Cytotoxic T-lymphocytes against malaria and tuberculosis: from natural immunity to vaccine design*

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ABSTRACT

1. *Mycobacterium tuberculosis* and the liver stage of *Plasmodium falciparum* are intracellular pathogens which are potentially susceptible to cytotoxic T-lymphocytes, a crucial component of the protective immune response to viral infections. Evidence from animal models points to a protective role for cytotoxic T-lymphocytes against *M. tuberculosis* and *P. falciparum*, but cytotoxic T-lymphocytes specific for these pathogens have been difficult to identify in man.

2. Using a reverse immunogenetic approach, candidate epitopes from selected antigens of *P. falciparum* and *M. tuberculosis* were used to detect peptide-specific cytotoxic T-lymphocyte responses in individuals exposed to these pathogens. Cytotoxic T-lymphocyte activity was detected by the ⁵¹Cr release cytotoxicity assay and a sensitive ELISPOT assay for single-cell interferon-γ release.

3. In naturally exposed, partially immune Africans in The Gambia, eight largely conserved cytotoxic T-lymphocyte epitopes in *P. falciparum*, restricted by several different HLA class I alleles, were identified. Several epitopes were also recognized in Tanzanians and cytotoxic T-lymphocytes recognized endogenously processed antigen.

4. In tuberculosis patients with HLA-B52, a CD8⁺ cytotoxic T-lymphocyte epitope was identified in ESAT-6, a secreted antigen specific for *M. tuberculosis* complex but absent in BCG. Cytotoxic T-lymphocytes exhibited HLA-B52-restricted peptide-specific interferon-γ release and lytic activity and recognized endogenously processed antigen.

5. These studies demonstrate that CD8⁺ cytotoxic T-lymphocytes specific for mycobacterial and protozoal antigens are induced during natural infections in humans. The identification of these T-cells endorses current strategies to develop cytotoxic T-lymphocyte-inducing vaccines against *P. falciparum* and *M. tuberculosis* and highlights candidate antigens for inclusion in subunit vaccines.

INTRODUCTION

*Plasmodium falciparum* and *Mycobacterium tuberculosis* each account for more deaths worldwide than any other infectious pathogens [1]. The last decade has witnessed a global resurgence of malaria and tuberculosis and the rapid spread of multidrug resistance in both *P. falciparum* and *M. tuberculosis*. There is thus an urgent need for...
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Effective vaccines against these diseases, and the HIV pandemic has further heightened the requirement for an effective vaccine against tuberculosis suitable for use in immunocompromised individuals. The majority of people naturally exposed to these pathogens acquire protective immunity but the mechanisms underlying this are largely unknown. However, an understanding of the components of naturally acquired protective immune responses and their antigenic targets is a prerequisite for rational vaccine design.

*P. falciparum* and *M. tuberculosis* are intracellular pathogens and thus might potentially be amenable to recognition by CD8+ cytotoxic T-lymphocytes (CTLs), which constitute an essential element of host defence against viruses. In virus-infected host cells, viral proteins are made by the cell’s protein synthetic pathway and are processed through the MHC class I antigen processing pathway (Figure 1a). Short virus-derived peptides, 8–10 amino acids in length, are generated and presented in association with MHC class I molecules at the cell surface. CD8+ CTLs bearing T-cell receptors of appropriate specificitv recognize these peptide–MHC class I molecule complexes through their T-cell receptors and are thus triggered to destroy (or otherwise influence) infected host cells. In contrast, when pre-formed extracellular pathogen-derived proteins are endocytosed by monocyte-derived antigen presenting cells (e.g. macrophages, Kupffer cells, dendritic cells), they are processed through the MHC class II antigen processing pathway and the resultant peptides (13–20 amino acids in length)

are presented in association with MHC class II molecules at the cell surface (Figure 1b). However, in certain circumstances, extracellular proteins taken up by monocyte-derived cells can, despite their intralysosomal location, give rise to short peptides that are presented in association with MHC class I molecules at the cell surface [2,3]. *M. tuberculosis* and *P. falciparum*, in contrast to viruses, live within specialized intracellular cytoplasmic membrane-bound vacuoles where they synthesize proteins which are processed predominantly through the MHC class II antigen processing pathway. However, given the role of MHC class I-restricted CTLs in animal models of malaria and tuberculosis (see below), it is believed that, additionally, short peptides derived from *M. tuberculosis* and *P. falciparum* antigens are presented in association with MHC class I molecules at the infected cell surface.

Much evidence from animal models for both malaria and tuberculosis points to a protective role for MHC class I-restricted CTLs [4–8]. In humans, indirect evidence indicates that CTLs may mediate protection from disease [9–11], but identification of MHC class I-restricted CTLs specific for *P. falciparum* and *M. tuberculosis* in naturally exposed humans has proved difficult. We have adopted an HLA-based approach, using candidate peptide epitopes from selected antigens, to identify CTLs specific for *P. falciparum* and *M. tuberculosis*. Sensitive assays of effector function enabled us to detect CTLs in the peripheral blood of naturally exposed subjects.

Induction of MHC class I-restricted CD8+ CTLs with new generation CTL-inducing vaccines is now feasible. Our studies have identified antigens in *P. falciparum* and *M. tuberculosis* which are targeted by naturally acquired CTLs; these antigens could thus form the basis of new CTL-inducing vaccines. We have also applied these techniques to analyse the induction of cellular immune responses by candidate malaria vaccines in clinical trials.

### Selection of Target Antigens

The liver stage of the plasmodial life cycle is the point at which *P. falciparum* antigens are accessible to the MHC class I antigen processing pathway, through which pathogen-derived peptides could be presented to CD8+ CTLs. The pre-erythrocytic antigens which had been fully sequenced [12–15] were therefore selected as potential targets of CTLs: circumsporozoite protein (CSP), thrombospondin-related anonymous protein (TRAP), liver stage antigen-1 (LSA-1) and sporozoite threonine/asparagine-rich protein (STARp).

For *M. tuberculosis*, secreted antigens are strongly implicated as the targets of protective immunity [16] and two of these, ESAT-6 and the Antigen 85 Complex, were
selected. The *M. tuberculosis* complex-specific antigen ESAT-6 is a major target of CD4+ memory immune responses in immunized mice [17], whereas vaccination of mice with a DNA construct encoding Antigen 85B (one of three components of the Antigen 85 Complex) confers partial protection against challenge with live *M. tuberculosis* [18].

**PREDICTION OF CANDIDATE EPITOPES FROM MHC CLASS I PEPTIDE MOTIFS**

Pool sequence analysis of naturally occurring peptides eluted from MHC class I molecules reveals a strong bias for particular amino acids at certain positions in the MHC class I peptide-binding groove, and this bias is relatively specific for the MHC class I allele [19]. Analysis of pool sequence data identifies ‘anchor’ residues, usually at positions 2 and 9 in the peptide-binding groove; only certain amino acids are permissible at these residues in the peptide sequence if the peptide is to bind in the groove. These constraints on the sequence thus define a peptide motif for each class I molecule [19]; whether a given peptide is likely to bind a given MHC class I molecule can be broadly predicted by the extent to which the peptide sequence conforms to this motif. The identification of peptide motifs from an ever increasing number of HLA class I molecules enabled us to select eight HLA class I alleles with known peptide motifs that are common in the study population. The primary amino acid sequences of each of the selected antigens were scanned with the peptide motifs corresponding to HLA-A2.01, -A2.02, -B7, -B8, -B17, -B35, -B53 and, for the *M. tuberculosis* antigens, additionally HLA-B52. Peptides 8–10 residues in length that were congruent with any of these motifs were then synthesized or purchased. For malaria antigen-derived peptides, HLA assembly assays [20] were carried out to test which of the peptides (approximately 100 in total) actually bound their respective HLA class I molecule *in vitro*; peptides which bound were then used as candidate epitopes. For the *M. tuberculosis* antigen-derived peptides, *in vitro* binding was not assessed and all 49 peptides were used as candidate epitopes for further analysis.

**DETECTION OF CTLs FROM PERIPHERAL BLOOD BY USING CANDIDATE EPITOPES**

The use of candidate epitopes to identify CTLs is based on the rationale that if CTLs from the peripheral blood of humans naturally exposed to *P. falciparum* or *M. tuberculosis* recognize a peptide *in vitro* then these CTLs must have been previously primed by an encounter with this peptide *in vivo*. This in turn implies that the antigen from which the peptide epitope is derived has been processed through the MHC class I antigen processing pathway and presented by HLA class I molecules to CD8+ CTLs *in vivo*, thus identifying the antigen as a target of MHC class I-restricted CD8+ CTLs.

When a CD8+ T-cell encounters its cognate HLA class I molecule–peptide complex, the interaction of the T-cell receptor complex with its specific ligand may trigger the T-cell to display effector function. The major effector functions of CD8+ T-cells are lysis of the target cell and cytokine secretion. Two distinct assays were used to detect these two different kinds of effector function.

**CHROMIUM RELEASE CYTOTOXICITY ASSAY AND GENERATION OF CTLs IN VITRO**

The chromium release cytotoxicity assay detects lytic activity of *in vitro*-expanded CTLs. The read-out is based on the detection of radioactive ⁵¹Cr released by ⁵¹Cr-labelled target cells which have been lysed by CTLs during a 4–6 h incubation *in vitro*. The target cells are pulsed with the peptide that was used to stimulate and expand the CTLs *in vitro*. Lysis of such peptide-pulsed targets, compared with non-specific background lysis of negative control targets that have not been pulsed with peptide, represents the peptide-specific lytic activity of the CTL line being tested. Heterologous B-cell lines, matched at a single HLA class I allele with the donor from whom CTLs are generated, are used as targets. This allows detection of MHC class I-restricted, peptide-specific lytic activity.

Low levels of antigen-specific CTL precursors in peripheral blood cannot be directly detected by the chromium release cytotoxicity assay. Rather, these precursors must be expanded (and activated) *in vitro* to much higher numbers. Traditionally, this has been achieved using the pathogen itself (*e.g.* influenza virus) or vaccinia viruses recombinant for the antigen of interest. Our initial work, however, showed that this *in vitro* stimulation of antigen-specific CTLs is best achieved with cognate peptide [21]. CTLs were therefore generated with candidate peptide epitopes and the efficiency of *in vitro* stimulation was progressively optimized during the course of these studies [22].

**IDENTIFICATION OF CD8+ CTL EPITOPES IN PRE-ERYTHROCYTIC ANTIGENS OF *P. FALCIPARUM***

**West Africa**

Naturally exposed, healthy Gambians without acute malaria were studied and laboratory work was carried out at the MRC Laboratories in Fajara, The Gambia. *P. falciparum* transmission in The Gambia is seasonal and
Table 1 Peptides identified as CTL epitopes in P. falciparum in The Gambia

<table>
<thead>
<tr>
<th>HLA type</th>
<th>Label</th>
<th>Sequence</th>
<th>Antigen</th>
<th>Position (amino acids)</th>
<th>Variability</th>
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</thead>
<tbody>
<tr>
<td>HLA-B8</td>
<td>cp43</td>
<td>LRKPKHKKL</td>
<td>CSP</td>
<td>105–113</td>
<td>Conserved</td>
</tr>
<tr>
<td></td>
<td>tr42</td>
<td>ASKNKEKAL</td>
<td>TRAP</td>
<td>107–115</td>
<td>Conserved</td>
</tr>
<tr>
<td></td>
<td>tr43</td>
<td>KNKEKALII</td>
<td>TRAP</td>
<td>109–117</td>
<td>Conserved</td>
</tr>
<tr>
<td>HLA-B17</td>
<td>l53</td>
<td>KSLYDEHI</td>
<td>LSA-1</td>
<td>1854–1861</td>
<td>Conserved</td>
</tr>
<tr>
<td>HLA-B7</td>
<td>cp6</td>
<td>MPNDPNRNV</td>
<td>CSP</td>
<td>300–308</td>
<td>Variable</td>
</tr>
<tr>
<td></td>
<td>tr26</td>
<td>HLGNVKYLV</td>
<td>TRAP</td>
<td>7–11</td>
<td>Conserved</td>
</tr>
<tr>
<td></td>
<td>tr39</td>
<td>GIAGGLALL</td>
<td>TRAP</td>
<td>500–508</td>
<td>Conserved</td>
</tr>
<tr>
<td>HLA-A2.1</td>
<td>st8</td>
<td>MINAYLDKL</td>
<td>STARP</td>
<td>523–531</td>
<td>Undetermined</td>
</tr>
<tr>
<td>HLA-A2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

it is estimated that each Gambian receives 1–3 infectious mosquito bites per year (entomological inoculation rate = 1–3) (Greenwood, B. M., personal communication). Pioneering work in The Gambia had recently identified that the HLA class I allele, HLA-B53, is associated with protection from severe malaria [9], thereby implicating MHC class I-restricted CTLs as an important component of acquired protective immunity. Molecular analysis of this association demonstrated, for the first time, the presence of P. falciparum-specific CTLs in naturally exposed humans [10]. These HLA-B53-restricted CTLs were specific for the peptide ls6 in LSA-1 [10]. Our studies were carried out in 1994 on residents of the village Brefet, the same population that had been studied 2 years earlier. Candidate peptide epitopes from four pre-erythrocytic antigens (CSP, TRAP, LSA-1 and STARP) were now used to stimulate CTLs from the peripheral blood of naturally exposed donors with any of the seven HLA class I alleles we had selected. Forty naturally exposed, partially immune adults were studied and eight new CTL epitopes were identified (Table 1) [23]. Epitopes were present in each of the antigens studied indicating that, for the population studied, a range of pre-erythrocytic antigens is targeted by naturally acquired CTLs. Although levels of CTLs were low, the observation of HLA class I differential susceptibility to severe malaria suggests that these low levels of CTLs contribute to protective immunity; higher levels of CTLs which could be induced by immunization might be particularly effective [23].

East Africa

The low entomological inoculation rate in The Gambia of 1–3 would result in only 10–30 infected host cells annually through which to prime CTLs. Thus the number of infected host cells for priming CTLs is very low perhaps accounting for the observed low levels of CTLs induced. If this were the limiting factor, then P. falciparum-specific CTL activity should rise in areas with more intense transmission. CTL responses to the epitopes listed in Table 1 were therefore studied in Ifakara, Tanzania, an area with an entomological inoculation rate of over 300 [24], one of the highest recorded in the world. Thirty-five healthy adult residents of Ifakara were studied. Several conserved CTL epitopes were recognized and overall levels of CTL activity were similar to but slightly higher than those seen in The Gambia [25]. This indicates that the level of CTL activity (as measured by the $^{51}$Cr release cytotoxicity assay) induced by natural infection does not rise in direct proportion to the inoculum of sporozoites. A new epitope in TRAP, tr29 (LLMDCGSGI), was identified in this study and, using vaccinia viruses recombinant for the TRAP gene, it was shown for the first time that TRAP epitopes are endogenously processed and presented to CTLs [25].

Polymorphism in immunodominant malaria antigens is thought to pose a major problem for effective vaccine development. Importantly, the majority of epitopes identified in The Gambia are conserved and most of these are also recognized in partially immune Tanzanians [25]. Among the Tanzanians studied the prevalence of the seven common HLA class I alleles for which epitopes had been identified is little different to that in The Gambia and other sub-Saharan populations. The HLA class I types for which CTL epitopes were identified collectively cover about 70% of Tanzanians and Gambians. This, together with the fact that conserved epitopes were recognized by donors in both East and West Africa, suggests that with an array of epitopes covering a broad enough range of common HLA types, MHC restriction of immune responsiveness will not be a barrier to the use of an epitope-based CTL-inducing malaria vaccine in a variety of populations across the continent of Africa [25,26].

**DEVELOPMENT OF A MORE SENSITIVE, MORE PHYSIOLOGICALLY RELEVANT ASSAY FOR MEASURING CD8 + CTL ACTIVITY**

_in vivo_, cytokine secretion, in particular interferon-$\gamma$ (IFN-$\gamma$), is a more important effector function of CTLs against pathogenic viruses than lytic activity [27]. Moreover, IFN-$\gamma$, a potent activator of macrophages, is
essential for host defence against mycobacteria, and evidence now suggests that IFN-$\gamma$ secretion by CD8+ CTLs in tuberculosis is of more protective relevance than target cell lysis [28,29]. This, together with the need for a more sensitive assay for detecting low-level CTL activity than the standard $^{51}$Cr release cytotoxicity assay, led us to investigate the ELISPOT assay for IFN-$\gamma$ which measures cytokine secretion at the single cell level.

Based on the principle of a sandwich capture ELISA (Figure 2), the immobilized high-affinity first antibody captures IFN-$\gamma$ molecules in the immediate vicinity of the cell from which they are secreted, while still at a relatively high concentration. Subsequent processing of the assay (Figure 2) results in the formation of discrete spots, with each spot representing the ‘footprint’ of a single IFN-$\gamma$-secreting cell [30]. We demonstrated that the assay is an order of magnitude more sensitive than the $^{51}$Cr release cytotoxicity assay for detecting low levels of in vitro-cultured CTLs while the specificity is generally equivalent to the $^{51}$Cr release cytotoxicity assay (A. Lalvani, R. Brookes and A. V. S. Hill, unpublished work).

**THE EX VIVO ELISPOT ASSAY FOR IFN-$\gamma$ DEMONSTRATES THAT CD8+ MEMORY T-CELLS ARE CAPABLE OF RAPID EFFECTOR FUNCTION**

The remarkable sensitivity of the ELISPOT assay prompted us to apply it in a novel way for the study of freshly isolated, uncultured antigen-specific CTLs directly from peripheral blood. This adapted ex vivo assay can detect very low frequencies of circulating epitope-specific CD8+ T-cells without the need for in vitro stimulation and expansion. Freshly isolated CD8+ T-cells can be seen to secrete IFN-$\gamma$ upon antigen contact even when the duration of the assay is reduced to just 6 h [31]. The behaviour of freshly isolated T-cells in this assay affords insight into the natural activation state of T-cells in vivo, since the quantitative and qualitative biases introduced by in vitro restimulation are circumvented.

This modified ex vivo assay was validated in the influenza virus system. Of 29 healthy human donors who had not been exposed to influenza virus for at least 5 months and who were thus in the memory state with respect to influenza infection, 22 had CD8+ T-cells that secreted IFN-$\gamma$ in short ex vivo assays in response to a range of six different HLA class I-restricted influenza epitopes [31]. These CD8+ T-cells were CD45RO+ and IFN-$\gamma$ secretion was MHC class I-restricted and peptide-specific [31]. This finding is contrary to the traditional view that CD8+ memory T-cells need to divide and differentiate over time upon antigen encounter in vivo in order to become effectors. Rather, it appears that a population of CD8+ T-cells circulates in a relatively activated state wherein they can rapidly display effector function on contact with cognate antigen.

Furthermore, the ex vivo ELISPOT assay for IFN-$\gamma$ enumerates peptide-specific CD8+ T-cells directly from peripheral blood, consistently giving frequencies higher than limiting dilution analysis performed in parallel on the same subjects (Table 2) [31]. This is probably because limiting dilution analysis detects only those cells with a capacity to proliferate in vitro; the ex vivo ELISPOT, in contrast, can detect effector cells that may lack such proliferative potential. The phenotype of these relatively activated CD8+ memory T-cells, which have previously been overlooked by conventional assays based on in vitro stimulation, suggests that they play a role in protective immunological memory in vivo [31].

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**Figure 2** Principles of the ELISPOT assay
Table 2  Peptide-specific T-cell frequencies enumerated by ex vivo ELISPOT for IFN-γ and corresponding precursor frequencies by limiting dilution analysis (LDA) for influenza virus epitopes in a series of individuals

LDAs were assayed at 14 days culture except where marked. ∗8-day LDA; †18-day LDA, following second restimulation on day 14. Wells were scored positive if peptide-specific lysis was > 10% above background. Spontaneous release was < 30% of maximal release for all assays, except donor no. 1 (11/96) ( = 40%).

<table>
<thead>
<tr>
<th>Donor</th>
<th>1 (9/96)*</th>
<th>1 (11/96)†</th>
<th>2†</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA class I restriction</td>
<td>HLA-A2.01</td>
<td>HLA-A2.01</td>
<td>HLA-A2.01</td>
<td>HLA-A2.01</td>
<td>HLA-B8</td>
<td>HLA-A3</td>
</tr>
<tr>
<td>Sequence</td>
<td>GILGFVFTL</td>
<td>GILGFVFTL</td>
<td>GILGFVFTL</td>
<td>GILGFVFTL</td>
<td>ELRSRYWAI</td>
<td>ELRSRYWAI</td>
</tr>
<tr>
<td>Effector frequency in PBMC by ELISPOT</td>
<td>1/15000</td>
<td>1/111000</td>
<td>1/6000</td>
<td>1/77000</td>
<td>1/43000</td>
<td>1/45000</td>
</tr>
<tr>
<td>Precursor frequency in PBMC by LDA</td>
<td>1/100000</td>
<td>1/250000</td>
<td>1/59000</td>
<td>1/333000</td>
<td>1/200000</td>
<td>1/420000</td>
</tr>
</tbody>
</table>

HUMAN CD8+ CTLs SPECIFIC FOR M. TUBERCULOSIS

Using the ex vivo ELISPOT assay for IFN-γ, 39 subjects with suitable HLA class I alleles were studied: these comprised healthy contacts and patients representing the full clinical spectrum of disease. Patients were provided by clinical collaborators at Northwick Park Hospital, London, and the Churchill Hospital, Oxford, and subjects comprised three major ethnic groups: Asians from the Indian subcontinent, Africans and white Caucasians, with the Asians constituting the largest group. If responses were detected in the ex vivo ELISPOT assay to any of the peptides from Antigen 85 or ESAT-6, peripheral blood lymphocytes were then repeatedly stimulated in vitro with the peptide in order to generate CTL lines and clones which could be further characterized. In this way, the epitope ES12 (LQNLARTI) from ESAT-6 (amino acid residues 69–76) was identified [32]. This peptide is recognized by CD8+ T-cells in an HLA-B52-restricted manner [32]. CTLs specific for this epitope were detected in the two HLA-B52-positive patients available for study: one was an Indian woman with tuberculous mediastinal lymphadenitis and the other an Afghan male with culture-positive tuberculous osteomyelitis of the hand. For both patients it was shown that CTL lines not only secreted IFN-γ but also lysed target cells in 51Cr-release cytotoxicity assays [32]. By using a vaccinia virus recombinant for the ESAT-6 gene to infect target cells it was demonstrated that the ES12-specific CTLs also recognize endogenously processed antigen (Figure 3) [32]. No CD8+ epitopes were identified in Antigen 85, although three CD4+ epitopes were detected and each of these lies within regions of Antigen 85 previously described as targets of CD4+ lymphoproliferative responses [32, 33].

Circulating activated ES12-specific CD8+ T-cells were detected by the ex vivo ELISPOT assay in the two patients with HLA-B52 at a frequency that approximates to that of influenza peptide-specific CD8+ T-cells in healthy subjects. The detection of these ES12-specific CD8+ T-cells directly from peripheral blood provides evidence that in humans an M. tuberculosis antigen is naturally processed in vivo through the MHC class I pathway leading to the induction of MHC class I-restricted effector T-cells [32]. The phenotype and specificity of the cells identified here endorses efforts to develop CTL-inducing vaccines against tuberculosis and also supports the candidacy of ESAT-6 as a component of such vaccines.

WHAT IS THE ROLE OF P. FALCIPARUM AND M. TUBERCULOSIS-SPECIFIC CTLs IN VIVO IN HUMANS?

The reverse immunogenetic approach is very powerful for detecting peptide-specific CTLs, but since it is, by definition, based on detecting CTLs specific for selected antigens restricted through a finite number of particular HLA class I HLA types, the CTLs that are identified represent only a small sample of the total population of pathogen-specific CTLs circulating in peripheral blood. The more antigens and HLA types that are studied, the more
complete will be the picture of CTL activity, but the approach will always tend to underestimate the overall breadth of CTL activity directed against a given pathogen in vivo. Moreover, the identification of antigen-specific CTLs does not tell us (i) how these cells interact with the whole, live pathogen and (ii) how they function in vivo.

For ES12-specific CTLs from tuberculosis patients we have recently demonstrated, in an ELISPOT assay for IFN-γ, direct recognition of live M. tuberculosis-infected macrophages (R. Brookes, A. Lalvani, A. A. Pathan and A. V. S. Hill, unpublished work). Analogous studies for malaria are not yet feasible since culture of the liver stage of P. falciparum in human hepatocytes cannot be reliably achieved.

Our results to date do not provide any direct evidence of how CTLs function in vivo, in particular as to whether or not CTLs are protective in humans. However, the fact that certain CTL epitopes in CSP exhibit allelic polymorphism, and some of these naturally occurring variants can escape recognition by CTLs while others can actually antagonize CTL responses to the index epitope (wild-type allele) [25,34], suggests that in vivo P. falciparum-specific CTLs exert a selective pressure on their target parasites. There is, as yet, no evidence for similar CTL escape mechanisms in tuberculosis but large numbers of field isolates of M. tuberculosis have not hitherto been genotyped for the recently identified CTL target antigen ESAT-6.

Further evidence for a protective role for CTLs is being sought by comparing frequencies of ESAT-6-specific CTLs between individuals with different clinical phenotypes of M. tuberculosis infection (e.g. exposed healthy contacts versus patients with pulmonary tuberculosis). In the case of malaria, we are searching for correlates of protective immunity in clinical vaccine trials where experimental vaccines have induced sterile protective immunity in a proportion of volunteers. This is discussed further below.

IMMUNOGENICITY STUDIES OF NOVEL VACCINES USING THE EX VIVO ELISPOT ASSAY FOR IFN-γ

Much evidence from animal models suggests that antiviral protective immunological memory in vivo is dependent on activated circulating T-cells capable of rapid effector function [35]. In contrast, levels of CTL precursors, estimated by techniques dependent on in vitro proliferation such as limiting dilution analysis, do not correlate with protection [35]. High levels of activated effector cells can be readily induced and measured in murine models but do not occur naturally in humans except during acute infections – and low levels of such cells are not detectable by conventional means [31]. This is probably because a proportion of these cells lack proliferative potential and thus are not amenable to in vitro expansion [31]. Protective efficacy of novel vaccines may depend on their ability to induce such effector cells yet the levels induced by vaccination may be too low to detect with standard assays. The ex vivo ELISPOT assay for IFN-γ is able to detect low levels of activated CD8+ and CD4+ T-cells directly from peripheral blood and is thus well suited to the analysis of the immunogenicity of new vaccines.

We have applied the ex vivo ELISPOT assay to track the induction of effector CD4+ and CD8+ CSP-specific T-cells in 10 malaria-naïve volunteers before and after each of three doses of the experimental recombinant malaria vaccine RTS,S. This fusion protein of hepatitis B surface antigen and the carboxy-terminal end of CSP is the most protective malaria vaccine candidate to date [36]. It is a potent inducer of humoral immune responses but these do not correlate with protection. Analysis of the antigen-specific cellular immune responses induced, and the temporal course over which effector cells appear after immunization and subsequently wane, is now helping to unravel the correlates of protective immunity to P. falciparum malaria.

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