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HLA-B*35-Restricted CD8$^+$-T-Cell Epitope in *Mycobacterium tuberculosis* Rv2903c

Michèl R. Klein,[1]* Abdulrahman S. Hammond,[1] Steve M. Smith,[2]† Assan Jaye,[1] Pauline T. Lukey,[3] and Keith P. W. J. McAdam[1]

TB Research Programme, MRC Laboratories, Fajara, The Gambia,[1] and Immunology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London,[2] and Respiratory Systems, GlaxoSmithKline R&D, Medicines Research Centre, Stevenage, Hertfordshire,[3] United Kingdom

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Few human CD8$^+$ T-cell epitopes in mycobacterial antigens have been described to date. Here we have identified a novel HLA-B*35-restricted CD8$^+$ T-cell epitope in *Mycobacterium tuberculosis* Rv2903c based on a reverse immunogenetics approach. Peptide-specific CD8 T cells were able to kill *M. tuberculosis*-infected macrophages and produce gamma interferon and tumor necrosis factor alpha.

There is mounting evidence that CD8 T lymphocytes are involved in the immune response against mycobacteria (16). The *Mycobacterium tuberculosis* H37Rv genome has been shown to contain at least 3,924 open reading frames (3). Given the large number of epitopes that have been identified in a small virus such as human immunodeficiency virus type 1 (HIV-1) (HIV Molecular Immunology Database 1999 [http://hiv-web.lanl.gov/immunology/]), the mycobacterial proteome is expected to contain a myriad of T-cell epitopes. So far relatively few human CD8 T-cell epitopes have been reported, with those that have being derived mainly from secreted mycobacterial antigens (9, 10, 12, 15). This type of antigen has been shown to induce strong cell-mediated immune responses in guinea pigs and to give substantial protection against aerosol challenge with virulent bacilli (6).

Here we set out to identify human CD8$^+$ T-cell epitopes in *M. tuberculosis* Rv2903c (3) as part of our continuing effort to identify potential T-cell epitopes in novel mycobacterial antigens that are restricted by common HLA types (9, 15).

Although functional data are currently lacking, we selected Rv2903c because sequence similarity suggests that this gene product belongs to the family of type I signal peptidases. These proteins are membrane-bound serine endopeptidases responsible for proteolytic removal of N-terminal signal sequences of presecretory proteins (13). Signal peptidases are essential for bacterial viability and may be potential targets for novel antibiotic compounds (13).

Rv2903c encodes a 294-amino-acid (- aa) polypeptide, and data from whole-genome DNA microarray experiments indicate that this open reading frame is expressed in several mycobacterial strains tested, including *M. tuberculosis* H37Rv, "Oshkosh" strain CDC1551, clinical isolate 1254, and *M. bovis* BCG Pasteur (Martin Voskuil [Stanford University, Stanford, Calif.], personal communication).

The amino acid sequence of Rv2903c was searched for HLA-B*3501 peptide-binding motifs using a computer algorithm (14). This class I restriction element was chosen because it is one of the common HLA-B types in West Africa and part of the HLA-B7 supertype family (1). Five peptides with the highest score were selected and tested for binding to HLA-B*3501 (Fig. 1). Binding assays were performed as previously described using Tap-deficient RMA-S cells transfected with HLA-B*3501 (generously provided by Masafumi Takiguchi, Center for AIDS Research, Kumamoto, Japan) (9, 19–21). Cells were cultured for 16 h at 26°C to allow "empty" HLA class I molecules to accumulate on the cell surface. Cells were

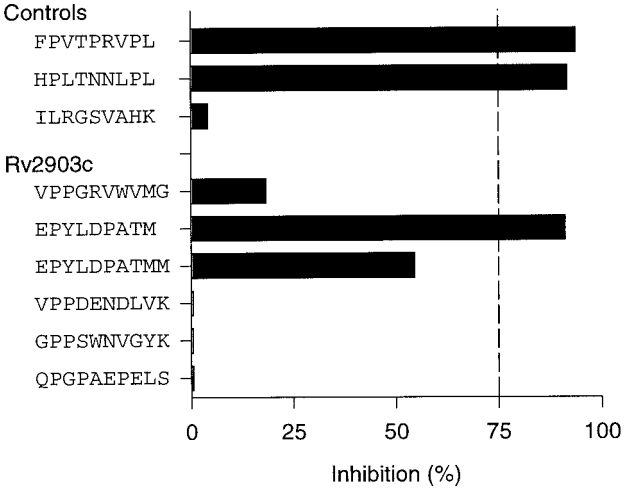

FIG. 1. Screening of Rv2903c-derived peptides for binding to HLA-B*3501. RMA-S B*3501 cells were pulsed with 50 μM unlabeled competitor peptide and 1 μM FL-labeled reference peptide. Results are expressed as percent inhibition of the maximum signal of the FL-labeled reference peptide. The vertical dotted line identifies peptides that inhibited the maximum signal by >75%. Positive controls were peptides HIV-1_{SF2} $Nef_{72–80}$ (aa 72 to 80) (4T6R), FPVTPRVPL, and EBV TEGU (aa 1974 to 1982), HPLTNNLPL. Peptide Flu-A NP (aa 265 to 273), ILRGSVAHK, was used as a negative control.

* Corresponding author. Present address: Department of Microbiology and Immunology, School of Medicine, Stanford University, 279 Campus Dr., Beckman Center B239 (Schoolnik Lab), Stanford, CA 94305-5323. Phone: (650) 723-7026. Fax: (650) 723-1399. E-mail: petiet@stanford.edu.

† Present address: Department of Pathology and Microbiology, University of Bristol, Bristol, United Kingdom.

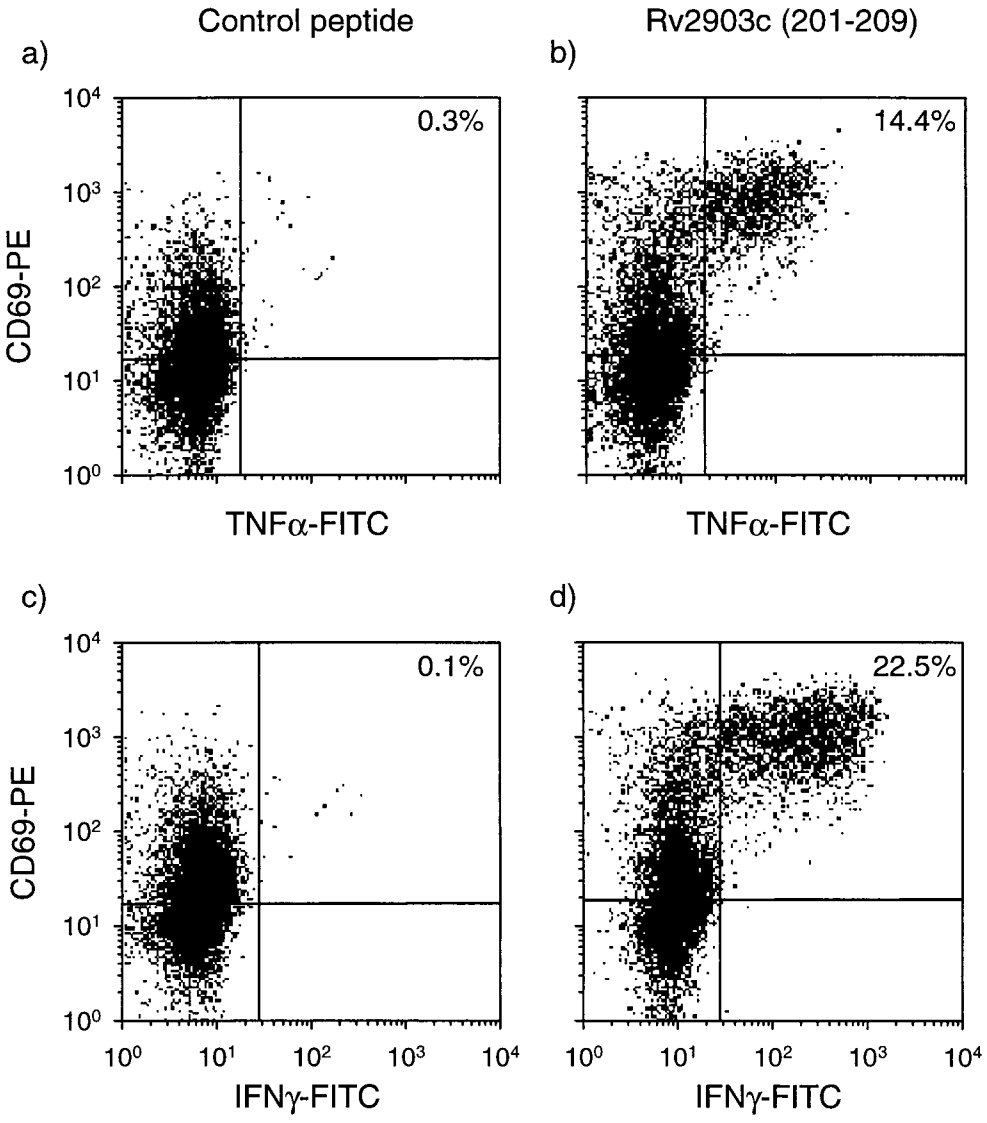

FIG. 2. HLA-B*3501-restricted cytokine production by activated CD8 T cells in response to mycobacterial peptides. Short-term cultures of PBMC stimulated with peptide Rv2903c (aa 201 to 209) were cocultivated for 6 h with RMA-S B*3501 cells pulsed with a control peptide, HIV-1_{SF2} $Nef_{72–80}$ (4T6R) (panels a and c), or the specific peptide (panels b and d). Cytokine secretion was blocked by brefeldin A. Surface markers CD8 and CD69 and intracellular IFN-γ (lower panels) and TNF-α (upper panels) were stained following fixation and permeabilization of cells. Dot plots show log fluorescence intensity. $CD8^+$ bright T cells were gated in the fluorescence-3 channel versus side scatter light plots. Quadrant markers for CD69, IFN-γ, and TNF-α were set using the proper isotype control reagents. Frequencies of positive cells are given as a percentage of the gated $CD8^+$ T cells. Abbreviations: PE, phycoerythrin; FITC, fluorescein isothiocyanate.

pulsed for 1 h at 26°C with 1 μM fluorescein (FL)-labeled reference peptide FPVTPCK(FL)VPL together with different concentrations of unlabeled peptide. Cells were then incubated for 3 h at 37°C, and the geometric mean fluorescence intensity was measured using a FACS Calibur flow cytometer (Becton & Dickinson, Mountain View, Calif.).

The positive control peptide, HLA-B*3501-restricted epitope HIV-1_{SF2} Nef aa 72 to 80 ($Nef_{72–80}$) (4T6R), FPVT PRVPL, gave reproducible inhibition of the maximum signal of the FL-labeled reference peptide. The concentrations needed to reduce the maximum signal by 50% were 0.6 to 1.3 μM. The 10-mer sequence Rv2903c (aa 201 to 210) and its 9-mer variant SP201/9 were the only ones to significantly inhibit the reference signal. Titration of the 9-mer peptide resulted in reproducible dose-response curves with 50% inhibitory concentrations of 1.6 to 2.1 μM. The half-life of complexes at 37°C of peptide SP201/9 and HLA-B*3501 was more than 7 h, similar to what we observed for peptide 4T6R (data not shown) (9). The affinity for binding to HLA-B*3501 and stability of peptide-MHC complexes were in the same range as previously reported for other immunogenic peptides (9, 20, 21).

We subsequently tested whether peptide SP201/9 is immunogenic in BCG-vaccinated healthy blood donors. Peripheral blood mononuclear cells (PBMC) were obtained from an HLA-B*3501-positive, BCG-vaccinated (as proven by the

presence of a scar) healthy volunteer. Effector T cells were generated from PBMC using live *M. tuberculosis* as previously described (9, 15, 17, 18). After 7 days effector T cells were tested for cell-mediated cytotoxicity in standard 6-h ^{51}chromium-release assays as previously described (9, 15, 17, 18).

Effector T cells efficiently killed macrophages pulsed with peptide SP201/9 as well as macrophages infected with *M. tuberculosis*, indicating that this peptide is naturally processed from its endogenous source. Lytic activity, expressed as percentage lysis (mean ± standard deviation) at an effector-to-target (E/T) ratio of 50:1, was 1.1% ± 0.3% for uninfected control targets and 23.% ± 2.8% for *M. tuberculosis*-infected macrophages ($P < 0.005$, Student's t test). At the same E/T ratio, lysis of macrophages pulsed with 10 μM synthetic peptide SP201/9 was 66.1% ± 6.1%. At the lower E/T ratio of 5:1 only minimal killing of infected macrophages was observed.

Subsequently we generated short-term bulk cultures of PBMC stimulated with synthetic peptide SP201/9 as previously described (9, 11). Briefly, PBMC were pulsed with 50 μM peptide SP201/9 for 1 h at room temperature and cultured in RPMI 1640 supplemented with 10% autologous plasma, antibiotics, and 5 ng of recombinant human interleukin 7 (R&D Systems Europe Ltd., Abingdon, United Kingdom) per ml. On day 3 and every 2 to 3 days thereafter complete medium containing recombinant human interleukin 2 (10 U/ml; generously provided by Ronald Rombouts, Chiron Benelux B.V., Amsterdam, The Netherlands) was added. Short-term bulk cultures were further expanded for 2 to 4 weeks by restimulation with peptide-pulsed and irradiated autologous PBMC or Epstein-Barr virus (EBV)-transformed B-LCL.

Cells that were stimulated with peptide SP201/9 were analyzed for intracellular cytokine production as previously described (7, 9). Briefly, effector cells were harvested and 2×10^6 cells were incubated for 6 h at 37°C with 5% CO_2 with 10^6 target cells pulsed with peptide SP201/9 or control peptide 4T6R. Cytokine secretion was blocked by brefeldin A (10 μg/ml; Sigma Chemical Co., Poole, United Kingdom). Becton Dickinson Immunocytometry Systems (San Jose, Calif.) FastImmune reagents and protocols were used to detect intracellular cytokines. The number of activated (i.e., $CD69^+$) CD8 cells that produced gamma interferon (IFN-γ) was 22.5% when tested against HLA-B*3501-transfected RMA-S cells pulsed with 10 μM peptide SP201/9, whereas only 0.1% of cells were positive when tested on target cells pulsed with control peptide 4T6R (Fig. 2). In addition, we observed 14.4% of activated $CD8^+$ T cells producing tumor necrosis factor alpha (TNF-α) in response to the specific peptide and only 0.3% in response to the control peptide 4T6R. Similar results were obtained when we used the autologous EBV-transformed B-LCL as targets (data not shown).

PBMC of two other HLA-B*35-positive, BCG-vaccinated (as shown by presence of scar), healthy volunteers were also tested for reactivity towards peptide SP201/9 using ELISPOT assays for IFN-γ (9, 10, 15). Recall responses to this peptide were assessed after 10 days of stimulation with peptide SP201/9. Numbers of IFN-γ spot-forming cells (SFC)/10^6 cells specific for peptide SP201/9 were 50 SFC/10^6 cells for one donor and 72 SFC/10^6 cells for the other donor. Direct ex vivo responses against this peptide were not detected in these healthy donors.

Taken together, we have identified a novel HLA-B*35-restricted CD8 T-cell epitope in a previously unknown antigen of *M. tuberculosis*. CD8 T cells responding to this peptide kill infected macrophages and produce IFN-γ and TNF-α. The latter is in agreement with previous observations for other mycobacterial CD8 T-cell epitopes that have been identified so far (reviewed in references 8 and 16). Our observations are also consistent with the existing body of evidence for the importance of type 1 cytokines in the control of mycobacterial infections (4, 5). However, formal proof that cytokine production or killing by CD8 T cells protects against tuberculosis (TB) in humans remains to be established further.

The finding of a novel human CD8 T-cell epitope in a potential membrane-bound mycobacterial protein, together with published epitopes in other somatic proteins (2), strongly suggests that the spectrum of antimycobacterial immune responses may be much broader than that previously anticipated (8). Further characterization and identification of *M. tuberculosis*-specific CD8 T-cell responses at the epitope level may help to elucidate the exact role of CD8 T cells in protection against TB in humans and, we hope, may assist in the development of novel TB vaccines.

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