

Review

CD8⁺ T Lymphocytes Against *Mycobacterium tuberculosis*

MICHÈL R. KLEIN and KEITH P. W. J. McADAM

Tuberculosis Research Programme, Medical Research Council (MRC) Laboratories, Atlantic Road, Fajara, PO Box 273, Banjul, Gambia, West Africa

Abstract. Tuberculosis (TB) is again a global health problem. Identification and characterization of the correlates of protective immunity against TB is critical for the rationale design of novel TB vaccines. There is accumulating data that CD8⁺ T lymphocytes are involved in the immune response against mycobacteria. Here the current state of the art is reviewed with respect to phenotype, specificity and effector mechanisms of mycobacteria-specific CD8⁺ T lymphocytes. In addition, the first listing is presented of mycobacteria-derived CD8⁺ cytotoxic T lymphocyte (CTL) epitopes containing sequences published up to the end of 1998.

Key words: tuberculosis; mycobacterium; CD8; cytotoxic T lymphocyte; epitope; vaccine.

Tuberculosis (TB) has been known since ancient times. In humans this disease is mainly caused by mycobacterial species of the *Mycobacterium tuberculosis* complex (i.e. *M. tuberculosis*, *M. africanum* and *M. bovis*). The World Health Organization (WHO) has estimated that about one third of the world's population is infected with *M. tuberculosis*, with around 3% of people suffering from active disease. In 1991 the global incidence of TB was about 8 million with approximately 3 million deaths and this has not fallen over the last 7 years^{31, 48}. The good news is that the majority of *M. tuberculosis*-infected subjects will never develop TB. It has been estimated that *M. tuberculosis*-infected but otherwise immune competent individuals have only a 10% lifetime risk of developing TB. The bad news is that, from the mid-1980's, there has been a steady increase in the number of TB cases. This is mainly the result of the spreading AIDS epidemic, as HIV-1 infected patients have an additional risk of 8% per year of developing TB³. Also, the emergence of multi-drug-resistant mycobacterial strains is becoming increasingly problematic for the control of TB³. The only TB vaccines currently available for human use are attenu-

ated strains of *M. bovis*, termed bacillus Calmette-Guérin (BCG), which were introduced in the 1920's. Although BCG vaccination appears to protect against severe forms of extra-pulmonary TB in children, protection against contagious pulmonary TB in adults seems limited and varies from 0–80% in different studies and in different populations¹⁹. Clearly, more effective vaccines are needed to provide an adequate solution to the global threat of TB^{19, 20, 38}.

It is generally accepted that protective immunity against TB can be acquired after exposure and infection with *M. tuberculosis*, but the exact correlates of immune-mediated protection are largely unknown. Comprehensive reviews have been published before addressing many different aspects of the immune response against *M. tuberculosis*. These include different T cell subsets (conventional CD4⁺ T helper cells and CD8⁺ T cells, as well as CD1 restricted CD4⁺CD8⁺ double negative (DN) T cells, $\gamma\delta$ T cells and NK-T cells) and cytokine networks^{10, 15, 44}. The first reports of CD8⁺ T cells in mycobacterial infections were reported over a decade ago and their potential role in protective immunity against TB has been suggested³⁰.

The current review will further address this issue and will give an update of *M. tuberculosis*-derived CD8⁺ T cell epitopes.

Human CD8⁺ T Cells in Mycobacterial Infections

Cellular infiltrates of bronchoalveolar lavage (BAL) and pleural effusions from TB patients have been shown to contain mycobacteria-specific CD8⁺ T cells^{18, 49, 51}. Several reports have shown that CD8⁺ cytotoxic T cells (CTLs) can readily be expanded from PBMC of BCG vaccinated individuals, TB patients, healthy occupational contacts and tuberculin skin test-positive persons^{17, 29, 42, 58, 61}.

Currently there is only limited information available on the role of CD8⁺ CTLs in protection against TB in humans. BOTHAMLEY et al.⁵ observed a man with persistent and recurrent pulmonary TB despite full appropriate chemotherapy. There was no evidence of HIV-1 infection or any other immune-related disorders other than an isolated CD8⁺ T lymphopenia. This anecdotal report is the only indication so far that CD8⁺ T cells could be relevant for controlling mycobacterial disease in humans. Additional support for CTLs is coming from *in vitro* studies on effector mechanisms and from experimental mouse models for TB (see below).

Historical Paradox of CD8⁺ T Cells Against Soluble Antigens

Historically, research on CD8⁺ T cells in mycobacterial diseases has drawn relatively little attention. One of the reasons for this was the generally held view that soluble antigens in endocytic compartments are preferably processed through the MHC class II pathway and exclusively elicit CD4⁺ T cell responses. However, there is accumulating evidence that under certain conditions extracellular antigens, as well as proteins derived from intracellular pathogens such as mycobacteria, have access to the MHC class I-processing pathway and can be presented to CD8⁺ T cells (reviewed in ref.²⁰). One of the early studies by REES et al.⁴⁹ on CD8⁺ T cells against mycobacteria have shown that these cells can respond to target cells pulsed with exogenous antigens, mycobacterial soluble extracts and purified antigens. More recently, several studies have shown that CD8⁺ T cells can respond to target cells pulsed with *M. tuberculosis* purified protein derivatives (PPD)^{58, 61} and culture filtrate antigens (CFAg)¹³.

Phenotype and Effector Functions of CD8⁺ T Cells

Mycobacteria-specific CD8⁺ T cells recognise their target antigen when it is presented in the context of self-molecules, the so-called restriction elements, as they largely determine the specificity of the response. CD8⁺ T cells have a broad arsenal of effector functions at their disposal which would be desirable to mobilize with novel TB vaccines (Fig. 1). CD8⁺ T cells can cause cytolysis of *M. tuberculosis*-infected cells and can produce a variety of soluble factors. This may either result in some degree of undesired immunopathology, i.e. tissue destruction (necrosis), inflammatory side effects and further spread of bacteria, or in apoptosis and bactericidal activity. The currently available data seem to favor the latter.

Restriction Elements of the CD8⁺ T Cell Response

Mice in which the β 2-microglobulin gene has been disrupted (β 2m $-/-$) suffer from severe and fatal pathology following i.v. injection of *M. tuberculosis*²³ and to a lesser extent, from disease after infection with BCG vaccination^{23, 33}. As no functional CD8⁺ T cells are present in β 2m $-/-$ knockout mice, these observations have, in general, been taken as evidence for a role of MHC class I restricted CD8⁺ CTLs in the control of mycobacterial disease. Both in mice and in humans several MHC class I antigens have been identified that restrict CD8⁺ CTL responses to mycobacteria (see Table 1). REES et al.⁴⁹ were among the first to show in humans that CD8⁺ T cell responses against certain mycobacterial antigens could be restricted by HLA-B8.

MOHAGHEGHPOUR et al.³⁹ have identified a series of HLA-A2 restricted CTLs, with at least one that was capable of recognising endogenously processed antigen. LALVANI et al.³⁴ reported human CD8⁺ CTLs which recognised short peptides presented in the context of HLA-B52 or -A2. Cytolysis was said to result in suppression of mycobacterial growth.

With current knowledge, results with the β 2m $-/-$ knockout mice could also support a role for CD1 restricted CD8⁺ T cells responses against mycobacteria. Indeed, STENGER et al.⁵⁷ recently identified human CD8⁺ CTLs that recognized mycobacterial lipid antigens in the context of CD1b. In this case, cytolysis of *M. tuberculosis*-infected macrophages was shown to reduce the number of viable mycobacteria by 35 to 50%.

LEWINSOHN et al.²⁷ identified CD8⁺ CTLs that killed *M. tuberculosis*-infected dendritic cells. Recognition in

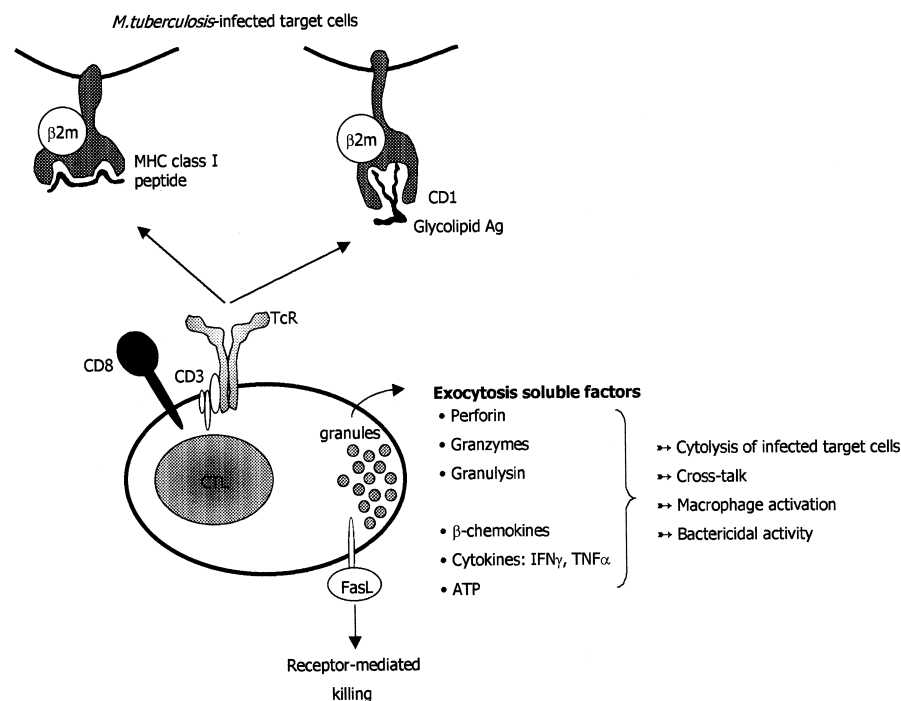


Fig. 1. The arsenal of CD8⁺ T cell effector mechanisms. Mycobacteria-specific CD8⁺ CTLs can recognize short peptides in the context of MHC class I molecules or glycolipid antigens presented by CD1 molecules. Consequently a series of events occur leading up to cytolysis of the infected target cell. Cell-mediated cytotoxicity involves Ca²⁺-dependent release of cytolytic granules, which results in apoptosis of the target cell as well as in direct or indirect mycobactericidal activity. In addition, cytolysis may also result from Fas-FasL interaction. However, in this case it seems that the effect on the viability of the mycobacteria is limited. CD8⁺ T cells are also capable of secreting other soluble factors and cytokines such as IFN-γ and TNF-α. These soluble messenger molecules allow CD8⁺ T cell to communicate with other effector cells as well as to activate macrophages for intracellular killing of mycobacteria

this case appeared not to be restricted by HLA-A, -B, or -C alleles nor by CD1a-c, but was clearly inhibited by the anti-MHC class I monoclonal antibody W6/32. This suggests involvement of other non-polymorphic class I-like molecules. Similar results have recently been reported by CANADAY et al.⁶, who also showed that presentation to mycobacteria-specific CD8⁺ T cells was blocked by anti-MHC class I antibodies, required proteasomal processing, but was insensitive to brefeldin A treatment, which blocks the conventional MHC class I-processing pathway. Together, these observations suggest that Ag-recognition by mycobacteria-specific CD8⁺ T cells involve several complementary Ag-processing pathways with phenotypically distinct CD8⁺ T cell-subsets.

Mechanisms of cytolysis

When CD8⁺ T cells recognize their antigens in the context of self-molecules, a series of events occur leading up to the target cell's demise. Cell-mediated cytotoxicity involves two major pathways².

Classical cytolysis involves the Ca²⁺-dependent re-

lease of cytolytic granules containing perforin and granzyme proteases leading to apoptosis⁵⁷. In humans it has been shown that the killing of intracellular mycobacteria is clearly dependent on the release of cytolytic granules⁵⁷. Rather confusing, however, are observations in perforin- and granzyme B -/- knockout mice infected with *M. tuberculosis*, which do not seem to experience a significantly different course of mycobacterial disease than their control littermates^{9, 36}.

The other major pathway is receptor-mediated and involves the engagement of Fas-ligand (CD95L) in the T cell membrane with the target cell surface receptor Fas (CD95). This interaction also results in the programmed disintegration of the target cell. It has been shown that CD1-dependent cytolysis by human CD4⁻CD8⁻ DN T cells is mediated by the CD95-CD95L interaction, but this appears to have no effect on the viability of mycobacteria⁵⁷. Furthermore, the survival time of CD95L -/- knockout mice after moderate challenge with *M. tuberculosis* is hardly different from that of control mice³⁶.

The contribution of the two major cytotoxicity pathways in the early phase of mycobacterial infection

seems limited, at least in the experimental mouse models for TB. Alternatively, compensatory mechanisms may be operational in these mice that could mask the effect of deleted genes. Studies in the mentioned gene knockout mice could also point towards the involvement of other granule constituents with lytic- or bactericidal activity. For example, WAGNER et al.⁶³ have shown that upon antigen-specific triggering, HIV-1 specific CD8⁺ CTLs release β -chemokines (RANTES, MIP-1 α and MIP-1 β) bound to sulphated proteoglycans, which are major constituents of cytolytic granules. Another granule-associated molecule worth mentioning is protein 519 (Granulysin), which is also exocytosed after stimulation through the T cell receptor and which exhibits lytic activity⁴⁶. STENGER et al.⁵⁶ have recently shown that this molecule also has direct bactericidal activity. Granulysin killed extracellular *M. tuberculosis* and, in combination with perforin, reduced the viability of phagocytosed *M. tuberculosis*. Production of extracellular adenosine 5'-phosphate (ATP) could also contribute to the bactericidal activity of human CD8⁺ CTLs⁴⁰. Exogenous ATP has been shown to induce the killing of intracellular mycobacteria within BCG-infected human macrophages through purinergic P2Z(P2X7) receptors³⁵.

Cytokines

Other effector functions of CD8⁺ T cells comprise the release of cytokines. The current dogma says that T cell-derived cytokines like IFN- γ and TNF- α are required to stimulate macrophages to kill or control the growth of intracellular mycobacteria. Mice in which these genes have been deleted have been shown to suffer more severely from experimental *M. tuberculosis* infection^{8, 21, 22}. BONATO et al.⁴ showed that the cells which conferred the highest level of protection in adoptive transfer experiments, as assessed by the number of mycobacteria in spleens of challenged mice, were IFN- γ -producing CD8⁺ CTLs. Using IFN- γ $-/-$ knockout mice, TASCON et al.⁶⁰ showed that IFN- γ -production by primed CD8⁺ T cells was required in order for these cells to exert their bactericidal effect.

In vitro studies have shown that mycobacteria-specific CD8⁺ CTLs from humans can also secrete IFN- γ upon antigen triggering^{6, 34, 37}. The relevance of the type-1 cytokine pathway for the control of mycobacterial infections in humans has been demonstrated by NEWPORT et al.⁴³. They observed in 4 children with severe mycobacterial infections a point mutation in the IFN- γ -receptor 1 gene that led to non-functional expression. Several other mutations have now been identified that

affect the ability to produce or to respond to IFN- γ , showing the relevance of the type-1 cytokine network for the control of mycobacterial diseases (reviewed in ref. ⁴⁵).

MHC Class I Restricted CD8⁺ CTL Epitopes

The number of published mycobacteria-derived CD8⁺ CTL epitopes is steadily increasing (see Table 1). Most studies so far have been done in mice and, by the end of 1998, only two studies have been published in which epitopes were identified for human CD8⁺ CTL^{34, 39}. The most widely explored method for identifying epitopes in target antigens is the so-called „reverse-genetics“ approach which use MHC class I peptide-binding motifs to identify potential epitopes within a given sequence⁴⁷.

The list of identified CD8⁺ CTL epitopes represents the current state-of-the-art and is by no means based on a systematic screening of the approximately 4000 predicted proteins in the genome of *M. tuberculosis*⁷. Currently, CTL epitopes have only been described in 7 of these: in some heat shock proteins (HSP) and in a handful of secreted antigens (see Table 1). The importance of secreted antigens for protective immunity against TB has been discussed previously¹. Several studies have focussed on CD8⁺ T cell responses against mycobacterial HSP^{52, 54, 68}. Under stress conditions human cells express similar proteins and mycobacteria HSP65-specific CD8⁺ CTLs have been demonstrated that can kill „stressed“ macrophages³². Although usage of „self“ proteins may prompt autoimmunity, it has been shown that the whole-cell component of the early-childhood pertussis vaccine primes the immune system to HSP, but apparently without major side effects¹².

Further identification of immune dominant proteins and epitopes may be useful for subunit vaccine development (see below). Special attention should be given to situations where people have been exposed and infected with *M. tuberculosis* but have not developed TB, or to patients with TB who self-cured their disease without specific treatment. Studying those people may give us vital clues on how to induce protective immunity against TB.

TB Vaccines

The ideal vaccine would be given early in life and would confer long-lasting protection against TB. Alternatives may include „booster“ vaccines given to individuals already infected with *M. tuberculosis* to enhance or to modify the immune response in order to achieve more effective immunity^{4, 14}.

Table 1. Overview *M. tuberculosis*-derived CD8⁺ CTL epitopes (update 1998)

<i>M. tuberculosis</i> protein ^a	Amino acid sequence ^b	MHC class I restriction	Reference
Human CTL epitopes			
Secreted Ag			
ESAT-6 (aa. 69–76)	LQNLARTI	HLA-B52	LALVANI et al. ³⁴
ESAT-6 (aa. 82–90)	AMASTEGNV	HLA-A*0201	LALVANI et al. ³⁴
19 kDa LP (aa. 88–97)	VLTDGNPPEV	HLA-A*0201	MOHAGHEGHPOUR et al. ³⁹
19 kDa LP (aa. 101–108) ^c	GLGNVNGV	HLA-A*0201	MOHAGHEGHPOUR et al. ³⁹
19 kDa LP (aa. 131–139) ^c	KITGTATGV	HLA-A*0201	MOHAGHEGHPOUR et al. ³⁹
Murine CTL epitopes			
Secreted Ag			
19 kDa LP (aa. 69–78) ^c	TAAGNVNIAI	H-2D ^b	VORDEMEIER et al. ⁶²
19 kDa LP (aa. 102–110) ^c	LGNVNGVTL	H-2D ^b	VORDEMEIER et al. ⁶²
38 kDa LP (aa. 129–137)	AQQVNYNLP	H-2D ^b	ZHU et al. ⁶⁶
38 kDa LP (aa. 166–175)	IAALNPGVNL	H-2D ^b	ZHU et al. ⁶⁶ , DA FONSECA ¹¹
38 kDa LP (aa. 191–199)	DTFLFTQYL	H-2K ^b	ZHU et al. ⁶⁶
38 kDa LP (aa. 225–234)	ALGENGNGGM	H-2D ^b	ZHU et al. ⁶⁶
38 kDa LP (aa. 243–250)	GCVAYIGI	H-2K ^b	ZHU et al. ⁶⁶
38 kDa LP (aa. 309–318)	YPIINYEYAI	H-2D ^b	ZHU et al. ⁶⁶ , DA FONSECA ¹¹
38 kDa LP (aa. 312–319)	INYEYAIIV	H-2K ^b	ZHU et al. ⁶⁶ , VORDEMEIER et al. ⁶²
38 kDa LP (aa. 317–325)	AIVNNRQKD	H-2D ^b	ZHU et al. ⁶⁶
Ag 85A (aa. 104–123) ^d	YDQSGLSVVMVGGQSSFYS	H-2K ^d /L ^d	DENIS et al. ¹⁴
Ag 85A (aa. 184–203) ^d	QQFVYAGAMSGLLDPSQAMG	H-2K ^d	DENIS et al. ¹⁴
Ag 85A (aa. 204–223) ^d	PTLIGLAMGDAGGYKASDMW	H-2L ^d	DENIS et al. ¹⁴
Heat shock proteins			
10 kDa (aa. 70–79) ^c	IYSKYGGTEI	H-2K ^d	VORDEMEIER et al. ⁶²
10 kDa (aa. 82–89) ^c	NGEYLYLIL	H-2K ^b	VORDEMEIER et al. ⁶²
16 kDa (aa. 24–31) ^c	AFPSFAGL	H-2K ^b	VORDEMEIER et al. ⁶²
65 kDa (aa. 6–15)	AYDEEARRGL	H-2K ^d	TASCON et al. ⁵⁹
65 kDa (aa. 64–72) ^c	PYEKIGAEI	H-2K ^d	VORDEMEIER et al. ⁶²
65 kDa (aa. 278–287)	GFGDRRKAML	H-2K ^d	TASCON et al. ⁵⁹
65 kDa (aa. 478–485) ^c	QTGVYEDL	H-2K ^b	VORDEMEIER et al. ⁶²
65 kDa (aa. 499–508)	SALQNAASIA	H-2D ^b	SCHOEL et al. ⁵²

^a Numbering is according to sequences listed in the database of predicted proteins of *M. tuberculosis* H37Rv⁷ (see http://www.sanger.ac.uk/Projects/M_tuberculosis/): ESAT-6 (Rv3875), 19 kDaLP (Rv3763), 38 kDa LP (Rv0934), antigen 85A (Rv3804c), 10 kDa HSP (Rv3418c), 16 kDa HSP (Rv2031c), 65 kDa HSP (Rv0440).

^b Putative anchor residues for binding to MHC class I are indicated in bold⁴⁷.

^c Peptides identified in MHC class I binding assays. No *in vitro* or *in vivo* CTL responses have been shown so far that are directed against these sequences.

^d These 20-mer peptides are recognized by murine CD8⁺ CTL when presented on peptide pulsed P815 (H-2^d) targets. The putative anchor residues are indicated in bold. The shorter peptides aa. 113–122 and 187–196 are also recognized in the context of H-2^d alleles¹⁴.

Live attenuated and recombinant mycobacteria

Live attenuated vaccines are thought to mimic most aspects of natural infection, and may provide the broadest range of pertinent stimuli to the immune system. Knockout mutants of *M. tuberculosis* are being constructed from which virulence factors or genes for prolonged survival in macrophages have been deleted²⁴. However, as the function of the vast majority of genes of *M. tuberculosis* is currently unknown, it will be difficult to predict whether these mutants are safe for long-term use in humans. An alternative approach would be to express private and immunogenic antigens of

M. tuberculosis in vaccine strains of BCG. Theoretically these recombinant BCG strains would be safe, and with the added feature that they might also be able to enhance protective immunity against TB.

It has been shown that vaccination with BCG and saprophytic species such as *M. vaccae* as well as undefined exposure to non-pathogenic environmental mycobacteria can elicit CD8⁺ T cell responses^{17, 55}. However, induction or boosting of mycobacteria-specific CD8⁺ CTLs under these conditions may not be optimal. Recently, HESS et al.²⁷ described the construction of recombinant BCG strains that secrete listeriolysin of *Listeria monocytogenes*. Listeriolysin is a pore-forming

protein essential for the release of *L. monocytogenes* bacteria from phagosomal vacuoles into the cytoplasm of infected cells. The rationale behind this recombinant BCG construct is to improve the access of mycobacterial proteins to the MHC class I Ag-processing pathway in order to enhance stimulation of CD8⁺ CTL responses.

Subunit vaccines

Much effort is also currently being devoted to designing so-called subunit vaccines. Several of these subunit preparations appear to induce mycobacteria-specific CD8⁺ T cell responses. These include purified- and recombinant-mycobacterial antigens^{13, 18, 41, 58}, different expression vectors like "naked" recombinant-DNA vaccines^{4, 28, 59, 66, 67} and recombinant vaccinia viruses⁶⁵ and synthetic peptides^{11, 62, 64}.

Currently the most promising subunit vaccines are naked recombinant DNA vaccines^{4, 28, 59, 66, 67}. Challenge experiments with virulent bacilli have shown reduced bacterial loads in internal organs of vaccinated mice. Although in all cases mycobacteria-specific CD8⁺ CTLs were induced, the causal relationship between CD8⁺ CTL responses and true protection to disease formally remains to be proven. Suggestive data comes from the study of ERB et al.¹⁶, who used DNA vectors expressing 19 kDa and AhpC proteins. No specific CD8⁺ CTL responses were detected after DNA-injection and no effect on bacterial load was observed. In addition, BONATO et al.⁴ showed that, after adoptive transfer of CD8⁺ CTLs from HSP65 DNA vaccinated mice into naive mice, the number of mycobacteria in the spleens of recipient mice was reduced by almost one log after challenge with *M. tuberculosis*.

Subunit vaccines may allow for better quality control of vaccine preparations and may aid the immune system in focusing on more relevant antigens. The repertoire of T cells, which are recruited during natural infection with *M. tuberculosis*, may not always be the best repertoire available. DENIS et al.¹⁴ have shown in mice that Ag85A-DNA vaccination resulted in the recognition of particular peptides which were not recognized by CD8⁺ T cells during infection with live *M. tuberculosis*. Other Ag-delivery systems that are being explored include the use of recombinant poxvirus vectors. WILKINSON et al.⁶⁵ showed that recombinant vaccinia virus expressing the 38 kDa antigen of *M. tuberculosis* induced CD8⁺ CTL responses. Vaccination with a combination of attenuated poxvirus vectors and DNA vaccines may prove to be a very powerful strategy for inducing and boosting mycobacteria CD8⁺ CTL responses^{25, 26}.

Vaccines based on synthetic peptides may be inexpensive and easy to manufacture. However, the general use of peptide-based vaccines in outbred human populations may be limited, as only a fraction of persons will have the required HLA class I type to present the selected peptides. A way forward would be to focus on common HLA types, like HLA-A2³⁹, or on HLA alleles, which have overlapping peptide-binding motifs⁵³. Grouping according to these so-called "supermotifs" may dramatically increase the number of people able to respond to particular peptides⁵³. Furthermore, the ways in which peptides are being formulated in vaccine preparations appear to be important for induction of CD8⁺ CTL responses. DA FONSECA et al.¹¹ compared peptide variants from the 38 kDa lipoglycoprotein carrying lipid tails of palmitic acid with their "bare" counterparts. Lipopeptides in combination with Freund's adjuvant induced CD8⁺ CTL responses in mice that were stronger than when using the regular peptides.

Concluding Remarks

It is now well established that CD8⁺ T cells are induced during mycobacterial infections. There is indirect evidence that mycobacteria-specific CD8⁺ T cells are relevant for protection against disease. CD8⁺ CTLs have been shown not only to kill *M. tuberculosis*-infected cells, but also to exert bactericidal activity through the release of soluble factors. Induction or boosting of mycobacteria-specific CD8⁺ CTL responses via vaccination seems, therefore, desirable. However, more systematic studies of mycobacteria-specific CD8⁺ CTL responses during the natural history of *M. tuberculosis* infection are still required to narrow down the correlates of protective immunity. Further identification and characterization of mycobacteria-specific CD8⁺ CTLs is warranted in order to exploit the effector mechanisms of these cells on a more rational basis in novel TB vaccine strategies.

Acknowledgment. The authors would like to thank Hazel Dockrell and Arnaud Marchant for helpful comments. Our studies are financially supported by GlaxoWellcome, Stevenage, UK.

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Received in February 1999

Accepted in April 1999