes may be useful for immunoprophylaxis against TB infection.

**Acknowledgment**

We thank Vaclav Horejsi, Institute of Molecular Genetics, Academy of Science of the Czech Republic, Prague, for providing the monoclonal antibody MEM31.

**References**

1. Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol **2001**; 19: 93–129.
2. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I–restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci USA **1992**; 89:12013–17.
3. Liebana E, Girvin RM, Welsh M, Neill SD, Pollock JM. Generation of CD8(+) T-cell responses to *Mycobacterium bovis* and mycobacterial antigen in experimental bovine tuberculosis. Infect Immun **1999**; 67:1034–44.
4. Koga T, Wand-Wurttenberger A, DeBruyn J, Munk ME, Schoel B, Kaufmann SH. T cells against a bacterial heat shock protein recognize stressed macrophages. Science **1989**; 245:1112–5.
5. Stenger S, Mazzaccaro RJ, Uyemura K, et al. Differential effects of cytolytic T cell subsets on intracellular infection. Science **1997**; 276:1684–7.
6. Stenger S, Hanson DA, Teitelbaum R, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science **1998**; 282:121–5.
7. Agrewala JN, Wilkinson RJ. Differential regulation of Th1 and Th2 cells by p91–110 and p21–40 peptides of the 16-kDa $\alpha$-crystallin antigen of *Mycobacterium tuberculosis.* Clin Exp Immunol **1998**; 114:392–7.
8. Dieli F, Singh M, Spallek R, et al. Change of Th0 to Th1 cell-cytokine profile following tuberculosis chemotherapy. Scand J Immunol **2000**; 52:96–102.
9. Ramensee HG, Bachmann J, Emmerich NPN, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics **1999**; 50:213–9.
10. Saulquin X, Ibisch C, Peyrat MA, et al. A global appraisal of immunodominant CD8 T cell responses to Epstein-Barr virus and cytomegalovirus by bulk screening. Eur J Immunol **2000**; 30:2531–9.
11. Plebanski M, Allsopp CE, Aidoo M, Reyburn H, Hill AV. Induction of peptide-specific primary cytotoxic T lymphocyte responses from human peripheral blood. Eur J Immunol **1995**; 25:1783–7.
12. Dieli F, Troye-Blomberg M, Ivanyi J, et al. Granulysin-dependent killing of intracellular and extracellular *Mycobacterium tuberculosis* by V$\gamma$9/V$\delta$2 T lymphocytes. J Infect Dis **2001**; 184:1082–5.
13. Kaufmann SHE. Immunity to intracellular bacteria. Annu Rev Immun **1993**; 11:129–63.
14. Ladel CH, Daugelat S, Kaufmann SHE. Immune response to *Mycobacterium bovis* bacille Calmette Guerin infection in major histocompatibility complex class I– and II–deficient knock-out mice: contribution of CD4 and CD8 T cells to acquired resistance. Eur J Immunol **1995**; 25:377–84.
15. Kaufmann SHE. CD8$^+$ T lymphocytes in intracellular microbial infections. Immunol Today **1988**; 9:168–74.
16. Canaday DH, Ziebold C, Noss EH, Chervenak KA, Harding CV, Boom WH. Activation of human CD8$^+$ $\alpha\beta$ TCR$^+$ cells by *Mycobacterium tuberculosis* via an alternate class I MHC antigen–processing pathway. J Immunol **1999**; 162:372–9.
17. Lalvani A, Brookes R, Wilkinson RJ, et al. Human catalytic and interferon-

γ–secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis.* Proc Natl Acad Sci USA **1998**;95:270–5.

18. Tan JS, Canaday DH, Boom WH, Balaji KN, Schwander SK, Rich EA. Human alveolar T lymphocyte responses to *Mycobacterium tuberculosis* antigens: role for $CD4^+$ and $CD8^+$ cytotoxic T cells and relative resistance of alveolar macrophages to lysis. J Immunol **1997**;159:290–7.
19. Cho S, Mehra V, Thoma-Uszynski S, et al. Antimicrobial activity of MHC class I–restricted $CD8^+$ T cells in human tuberculosis. Proc Natl Acad Sci USA **2000**;97:12210–5.
20. Geluk A, Van Meijgaarden KA, Franken KLMC, et al. Identification of major epitopes of *Mycobacterium tuberculosis* AG85B that are recognized by HLA-A*0201–restricted $CD8^+$ T cells in HLA-transgenic mice and humans. J Immunol **2000**;165:6463–71.
21. Klein MR, Smith SM, Hammond AS, et al. HLA-B*35–restricted CD8 T cell epitopes in the antigen 85 complex of *Mycobacterium tuberculosis.* J Infect Dis **2001**;183:928–34.
22. Pathan AA, Wilkinson KA, Wilkinson RJ, et al. High frequencies of circulating IFN-γ–secreting CD8 cytotoxic T cells specific for a novel MHC class I–restricted *Mycobacterium tuberculosis* epitope in *M. tuberculosis*–infected subjects without disease. Eur J Immunol **2000**;30:2713–21.
23. Vordermeier HM, Zhu X, Harris DP. Induction of $CD8^+$ CTL recognizing mycobacterial peptides. Scand J Immunol **1997**;45:521–6.
24. Betts MR, Casazza JP, Koup RA. Monitoring HIV-specific $CD8^+$ T cell response by intracellular cytokine production. Immunol Lett **2001**;79:117–25.
25. Da Fonseca DP, Joosten D, Van Der Zee R, et al. Identification of new cytotoxic T-cell epitopes on the 38-kilodalton lipoglycoprotein of *Mycobacterium tuberculosis* by using lipopeptides. Infect Immun **1998**;66:3190–7.
26. Lalvani A, Hill AVS. Cytotoxic T-lymphocytes against malaria and tuberculosis: from natural immunity to vaccine design. Clin Sci **1998**;95:531–8.
27. De Libero G, Flesh I, Kaufmann SHE. Mycobacteria-reactive $Lyt\text{-}2^+$ T cell lines. Eur J Immunol **1988**;18:59–66.
28. Stenger S, Rosat JP, Bloom BR, Krensky AM, Modlin RL. Granulysin: a lethal weapon of cytolytic T cells. Immunol Today **1999**;20:390–4.
29. Ernst WA, Thoma-Uszynski S, Teitelbaum R, et al. Granulysin, a T cell product, kills bacteria by altering membrane permeability. J Immunol **2000**;165:7102–8.