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CD8$^+$-T-Cell Responses of Mycobacterium-Infected Mice to a Newly Identified Major Histocompatibility Complex Class I-Restricted Epitope Shared by Proteins of the ESAT-6 Family

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Here we describe the identification of a new CD8$^+$-T-cell epitope, the GYAGTLQSL nonamer, shared by the TB10.3 and TB10.4 proteins of the *Mycobacterium tuberculosis* ESAT-6 family. Cytotoxic T cells from mycobacterium-infected mice efficiently recognized this epitope. GYAGTLQSL-specific T-cell hybridomas, which were able to recognize *Mycobacterium bovis* BCG-infected macrophages, were generated and now allow investigation of mycobacterial-antigen processing through the major histocompatibility complex class I pathway.

The contribution of CD8$^+$ T cells to the control of infection with *Mycobacterium tuberculosis* is supported by a marked emergence of gamma interferon-producing CD8$^+$ T lymphocytes in the lungs of infected mice (5, 9, 10) and by the increased susceptibility of mice deficient in genes involved in the major histocompatibility complex class I (MHC-I) presentation pathway to this infection (13). However, the specificity of antimycobacterial CD8$^+$ T cells and the mechanisms of mycobacterial-antigen presentation by MHC-I molecules are poorly understood. Only a few MHC-I-restricted epitopes, i.e., 38-kDa: 225-234 (17), MPT64:190-198 (5), and antigen 85A (Ag85A):144-152 (3) were shown to be recognized by antimycobacterial CD8$^+$ T cells. The MHC-I presentation of mycobacterial antigens may thus be relatively inefficient, and CD8$^+$-T-cell responses to this infection may be induced by substantial expansion of clones with limited specificities (5). Therefore, the identification of MHC-I-restricted mycobacterial epitopes and generation of tools able to detect their presentation in the MHC-I context can be of considerable interest for the investigation of CD8$^+$-T-cell immunity during *M. tuberculosis* infection.

We applied the SYFPEITHI program (http://syfpeithi.bmi-heidelberg.com/) to 400 open reading frames (ORFs) overlapping the first 10% (Rv0001 to Rv0400) of the *M. tuberculosis* H37Rv genome (http://genolist.pasteur.fr/TubercuList/) (2). The scan of the first 10% of the genome was performed to obtain a reasonable number of peptides to be synthetized and tested by cytotoxic-T-lymphocyte (CTL) assay. One hundred fifty nonamers which possess consensus H-2K^d-binding motifs were selected. Based on the computed scores of known H-2K^d-restricted peptides, out of these 150 nanomers, only 84 with SYFPEITHI scores of ≥23 were considered potentially immunogenic. Moreover, since hydrophobic epitopes may reach an alternative MHC-I pathway (6), we selected only hydrophilous H37Rv peptides (11 out of the 84) (Table 1). As a positive peptide control, we used a previously described H-2K^d epitope, Rv3804c (Ag85A):144-152 (3).

The selected H37Rv-derived peptides were synthesized by Pepscan Systems (Lelystad, The Netherlands) or Neosystem (Strasbourg, France) and monitored for their capacity to generate CTL responses. BALB/c (H-2^d) mice (Iffa Crédo, L'Arbresle, France) received two subcutaneous (s.c.) injections of 50 μg of individual peptides emulsified in incomplete Freund adjuvant at a 2-week interval. Eight days following the last injection, their lymph node cells were stimulated in vitro for 5 days with the homologous peptides and their CTL activity was measured as previously described (4). The Rv0140:79-87 peptide generated only a weak CTL response, while the GYAGTLQSL epitope, shared by the Rv0288 (TB10.4) and Rv3019c (TB10.3) proteins (referred to as TB10.3/4:20-28) and

TABLE 1. *M. tuberculosis* (H37Rv)-derived H-2K^d epitopes predicted by use of the SYFPEITHI algorithm

H37Rv proteins[a]	Putative H-2K^d-binding amino acid sequence[b]	Epitope positions	Computed score for H-2K^d
Membrane proteins (no. of transmembrane domains)			
Rv0051 (9)	IYRPGRDLV	487–495	23
Rv0111 (10)	PYRYIGQTL	509–517	23
Rv0194 (9)	LYTPIGELA	894–902	23
Rv0219 (4)	SYGSGPLHL	11–19	25
Secreted proteins			
Rv0288 (TB10.4), Rv3019c (TB10.3)	GYAGTLQSL	20–28, 20–28	27
Rv3804c (Ag85A)	VYAGAMSGL[c]	144–152	25
Proteins with unknown localizations			
Rv0065	RYPHSPRLI	76–84	24
Rv0127	WYAGRNREL	22–30	24
Rv0140	SYYSVTTDA	79–87	27
Rv0161	RYRGRASAL	31–39	26
Rv0164	RYASRMPVL	11–19	24
Rv0393, Rv0397	TYVTTPGSA	350–358, 27–25	25

[a] Predicted proteins of *M. tuberculosis* H37Rv (http://genolist.pasteur.fr/TubercuList/).
[b] Underlined letters represent H-2K^d consensus binding motifs.
[c] A previously described Ag85A-derived epitope restricted by H-2K^d (3).

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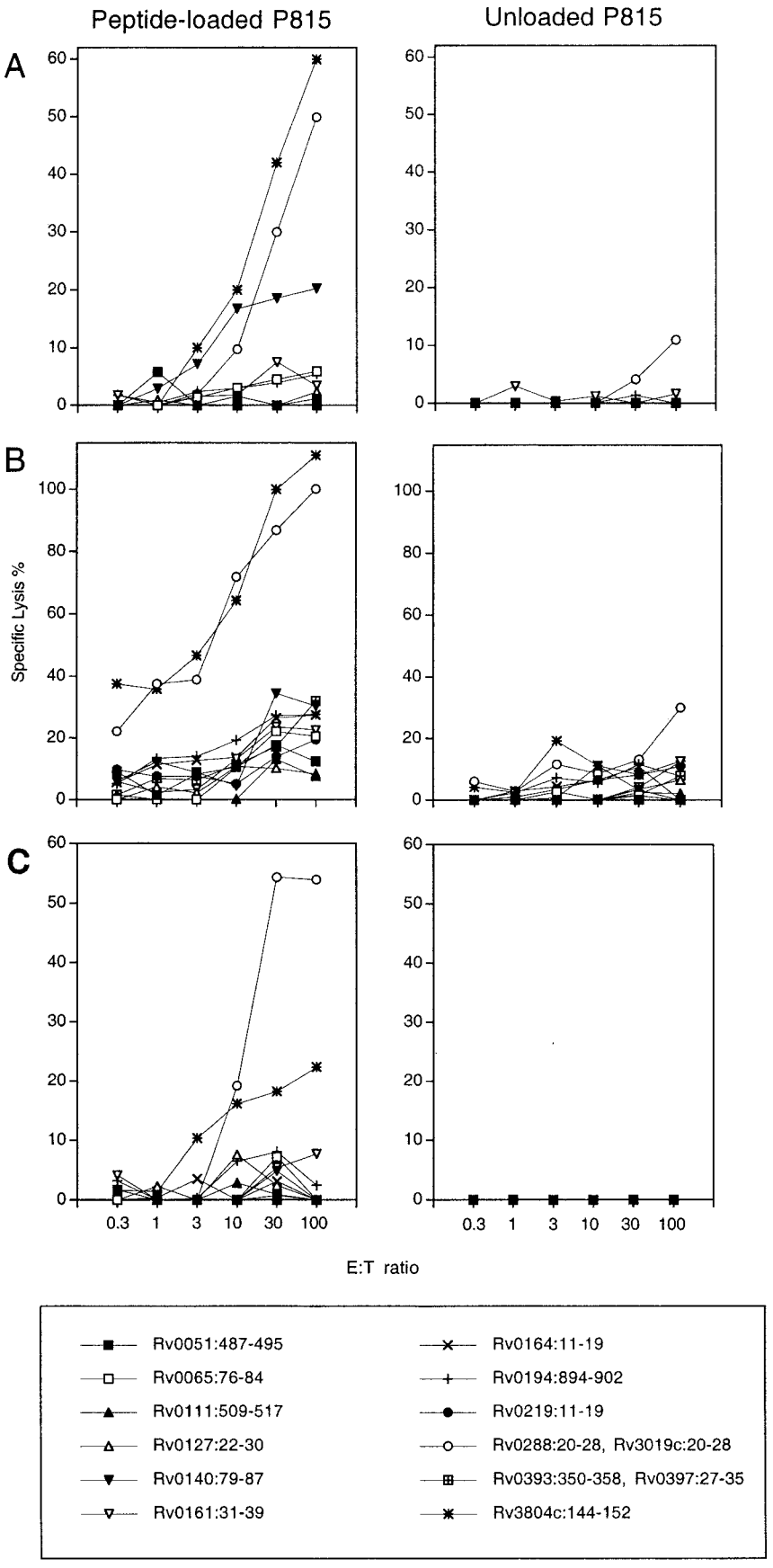
Peptide-loaded P815
Unloaded P815
A
B
C
Specific Lysis %
E:T ratio
0.3 1 3 10 30 100
Rv0051:487-495
Rv0065:76-84
Rv0111:509-517
Rv0127:22-30
Rv0140:79-87
Rv0161:31-39
Rv0164:11-19
Rv0194:894-902
Rv0219:11-19
Rv0288:20-28, Rv3019c:20-28
Rv0393:350-358, Rv0397:27-35
Rv3804c:144-152

```
                10        20        30        40        50
Rv0288   MSQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGIT
         ::::::::::.:::::::::::::::::.:: :::.:.::::::::::
Rv3019c MSQIMYNYPAMMAHAGDMAGYAGTLQSLGADIASEQAVLSSAWQGDTGIT
                10        20        30        40        50

                60        70        80        90
         YQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGG
         ::.::.:::::.::::::::..::.:::.::::.::: :::::::
         YQGWQTQWNQALEDLVRAYQSMSGTHESNTMAMLARDGAEAAKWGG
                60        70        80        90
```

FIG. 2. Alignment of the protein sequences of Rv0288 (TB10.4) and Rv3019c (TB10.3). The sequence of the TB10.3/4:20-28 epitope is underlined.

the Rv3804c (Ag85A):144-152 positive-control peptide, elicited CTLs able to efficiently lyse peptide-loaded P815 targets (Fig. 1A). TB10.3 and TB10.4 are low-molecular-weight proteins with 84.4% sequence identity (Fig. 2) and constitute with TB12.9 (Rv3017c) (12) a subfamily of the highly immunogenic ESAT-6 protein family (1, 2, 15). TB12.9 does not share the GYAGTLQSL epitope. It is noteworthy that the actual knowledge of the *M. tuberculosis* genome does not allow us to precisely anticipate which mycobacterial proteins are secreted. However, among the first 400 ORFs, 6 encode proteins identified in the culture supernatant of *M. tuberculosis* and 6 others are putatively secreted. Among the ORFs selected here, only TB10.4 and TB10.3 are secreted proteins.

We then screened these peptides for their recognition by CTLs from mice infected with *Mycobacterium bovis* BCG or with *M. tuberculosis*. The effector cells of these mice specifically and efficiently lysed P815 cells pulsed with TB10.3/4:20-28 peptide or Rv3804c (Ag85A):144-152 positive-control peptide (Fig. 1B and C). The other H37Rv-derived peptides were unable to induce the lysis of target cells by such effectors. Splenocytes of controls injected with phosphate-buffered saline showed no TB10.3/4:20-28-specific CTL activity (data not shown), indicating that immunization with mycobacteria, rather than in vitro priming with TB10.3/4:20-28 peptide, induced this CTL response. The TB10.3/4:20-28 epitope is thus naturally processed and presented by MHC-I molecules in BCG- or *M. tuberculosis*-infected mice.

In this study, the frequency of immunogenic CTL epitopes among peptides predicted by SYFPEITHI was relatively low (1 out of 11). In a previous study using a similar bioinformatic approach on mycobacterial immunogens, this frequency was varied (4 out of 7 for H-2K^b and 1 out of 11 for H-2D^d) (16). However, recent studies show that a combination of the prediction of MHC-I ligand with information on proteasomal cleavages improves the efficiency of the identification of CD8$^+$-T-cell epitopes (8).

Heat-killed (20 min, 86°C) BCG lost its capacity to induce a

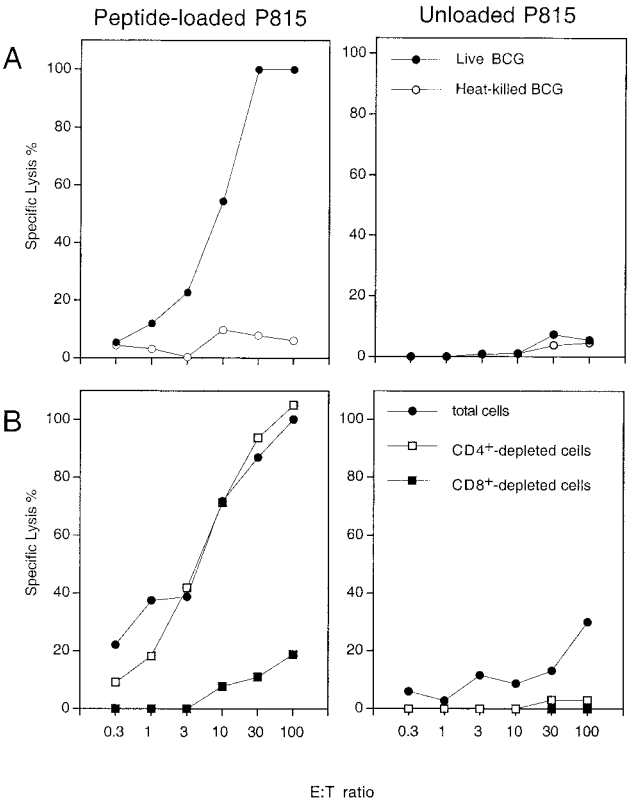

FIG. 3. Requirement of immunization with live BCG for induction of TB10.3/4:20-28-specific CD8$^+$ CTLs. (A) TB10.3/4:20-28-specific CTL responses. BALB/c mice were immunized by a single s.c. injection of 10^7 CFU of live BCG or by two injections at a 15-day interval of 10^7 CFU of heat-killed BCG. Splenocytes were harvested at day 21 for CTL assay. (B) TB10.3/4:20-28-specific CTL activity of total CD4$^+$- or CD8$^+$-T-cell-depleted splenocytes of BCG-immunized BALB/c mice. T-subset depletion was assessed by negative selection with biotinylated anti-CD4 (GK1.5) or anti-CD8 (H35-17-2) monoclonal antibodies and streptavidin-coupled magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

TB10.3/4:20-28-specific CTL response (Fig. 3A). This result is in agreement with the hypothesis that the most obvious way for mycobacteria to gain access to the host cell cytoplasm and its MHC-I pathway would be membrane permeabilization of the primary phagosomes inducible only by metabolically active mycobacteria (7, 14). The TB10.3/4:20-28-specific CTL activity of BCG-immunized mice was inhibited when effectors were depleted of CD8$^+$, but not CD4$^+$, T cells (Fig. 3B). Furthermore, TB10.3/4:20-28-loaded L fibroblasts transfected with H-2K^d, but not with H-2D^d or H-2L^d, were lysed by the CTLs of BCG-immunized mice (data not shown). Thus, immunization

FIG. 1. CTL responses to mycobacterial nonamers predicted to bind to H-2K^d. CTL activity in the lymph nodes of BALB/c mice (n = 3) immunized s.c. with individual peptides emulsified in incomplete Freund adjuvant (A), in the splenocytes of mice (n = 4) injected s.c. with 10^7 CFU of BCG Pasteur 1173P2 (B), or in the splenocytes of mice infected s.c. with 10^6 CFU of *M. tuberculosis* (H37Rv) (C) is shown. Eight days after the last injection of H37Rv-derived peptides or 4 weeks after infection with BCG or H37Rv, CTL activity against unloaded or peptide-loaded P815 targets was measured in a standard 5-h ^{51}Cr release assay run in duplicate. The percentage of specific lysis was calculated with the formula 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was obtained by the addition of 1% Triton X-405 to labeled target cells. Results are representative of at least two independent experiments. Standard deviations were always <5%.

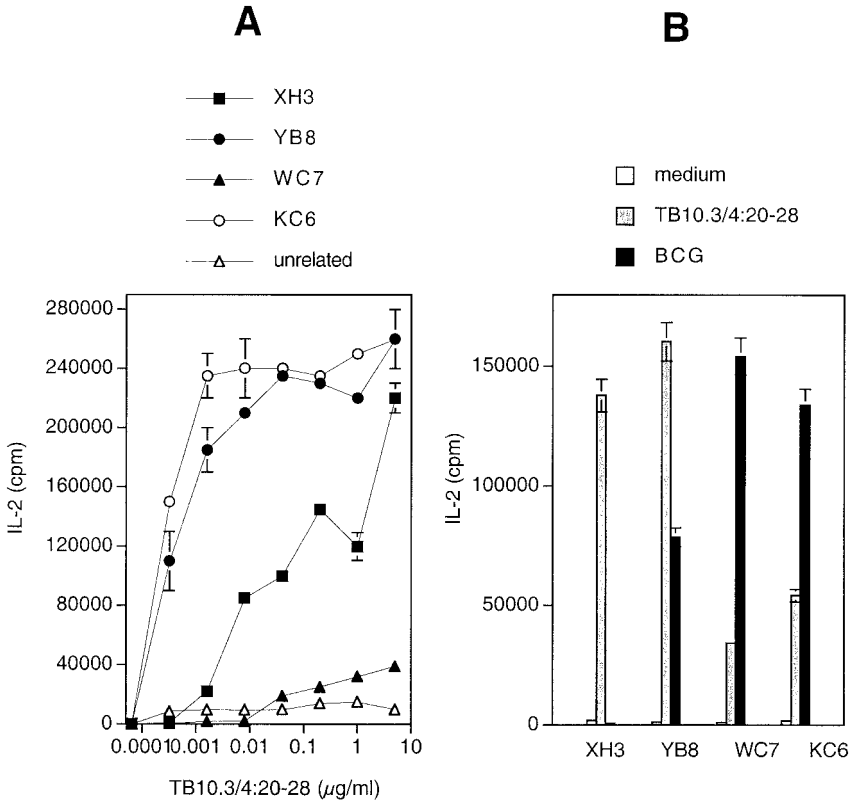

FIG. 4. Generation of H-2K^d-restricted TB10.3/4:20-28-specific T-cell hybridomas from BCG-immunized BALB/c mice. (A) Specific IL-2 production by four TB10.3/4:20-28-specific T-cell hybridomas (XH3, YB8, WC7, and KC6) (10^5 cells/well) subsequent to overnight stimulation with various concentrations of TB10.3/4:20-28 peptide in the presence of syngeneic bone marrow-derived dendritic cells (10^5 cells/well) used as an antigen-presenting cell. (B) Specific IL-2 production (monitored by CTLL-2 bioassay) by XH3, YB8, WC7, and KC6 T-cell hybridomas in response to stimulation by Raw 264.7 macrophages incubated with 1 μg of TB10.3/4:20-28 peptide per ml or previously infected with BCG at a final concentration of 5×10^5 CFU/ml under antibiotic-free conditions for 4 days.

of mice with live BCG generates H-2K^d-restricted TB10.3/4:20-28-specific CD8$^+$ CTLs.

Using splenocytes of BCG-immunized BALB/c mice, we generated a panel of 10 CD8$^+$-T-cell hybridomas specific for the TB10.3/4:20-28 epitope. As shown in Fig. 4A, four of these hybridomas, namely, XH3, YB8, WC7, and KC6, produce interleukin-2 (IL-2) when they are stimulated with TB10.3/4:20-28 peptide in the presence of H-2^d bone marrow-derived dendritic cells. Unrelated H-2K^d-restricted Rv3804c (Ag85A):144-152 or LCMV-NP:118-126 peptides did not stimulate these hybridomas (data not shown). Assays of TB10.3/4:20-28 presentation by L fibroblasts transfected with H-2K^d, H-2D^d, or H-2L^d demonstrated that XH3 and YB8 were restricted by H-2K^d but that WC7 and KC6 were restricted by both H-2K^d and H-2L^d (data not shown). Three out of these 10 TB10.3/4:20-28-specific T-cell hybridomas efficiently recognized BCG-infected Raw 264.7 (H-2^d) macrophages (Fig. 4B), indicating that the processing of mycobacteria leads to MHC-I presentation of the TB10.3/4:20-28 epitope. These hybridomas are the first-described stable CD8$^+$-T-cell clones specific for an MHC-I-restricted mycobacterial antigen and represent powerful tools which now allow investigation of cellular and molecular mechanisms of mycobacterial-antigen presentation via the MHC-I pathway.

Based on the low quantities of ESAT-6 family proteins within in vitro mycobacterial cultures despite the substantial host immune responses to these proteins, it has been suggested that their expression may be upregulated during intracellular growth in an infected host (11). Considering (i) this hypothesis, (ii) the fact that TB10.4 is strongly recognized by peripheral blood mononuclear cells of BCG-vaccinated or tuberculosis patients (11), and (iii) our data, it might be of particular interest to investigate the potential vaccinal interest of TB10.3 or TB10.4 proteins against infection with *M. tuberculosis.*

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