Antibody targeted MHC complex directed expansion of HIV-1 and KSHV-specific CD8 positive lymphocytes: a new approach to therapeutic vaccination.

Justin Stebbing1, Brian Gazzard1,2, Steve Patterson1, Mark Bower2, Dhayaneethie Perumal1, Mark Nelson3, Andrew McMichael1, Graham Ogg3, Agamemnon Epenetos4, Frances Gotch1 and Philip Savage4,5.

1. The Department of Immunology, Division of Investigative Science, Faculty of Medicine, Imperial College of Science, Technology and Medicine, The Chelsea and Westminster Hospital, 369 Fulham Road, London SW10 9NH, United Kingdom

2. The Departments of HIV Medicine and Oncology, The Chelsea and Westminster Hospital, 369 Fulham Road, London SW10 9NH, United Kingdom

3. Immunology Group, Weatherall Institute of Molecular Medicine, Oxford, OX3 9DS, United Kingdom

4. Alexis Biotech Ltd 81 Harley St, London W1N 9DE, United Kingdom

5. Department of Oncology, Velindre Hospital, Cardiff, CF14 2TL, United Kingdom

Address for correspondence:

Dr Philip Savage
Department of Oncology
Velindre Hospital
Whitchurch
Cardiff
CF14 2TL
United Kingdom
Tel: 029-20615888 x 2297
Fax: 029-20529625
E mail Philip.Savage@velindre-tr.wales.nhs.uk
Abstract

The ability of therapeutic vaccines to generate large numbers of CD8 positive T lymphocytes that have specificity for HIV-1 or other virally infected cells has enormous potential clinical value. However approaches to produce CTLs in vivo via vaccine technology have thus far been disappointing and the ex vivo production of cells for adoptive transfer is labour intensive and expensive. We describe the results of a two step antibody targeting system for the production of CD8 positive T lymphocytes specific for HIV-1 and Kaposi’s sarcoma-associated herpesvirus (KSHV), suitable for use in vivo. In 8 consecutive HLA-A2 positive HIV-1 infected individuals with Kaposi’s sarcoma, two cycles of this system resulted in greater than 1 Log increases of specific anti-HIV and anti-KSHV CD8 positive lymphocytes. These expanded cells have an effector phenotype which includes the ability to produce interferon-γ and CD45RA⁺/CD69⁺ staining. We have shown that antibody targeted B cells can function as effective antigen presenting molecules and lead to sustained specific T lymphocyte expansion from PBMCs of immunosuppressed individuals. This approach, which offers an easy and effective protocol for the amplification of specific anti-viral and anti-tumour CTLs, may offer significant advances for in vivo T cell immunotherapeutic protocols.

Keywords: HIV, therapeutic vaccine, KSHV, monomer, fusion protein.
Introduction

HIV presents unprecedented and formidable challenges to vaccine design \(^1\,^2\) and many consider the goal of sterilising immunity to be unrealistic \(^3\). As such, the development of more effective treatment strategies and approaches to long term infection has intensified. In comparison to normal cells, virally infected cells and many tumour cells have recognisable differences in the immunogenic peptides that they display in the grooves of their major histocompatibility complex (MHC) class I molecules. Such immunological differences and the description of many viral peptides \(^4\) may enable effective T cell mediated immunotherapy with a limited expanded population of autologous T cells \(^5\), the goal of such therapy being the immune clearance of virally infected cells.

It has been argued in HIV, that vaccines that induce such CTLs will prove efficacious, provided that responses are of high specificity and magnitude \(^6\). This hypothesis results from the evidence suggesting that following infection, CTLs are able, for some time, to control viral replication. Such data includes data that show: (i) that there is a rise in viral load occurring after CD8-blocking antibodies are infused into macaques infected with simian immunodeficiency virus (SIV) \(^7\), (ii) that viral mutation resulting in escape from CD8+ T cell recognition occurs in HIV and SIV infections \(^8\,^9\,\,^{10}\), (iii) that long term non progressors maintain good CD8 responses \(^11\,^12\) and, (iv) that sex workers who are frequently exposed to HIV and high viral loads seem to be resistant to progressive HIV infection and generate anti-HIV responses through CD8+ T cells \(^13\).

The current standard approach to generating antigen-specific CTLs involves their isolation and culturing \textit{in vitro} utilising significant quantities of autologous dendritic cells. Oelke \textit{et al.} have recently reported that human leukocyte antigen-immunoglobulin (HLA-Ig)-based artificial antigen presenting cells (aAPCs) support the expansion of both high and low affinity CTLs against cytomegalovirus and melanoma respectively \(^14\). Their system couples a soluble HLA-Ig fusion protein and CD28-specific antibody to beads resulting in antigen-specific CTL expansion \textit{in vitro} over successive rounds of
stimulation. These and other related approaches invariably involve expensive, labour intensive and cumbersome ex vivo production of large numbers of CTLs for potential adoptive transfer, a process susceptible to contamination at every step 15-19.

It has been previously demonstrated that HLA class I viral peptide complexes targeted to B cells can stimulate specific CTL expansion in vitro 20. Additionally, B cells which are present in large numbers even in immunocompromised patients can act effectively as antigen presenting cells inducing specific CTL responses in vivo 21,22. Here, using B cells as antigen presenting cells, we have examined the expansion of specific CTLs against conserved class I restricted HIV-1 and Kaposi’s sarcoma-associated herpesvirus epitopes using this two-step antibody HLA class I/peptide complex delivery system. This procedure, using ex vivo PBMCs obtained from HIV infected donors should generate large numbers of specific CD8 positive T lymphocytes and potentially be a system suited to the generation of such cells in vivo.

Patients and methods

Patients
Between February – July 2003, we recruited 8 consecutive HIV-1 infected individuals with AIDS-related Kaposi’s sarcoma, known to be HLA-A2 positive (Table 1). PBMCs were isolated from 40 mLs of venous blood using Ficoll-Histopaque® (Sigma, Poole, UK) density gradient centrifugation. All patients provided written informed consent and the study received the appropriate ethical approval.

Ex vivo immunisation procedure
5 x 10^6 PBMCs were incubated with the B9E9 scFvSA fusion protein (10 µg/mL) for one hour at 4°C. The B9E9 scFvSA fusion protein contains the single-chain variable region of the murine IgG2a anti-CD20 murine antibody B9E9 fused to the genomic streptavidin of Streptomyces avidinii. The protein is secreted into the periplasm of genetically engineered E. coli as monomeric subunits (43,400 Daltons) that spontaneously fold into
soluble tetramers with a molecular weight of 173,600 Daltons. The four antigen-binding and biotin-binding sites of the fusion protein retain the functional capabilities of the parent molecules. The fusion protein was a kind gift of Dr J Schultz (Neorx Corporation, Seattle, USA).

After washing in PBS, cells were incubated with the biotinylated HLA class I/peptide complex (0.5 µg/mL) for 30 minutes at room temperature. Biotinylated recombinant HLA-A2 class I monomers contained for each patient either the HLA-A*201 restricted Gag peptide (SLYNTVATL) or KSHV glycoprotein B peptide (LMWYELSKI) and were obtained from ProImmune Ltd (Oxford, UK). After further washing, cells were placed into 24 well plates at 2.5 x 10^6 PBMCs and cultured in RPMI with 10% fetal calf serum, L-glutamine and penicillin/ streptomycin (see cartoon in Figure 1). Interleukin-7 (R&D systems, Minneapolis, MN) was added on day 1 at 10ng/mL, and IL-2 (Chiron, Harefield, UK) was added at 10 U/mL on day 4, as described previously. For further stimulation, fresh PBMCs were obtained from the same patients at weekly intervals and treated as above. The new autologous ‘immunised’ cells were then mixed with the existing cells at a 1:2 ratio and culturing continued for a further 7 days in 5% CO₂ at 37°C.

Flow cytometry and ELIspot assays
Three colour flow cytometric analysis was performed on 5x10⁵ PBMC removed from the ex vivo culture. These were labelled with PE conjugated tetramers containing either the HIV-1 gag, KSHV glycoprotein B, or control peptides (ProImmune) and PercP conjugated anti- CD8 (BD Biosciences, Oxford, UK). Further phenotyping of cells was performed using FITC conjugated anti-CD27 and anti-CD45Ra (Pharminingen, Oxford, UK) and APC conjugated anti-CD27 (Pharminingen) anti-CD69 (Dako, Ely, UK). A minimum of 100,000 cells were acquired in the live gate and analysed on a Becton Dickinson FACScaliber using Cell Quest® software. Appropriate isotype control antibodies were used in each case.
IFN-γ ELIspot assays were carried out as previously described (MABTECH, Stockholm, Sweden)\(^{26,27}\), in triplicate for each patient tested. The HIV-1 gag and KSHV glycoprotein B peptides were incubated at 5 \(\mu\)M with 2 x 10^5 PBMCs. Results were considered positive if the number of spot forming cells (SFCs) per million PBMCs in peptide-stimulated wells was two-fold higher than the number of spots per million PBMCs in control wells and at least 50 spots per million PBMCs were present.

HIV-1 negative and positive individuals lacking HLA-A2 were used as controls and showed no tetramer positive staining. HLA class I typing was performed by the Anthony Nolan Trust (Royal Free Hospital, London, UK) using amplification refractory mutation system-PCR with sequence-specific primers \(^{28}\). Plasma HIV-1 viral loads were determined by the Bayer HIV-1 RNA 3.0 (bDNA) Assay (Berkshire, UK) or by PCR assay (Cobas Amplicor HIV-1 Monitor test version 1.5, Roche Diagnostics, UK) with a lower level of detection of 50 HIV-1 RNA copies/mL. Absolute CD4 and CD8 counts (cells/mm\(^3\)) were obtained by flow cytometry (BeckmanCoulter, Oxford, UK).

### Results and discussion

The lack of an optimal method to produce antigen specific CTLs has hindered the further development of adoptive immunotherapy. Here, using B cells targeted with HLA class I/peptide complexes as antigen presenting cells, we show the rapid expansion of specific CD8 +ve lymphocytes against known HIV and Kaposi’s sarcoma-associated herpesvirus epitopes using PBMCs from HIV-1 positive individuals. The first step involves the delivery of an anti-CD20 B9E9 fusion protein to the surface of B cells. Step 2 involves the delivery of recombinant biotinylated HLA class I peptide, containing either the HLA-A*201 restricted Gag peptide (SLYNTVATL) or KSHV glycoprotein B peptide (LMWYELSKI \(^{24}\)). Both steps are achieved using whole PBMCs without the need for any further cell separation (Figure 1).
In 8 consecutive patients with differing stages of AIDS-related Kaposi’s sarcoma (Table 1), two successive cycles resulted in the sustained generation of large numbers of CD8 +ve lymphocytes, specific for HIV or KSHV, in every case (Figure 2A – 1C). The numbers of KSHV specific cells increased from 2.1% (interquartile range 1.2 – 4.3) to 25.7% (16.3 – 36.1) and the numbers of HIV-1 specific cells increased from 1.3% (0.92 – 2.0) to 17.8% (12.0 – 42.1). We have further identified the HIV or KSHV-tetramer positive CD8 +ve lymphocytes as effector cells, based on their CD69+, CD45Ra+, CD27- and CD28- phenotype (Figure 2B) and these cells have functional activity, as assessed in IFN-γ ELIsps (Figure 3).

The specificity of the T cells produced was further investigated in tetramer and ELIspot analysis using control peptides not corresponding to the specific peptide used in the original monomer. In these experiments, the number of spot forming colonies in ELIspots did not rise above baseline levels supporting the specific expansion of T cells recognising only the HLA class I/peptide complex used in the expansion. Similarly staining with HLA-A2/tetramer containing control peptides showed no increase over pre-stimulation base line levels (data not shown).

The responses observed in the ELIspots, greater than a median of 700 IFN-γ ELISpots/10^6 PBMCs, are 2-3 times greater than the proposed peak responses required for an effective HIV vaccine (300 spots/10^6 PBMCs) based on previous data from non-human primates.

HIV is known to induce a wide array of B cell dysfunctions including ineffective B cell costimulatory function with associated low expression of the CD28 ligands, CD80 and CD86. However, it is clear from these results that the B cells targeted with the HLA class I/peptide complexes are able to function as effective antigen presenting cells suggesting that this two step system appears to overcome any effects of reported perturbations in B cell function.
The efficiency of CTL induction has previously been shown to be related to the stability of the MHC class I/peptide complex on the surface of antigen presenting cells. In this system, we have aimed to optimise the number and stability of expression of the complexes, by using fusion proteins and monomers with long half lives in the context of a high affinity binding system to a non-internalising B cell marker. In a number of CTL expansion systems, a relationship between the number of MHC peptide complexes and the level of the T cell response has been demonstrated and there are also data suggesting that excess levels of MHC class I/peptide complexes may also result in apoptosis rather than the CTL expansion as shown here.

In this system, CD20 is present at approximately 87,000 copies per B cell surface. Using the antibody used at our concentrations, its affinity constant and considering an average of 3.6 biotin binding sites per antibody, the potential for numbers of a single MHC class I/peptide complexes in the region of 200,000-300,000 on each B cell surface may occur. This is significantly higher than levels produced by peptide pulsing which results in peptide placement in a maximum of 5000 MHC class I molecules per cell, out of a total number of 100,000 on the surface. The functional presence of these recombinant complexes has been shown previously to extend to at least 72 hours and is significantly greater than the median half life of 2.5 to 4 hours of complexes produced with peptide pulsing. This enhance stability combined with their increased number suggests that this method could be expected to enhance the strength and duration of T cell activation.

This new approach to generating CD8 positive T lymphocytes for HIV therapy appears effective in vitro across individuals with a wide range of CD4-lymphopenia and HIV-viremia. Whilst to date we have we have only looked at this preliminary study ex vivo, the technology we describe lends itself to in vivo work as the B9E9 antibody has already been shown to be safe in patients and similar but non-targeted MHC/peptide complexes have been shown to be both immunogenic and non-toxic in mice. As we have only been able to show limited expansion to the first round of stimulation (Figure 2C), these data would also argue that clinical trials with this system should allow for at least 2
cycles. In addition to HIV and KSHV, this system that could produce the simple and effective expansion of CTLs *in vivo* may also be of value in other chronic viral infections treated with adoptive immunotherapy such as in CMV $^{44,45}$ and EBV $^{19}$ and also in diseases such as melanoma where currently *ex vivo* expansion of CTLs and subsequent re-infusion is showing potential $^{46}$.

In summary we have demonstrated that B cells targeted with HLA class I/peptide complexes can serve as effective antigen presenting molecules and the CD8 positive T lymphocytes expanded have an effector phenotype as demonstrated by interferon-γ production and CD27/28/45Ra/69$^+$ staining. Anti viral and anti-tumour vaccination procedures based on targeting HLA class I/ peptide complexes to B cells *in vivo* via the antibody delivery system could offer significant advances in both the applicability and effectiveness of such ‘CTL expanding’ approaches.
Figure legends:

**Figure 1**
A cartoon demonstrating the 2 step antibody targeted delivery of biotinylated HLA class I/peptide complexes to CD20 on the surface of B cells, as used in these experiments.

**Figure 2**

* a. Tetramer analysis from a single patient demonstrating the increase in specific CTLs to HIV-1 and KSHV over the 2 cycles of targeted therapy *in vitro*.

* b. The tetramer positive CD8 positive T cells (R2 cycle 2) were further phenotyped and found to express CD45Ra and CD69 with absent CD27 and CD28.

* c. The median percentage of tetramer positive CD8 positive cells at background, after one cycle and following 2 cycles of amplification (responses for HIV-1 Gag = red, KSHV glycoprotein B = blue; the median for all 8 patients is shown with the interquartile range).

**Figure 3**

Spot forming colonies per million PBMCs in IFN-γ ELISpot assays before and after 2 cycles. Median responses to gag (red) and KSHV gb (blue) peptides are shown (+SEM). Responses were specific as demonstrated by a lack of increased responses to other HLA-A2 restricted epitopes.
Table 1. Patient characteristics of 8 ‘heterogenous’ individuals used in this study with AIDS-related Kaposi’s sarcoma (KS). ACTG is the AIDS Clinical Trials Group Oncology Committee 47, where (briefly) tumor status (0 = cutaneous only, 1 = more than cutaneous disease) and immunological status (0 = CD4 count < 150, 1 = CD4 > 150 cells/mm\(^3\)) are used to stage KS. HAART = highly active anti-retroviral therapy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sites of KS</th>
<th>CD4 count cells/mm(^3)</th>
<th>CD8 count cells/mm(^3)</th>
<th>HIV-1 viral load copies/mL</th>
<th>Therapy</th>
<th>ACTG staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pulmonary</td>
<td>160</td>
<td>754</td>
<td>&lt; 50</td>
<td>HAART, paclitaxel</td>
<td>T1I0</td>
</tr>
<tr>
<td>2</td>
<td>Cutaneous</td>
<td>229</td>
<td>622</td>
<td>13,291</td>
<td>HAART</td>
<td>T0I1</td>
</tr>
<tr>
<td>3</td>
<td>Pulmonary</td>
<td>48</td>
<td>330</td>
<td>128,471</td>
<td>Paclitaxel</td>
<td>T1I0</td>
</tr>
<tr>
<td>4</td>
<td>Visceral and pulmonary</td>
<td>111</td>
<td>289</td>
<td>59,225</td>
<td>Liposomal daunorubicin</td>
<td>T1I0</td>
</tr>
<tr>
<td>5</td>
<td>Oral</td>
<td>354</td>
<td>754</td>
<td>&lt; 50</td>
<td>HAART, paclitaxel</td>
<td>T1I1</td>
</tr>
<tr>
<td>6</td>
<td>Cutaneous</td>
<td>50</td>
<td>99</td>
<td>510</td>
<td>HAART</td>
<td>T0I1</td>
</tr>
<tr>
<td>7</td>
<td>Cutaneous</td>
<td>505</td>
<td>358</td>
<td>&lt; 50</td>
<td>HAART</td>
<td>T0I0</td>
</tr>
<tr>
<td>8</td>
<td>Cutaneous</td>
<td>497</td>
<td>627</td>
<td>&gt; 500,000</td>
<td>None</td>
<td>T0I1</td>
</tr>
</tbody>
</table>

Acknowledgments

JS is an MRC funded PhD student (grant number G84/ 5631). We are very grateful to the patients who provided samples.
Figure 1.
Figure 2A. An increase in tetramer positive CD8 positive cells in an individual patient with AIDS-related KS. FL3 (x-axis) is CD8, FL2 (y-axis) corresponds to the tetramer.

Figure 2B. The cells in R2 above (cycle 2) are CD27 - CD28 - and express CD45Ra and CD69.

Figure 2C. % of gag (red) and KSHV gb (blue) tetramer positive CD8 positive CTLs with each cycle (median of 8 patients and interquartile range shown).
Figure 3. Spot forming colonies per million PBMCs in IFN-γ ELISpot assays before and after 2 cycles. Median responses to gag (red) and KSHV gb (blue) peptides are shown.


Connors M. HIV-specific CD8(+) T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat Immunol. 2002;3:1061-1068.


Antibody targeted MHC complex directed expansion of HIV-1 and KSHV-specific CD8 positive lymphocytes: a new approach to therapeutic vaccination

Justin Stebbing, Brian Gazzard, Steve Patterson, Mark Bower, Dhayneethie Perumal, Mark Nelson, Andrew McMichael, Graham Ogg, Agamemnon Epenetos, Frances Gotch and Philip Savage