CD4+ T cells specific for glycoprotein B from cytomegalovirus exhibit extreme conservation of T-cell receptor usage between different individuals

Laura Crompton,[1] Naeem Khan,[2] Rajiv Khanna,[3] Laxman Nayak,[4] and Paul A. H. Moss[1]

[1]Cancer Research UK (CRUK) Institute for Cancer Studies, University of Birmingham, Birmingham, United Kingdom; [2]Division of Immunology, School of Infection & Host Defence, University of Liverpool, Liverpool, United Kingdom; [3]Division of Infectious Diseases and Immunology, Queensland Institute Medical Research, Queensland, Australia; and [4]Department of Applied Gerontology, Selly Oak Hospital, Birmingham, United Kingdom

Antigen-specific CD8+ cytotoxic T cells often demonstrate extreme conservation of T-cell receptor (TCR) usage between different individuals, but similar characteristics have not been documented for CD4+ T cells. CD4+ T cells predominantly have a helper immune role, but a cytotoxic CD4+ T-cell subset has been characterized, and we have studied the cytotoxic CD4+ T-cell response to a peptide from human cytomegalovirus glycoprotein B presented through HLA-DRB*0701. We show that this peptide elicits a cytotoxic CD4+ T-cell response that averages 3.6% of the total CD4+ T-cell repertoire of cytomegalovirus-seropositive donors. Moreover, CD4+ cytotoxic T-cell clones isolated from different individuals exhibit extensive conservation of TCR usage, which indicates strong T-cell clonal selection for peptide recognition. Remarkably, this TCR sequence was recently reported in more than 50% of cases of CD4+ T-cell large granular lymphocytosis. Immunodominance of cytotoxic CD4+ T cells thus parallels that of CD8+ subsets and suggests that cytotoxic effector function is critical to the development of T-cell clonal selection, possibly from immune competition secondary to lysis of antigen-presenting cells. In addition, these TCR sequences are highly homologous to those observed in HLA-DR7+ patients with CD4+ T-cell large granular lymphocytosis and implicate cytomegalovirus as a likely antigenic stimulus for this disorder. (Blood. 2008;111:2053-2061)

Introduction

The cytotoxic T-cell response has evolved primarily to control viral infection through the recognition of peptides derived from intracellular proteins. CD8+ cytotoxic T cells recognize peptides presented by human leukocyte antigen (HLA) class I molecules, and the repertoire of peptides recognized by CD8+ T cells is generally much more restricted than those that elicit a CD4+ T-cell response.[1] In addition, comparison of T-cell receptor usage from CD8+ cytotoxic T-cell clones taken from different individuals often shows marked conservation of T-cell receptor (TCR) structure, indicating a high degree of clonal selection in vivo. The precise factors that determine such immunodominance remain uncertain but include selection of high-avidity T-cell clones through competition for antigen at the surface of the antigen-presenting cell. These findings have important implications for attempts to enhance CD8+ T-cell cytotoxicity through vaccination or adoptive T-cell transfer.

Cytotoxic CD4+ T cells have many phenotypic and functional features in common with CD8+ cells, including expression of perforin and granzyme, and are likely to play a role in the elimination of viral infection from antigen-presenting cells that express HLA class II.[2,3] At the current time, little is known as to the degree of clonal restriction within cytotoxic CD4+ T cells.

Cytomegalovirus (CMV) is a ubiquitous pathogen that infects the majority of the human population.[4] The virus is never cleared from the host, and viral latency results from sustained immune control of lytic viral replication. The CD8+ T-cell response to CMV is among the largest that has been documented to any antigen and can exceed 40% of the CD8+ T-cell repertoire in elderly individuals.[5] Less is known about the CD4+ cytotoxic T-cell response to CMV, although its role in both murine and human viral infection is now being investigated.[6-8]

CD4+ T cells usually recognize an antigen that has been taken up by the antigen-presenting cell rather than presented directly from cytosol. Thus, virally infected cells might be unable themselves to present antigens through HLA class II molecules, which could limit the sterilizing potential of a cytotoxic CD4+ T-cell response. However, recent evidence has indicated that CMV glycoprotein B (gB) can be presented directly from infected cells, and gB-specific CD4+ T cells are able to lyse infected HLA class II+ targets without the need for cross-presentation.[9,10] Characterization of these T-cell responses identified the immunodominant peptide epitope DYSNTHSTRYV from gB, hereafter referred to as DYS, which is restricted through HLA-DRB*0701.[10]

Here, we have studied the T-cell response to DYS peptide in healthy individuals and show extreme conservation of T-cell receptor usage in both the TCRα and TCRβ chains. This pattern of TCR selection was directly comparable with that seen in previous studies of CD8+ cytotoxic T-cell immune responses and is the first example of TCR clonal conservation within CD4+ T-cell immune responses. Moreover, this TCR sequence is extremely homologous to that observed in cases of large granular lymphocytosis (LGL) in patients with the HLA-DRB*0701 allele[11] and suggests that CMV can act as an antigenic stimulus for the initiation or maintenance of the LGL expansion.

Submitted April 4, 2007; accepted October 12, 2007. Prepublished online as *Blood* First Edition paper, November 6, 2007; DOI 10.1182/blood-2007-04-079863.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

An Inside *Blood* analysis of this article appears at the front of this issue.

Methods

Institutional Review Board approval was granted by the South Birmingham Regional Ethics committee, and informed consent was obtained in accordance with the Declaration of Helsinki.

Cell lines

Human fetal fibroblasts (HFFs) were obtained from the European Collection of Animal Cell cultures and were maintained in the media described above. HFFs were used for virus propagation in vitro.

Primary human fibroblasts derived from skin biopsies were grown in Dulbecco modified Eagle medium (Invitrogen, Paisley, United Kingdom) supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. Fibroblasts were activated to upregulate HLA expression by addition of 300 U/mL interferon-γ (IFN-γ; Sigma-Aldrich, Poole, United Kingdom) to culture medium 3 days prior to use as antigen-presenting cells.

Peripheral blood mononuclear cells (PBMCs) were separated from healthy, CMV-seropositive individuals by Ficoll-Hypaque centrifugation into RPMI 1640 medium (Invitrogen) supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. Where specified, PBMCs were depleted of $CD8^+$ T cells using anti-CD8 antibodies (BD Biosciences, Oxford, United Kingdom) and anti-phycoerythrin (anti-PE) microbeads (Miltenyi Biotech, Surrey, United Kingdom) and magnetic selection in accordance with the manufacturer's recommended protocol.

Dendritic cells (DCs) were prepared by 6-day culture of adherent PBMCs in the above medium supplemented with granulocyte-macrophage colony-stimulating factor (Peprotech, London, United Kingdom) and interleukin-4 (R&D Systems, Minneapolis, MN), and then maturation for 24 hours in 50 ng/mL tumor necrosis factor-α (Peprotech). Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines (LCLs) were prepared from PBMCs with prototype 1 strain B95.8 and were cultured in the medium described above with cyclosporin A.

Antigens and viruses

CMV strains AD169 and RV798 were kind gifts from Gavin Wilkinson (Cardiff, United Kingdom) and Tom Jones (Wyett Research Institute), respectively. CMV lysate was produced from AD169-infected HFFs after 2 weeks of infection in vitro. Recombinant modified vaccinia virus Ankara (MVA) expressing gB was created as described previously. DYS peptide was synthesized by 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience, University of Birmingham, Birmingham, United Kingdom) dissolved in dimethyl sulfoxide (DMSO) in 10-mg/mL stock solutions.

T-cell cloning

CD4 T-cell clones were generated by stimulating freshly isolated PBMCs with DYS peptide (5 μg/mL) for 3 hours. Antigen-specific cells were then selected using the IFN-γ Cytokine Capture Assay (Miltenyi Biotech) in accordance with the manufacturer's protocol. Magnetically selected cells were cloned by limiting dilution at 0.3 and 3 cells per well with autologous or HLA-matched, gamma-irradiated LCL loaded with DYS peptide (10^4/well) and allogeneic gamma-irradiated, phytohemagglutinin-treated PBMCs (10^5/well) in RPMI supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 100 U/mL interleukin-2 (Chiron, Emeryville, CA), and 10% human AB serum (HD Supplies, Aylesbury, United Kingdom).

Chromium release assays

$CD4^+$ T-cell clones and PBMCs were tested for cytotoxicity in 6-hour chromium release assays. Effector-target ratios were 2.5:1 unless otherwise stated. Targets were autologous, partially HLA matched or HLA mismatched LCLs, DCs, or IFN-γ–activated fibroblasts. Targets were pulsed with 2 μg/mL peptide for 2 hours prior to the assay (DMSO solvent was used for control targets) or pulsed overnight with CMV lysate before the assay. Virus infections with MVA-gB or MVA-pSC11 (at multiplicity of infection [MOI] of 2:1) were carried out 24 hours before assays, and infections with CMV strains AD169 or RV798 (at MOI 5:1) were for 48 hours prior to the assay. Supernatants were sampled and γ-emission quantified using a Packard Cobra gamma counter (Packard, Berks, United Kingdom). Results were expressed as percentage of specific lysis of the target cell line. For inhibition of perforin, DYS-specific clones were incubated with 100 nM concanamycin A (ConA; Sigma) for 30 minutes before the addition of target cells. The assay was then carried out as described.

ELISA of IFN-γ release

$CD4^+$ T-cell clones were incubated with autologous, HLA-matched or HLA-mismatched LCLs that had been preexposed for 2 hours to peptide (2 μg/mL, unless otherwise stated) or preexposed overnight to CMV lysate or infected with MVA-gB or MVA-pSC11 at an MOI of 2:1 at 24 hours prior to use. The supernatant medium was harvested after 18 hours and assayed for IFN-γ by ELISA (Endogen; Rockford, IL) in accordance with the manufacturer's recommended protocol.

TCR analysis

RNA was extracted from DYS-specific clones (1-5 × 10^6 cells) using the RNeasy kit (Qiagen, Crawley, United Kingdom) according to manufacturer's instructions. Complementary DNA synthesis and amplification were performed using the SMART RACE cDNA amplification kit (Clontech, Basingstoke, United Kingdom) with TCRα and TCRβ chain–specific primers. Polymerase chain reaction products were cloned into a TOPO vector (Invitrogen) and DNA sequencing was performed using a Bigdye Terminator cycle-sequencing kit (Applied Biosystems, Warrington, United Kingdom).

Cell staining and flow cytometry

Antigen-specific, IFN-γ–producing cells were detected by stimulating 1 × 10^6 PBMCs with DYS peptide (2 μg/mL) or CMV lysate for 6 hours, with the addition of 10 μg/mL Brefeldin A (Sigma-Aldrich) after 1 hour. Cells were washed and incubated with anti-CD4PC5 and anti-CD3PE (Beckman Coulter, High Wycombe, United Kingdom) or, for phenotypic analysis, anti-CD4PC5 and one of a panel of fluorescein isothiocyanate (FITC)–conjugated anti-CD45RA, anti-CD45RO, anti-CD28, anti-CD27 (all BD Biosciences, Oxford, United Kingdom), or anti-CD57 (Beckman Coulter). Cells were fixed and permeabilized using reagents from the Intraprep Kit (Beckman Coulter) according to the manufacturer's instructions before being incubated with PE or FITC-conjugated anti–IFN-γ (BD Biosciences).

For detection of intracellular perforin and granzyme B, clones were stained for surface antigens as before and then fixed by addition of 2% paraformaldehyde/phosphate-buffered saline. Anti–perforin-PE and anti–granzyme B FITC antibodies (BD Biosciences) were added to the cells in the presence of saponin buffer (0.5% saponin [Sigma], 5% fetal calf serum, phosphate-buffered saline).

For TCRβ-chain variable region gene analysis, indirect staining was performed using an anti–TRBV6–5 IgG antibody (Beckman Coulter) and a goat anti–mouse IgG FITC (DAKO Cytomation, Ely, United Kingdom) before staining with anti-CD4PC5. Cells were fixed, permeabilized, and stained with anti–IFN-γ FITC (BD Biosciences) as described. All samples were analyzed on a Coulter XL flow cytometer and later using WinMDI (version 2.8) software (Scripps Institute; http//facs.scripps.edu/software.html).

Results

Identification of DYS-specific cytotoxic $CD4^+$ T cells

PBMCs from 10 HLA-DRB*0701–positive donors were stimulated with DYS peptide from CMV gB or whole CMV viral lysate

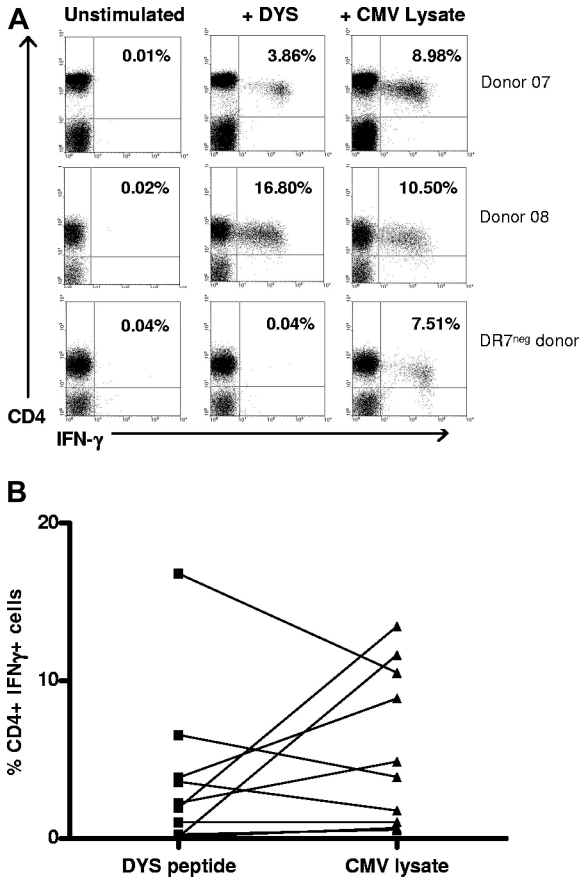

Figure 1. CD4+ T cells specific for DYS peptide are present at high frequency in peripheral blood. PBMCs from CMV-seropositive donors were stimulated with DYS peptide or CMV lysate, and antigen-specific CD4+ T cells were detected by intracellular IFN-γ expression. (A) Representative flow cytometric profiles from HLA-DRB*0701–positive donors (08 and 07) and an HLA-DRB*070–negative donor showing the DYS-specific CD4+ T-cell response. (B) DYS peptide and CMV lysate–specific CD4+ responses from the cohort of HLA-DRB*0701–positive, CMV-seropositive donors (n = 10). Horizontal lines link the DYS-specific and CMV lysate–specific responses from individual donors, each shown as a percentage of CD4 T cells.

prepared from infected fibroblast cultures. Antigen-specific responses were detected using cytokine flow cytometry (CFC) for detection of IFN-γ production within the CD4+ T-cell population (Figure 1A). No IFN-γ production was detected following stimulation of HLA-DRB*0701–positive, CMV-seronegative donors or with use of mock CMV lysate generated from uninfected fibroblasts (data not shown). Furthermore, stimulation of PBMCs from HLA-DRB*0701–negative, CMV-seropositive donors with DYS peptide showed no response. All HLA-DRB*0701–positive, CMV-seropositive donors mounted strong CD4+ T-cell responses to CMV lysate that averaged 5.72% of total CD4+ T cells (range, 0.54%-13.6%; Figure 1B). However, stimulation with the DYS peptide alone also induced a strong CD4+ T-cell response. As much as 16% of the total CD4+ T-cell population was specific for this peptide, and 7 of 10 donors showed epitope-specific responses of more than 1%, indicating marked focusing of the immune response (overall mean, 3.64%; range, 0.09%-16.8%). Interestingly, 3 of the 10 donors demonstrated a greater response to DYS peptide than to whole viral lysate. It is noteworthy that these individuals were among the 4 donors with the largest CD4+ T-cell response to DYS peptide, and it is likely that this finding reflects limiting amounts of DYS peptide within the lysate mixture.

DYS-specific CD4+ T cells have an effector memory phenotype

The magnitude of the DYS-specific immune response is the largest antigen-specific CD4+ T-cell response that has been detected in humans; therefore, we sought to characterize DYS-specific T cells in more detail by determining their cell surface phenotype. PBMCs from HLA-DRB*0701–positive, CMV-seropositive donors were stimulated with DYS peptide or CMV lysate using CFC and costained with antibodies specific for cell-surface markers CD45RA, CD45RO, CD28, CD27, and CD57. DYS-specific cells expressed high levels of CD45RO (mean, 86.9%; range, 66%-99%), with low expression of CD45RA (mean, 9.4%; range, 1.0%-27%) and CD27 (mean, 14.4%; range, 0.3%-73%; Figure 2A,B). Levels of CD28 expression were low for the majority of donors, but in some cases more than 50% of the responding population was CD28 positive (mean, 32%; range, 0.17%-97%). Expression of CD57, a characteristic marker of highly differentiated T cells, showed marked variation between donors (mean, 38%; range, 3%-91%). This phenotype is indicative of an effector memory T-cell subset[12] and was similar to the phenotype of CMV lysate–specific CD4+ T-cell clones from the same donors (Figure 2C). Interestingly, a small percentage of the antigen-specific T cells demonstrated CD45RA expression, indicating reversion from CD45RO expression similar to that described for CD8 T cells.[13-15] In order to assess the direct cytotoxic potential of DYS-specific T-cell clones without prestimulation, a cytotoxicity assay was performed using freshly isolated peripheral blood as the effector cell population. PBMCs were depleted of CD8+ T cells and used as effectors in a chromium release assay on LCL targets (Figure 2D). Significant lysis was observed against targets either pulsed with peptide or infected with MVA-gB, indicating that circulating DYS-specific cells are directly cytotoxic against virally infected target cells.

Specificity analysis of DYS-specific CD4 T-cell clones

To examine the functional properties of DYS-specific CD4+ T cells in more detail, we established DYS-specific T-cell clones from 5 donors using the IFN-γ–cytokine secretion assay. All clones showed specific recognition of peptide-pulsed LCL but were also able to recognize processed antigen when target cells were loaded with CMV lysate or infected with MVA-gB (Figure 3A). In order to determine whether endogenous gene transcription was required for antigen presentation, target cells were pulsed with ultraviolet-inactivated MVA-gB. These targets were not recognized by DYS-specific T cells, confirming that recognition was not due to exogenous uptake of gB protein. The HLA restriction of the clones was confirmed using HLA-DRB*0701–matched and mismatched target cells (Figure 3A).

The functional avidity of DYS-specific T-cell clones was assessed using peptide titration analysis, with serial dilutions of peptide ranging from 10^{-4} to 10^{-11} M. The amount of peptide required to produce 50% maximal recognition ranged from 10 nM to 100 nM for each of the 8 clones, comparable to other HLA class II–restricted responses[16,17] (Figure 3B).

Cytotoxic function of DYS-specific CD4 T-cell clones

The chromium release assay was used to measure cytotoxic activity of the CD4+ clones, which all demonstrated significant lysis of peptide-loaded LCL (Figure 4A). The mechanism of cytotoxicity was determined with use of the perforin inhibitor ConA, which reduced specific lysis to background levels (Figure 4A). This indicates that cytotoxicity is mediated through the perforin–granzyme system and is supported by detection of intracellular perforin and granzyme B (Figure 4B). In order

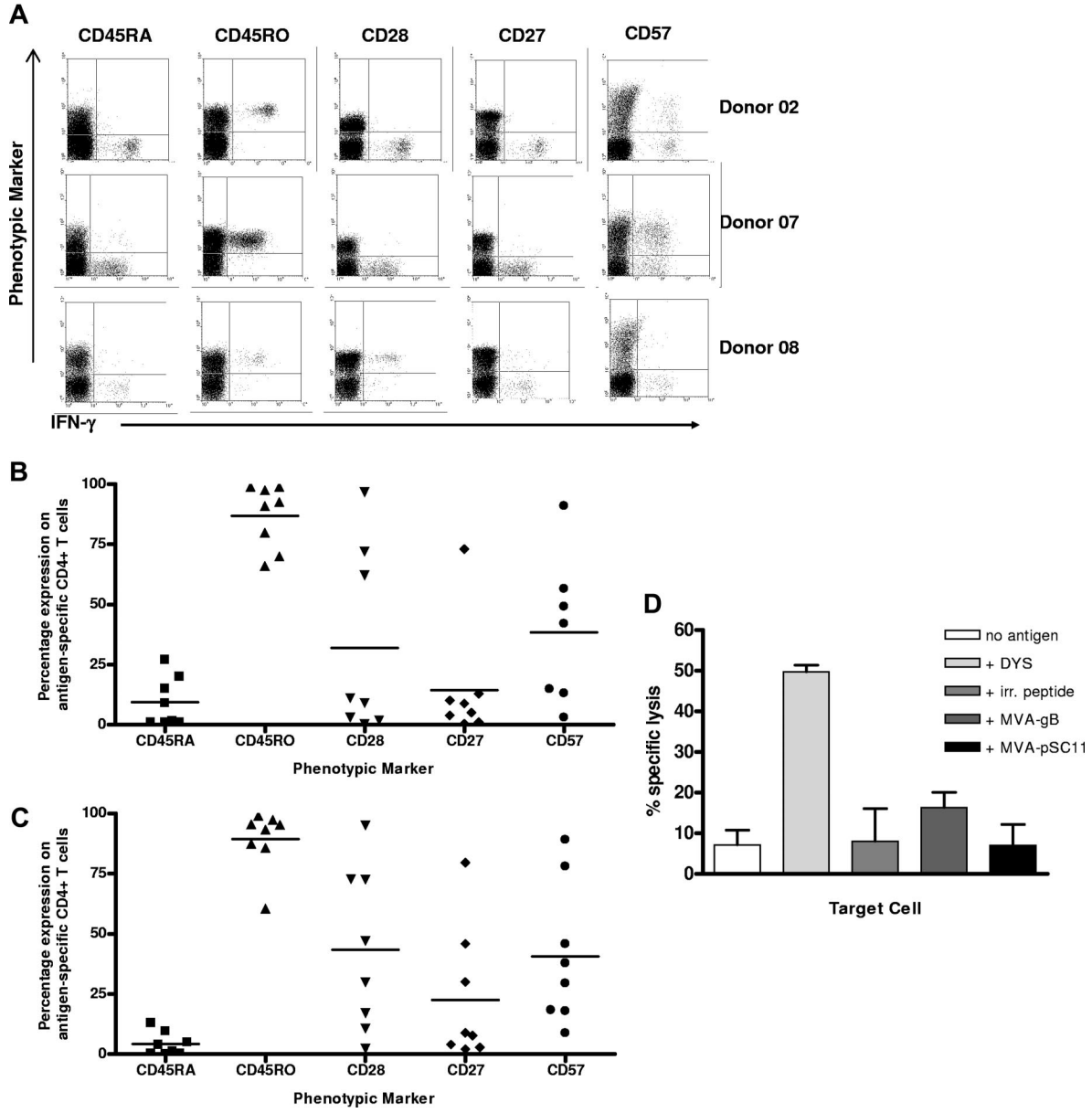

Figure 2. DYS-specific CD4$^+$ T cells have an effector memory membrane phenotype. PBMCs from HLA-DRB*0701–positive, CMV-seropositive donors were stimulated with DYS peptide or CMV lysate, and antigen-specific CD4$^+$ T cells were determined by intracellular IFN-γ expression. The expression of CD45RA, CD45RO, CD28, CD27, and CD57 then was determined on this population. (A) Representative flow cytometric profiles from 3 donors (07, 08, and 02) showing membrane phenotype of DYS-specific cells. The percentage of (B) DYS-specific or (C) CMV lysate–specific CD4$^+$ T cells expressing each marker is shown from individual donors. The mean value is represented by a horizontal line. (D) DYS-specific CD4$^+$ T cells demonstrate direct ex vivo cytotoxic activity. Freshly isolated PBMCs were CD8$^+$ depleted by magnetic selection and incubated with autologous LCL targets at an effector-target ratio of 25:1 in chromium release assays. Target cells were CMV lysate pulsed, peptide pulsed, or unpulsed; MVA-gB infected or control MVA infected.

to investigate the potential biologic spectrum of antigen presentation to DYS-specific CD4$^+$ T cells, we infected fibroblasts and DCs with CMV strain AD169 prior to cytotoxicity analysis. CMV-encoded immune evasion genes carry the potential to downregulate HLA class II–restricted peptide presentation, and so the recombinant RV798 viral strain, which carries a deletion in the US2-US11 region, was also studied. Fibroblasts were pretreated with IFN-γ to induce expression of MHC class II prior to infection with AD169 or RV798, or were pulsed with DYS peptide. Fibroblast targets were effectively killed in all cases and demonstrated a high degree of lysis (Figure 4C). DCs express constitutively high levels of MHC class II and were efficiently lysed by DYS-specific cytotoxic T lymphocytes (CTLs) following infection with AD169 or RV798 (Figure 4D). Indeed, cytotoxicity following direct infection with virus was substantially greater than that seen following pulsing of DCs with exogenous DYS peptide and contradicts the idea that human CMV infection of DCs blocks T-cell recognition. Thus, DYS-specific CTLs are able to lyse antigen-presenting cells with either constitutive or inducible expression of HLA-DRB*0701. In addition, the immune evasion genes US2-11 have limited effects on the presentation of this peptide, since recognition of targets infected with RV798 was no greater than recognition of targets infected with AD169.

DYS-specific CD4$^+$ cytotoxic T cells show extreme conservation of T-cell receptor usage

Cytotoxic CD8$^+$ T cells frequently demonstrate highly conserved T-cell receptor gene usage between different individuals. In order to determine whether a similar phenomenon was present in DYS-specific CD4$^+$

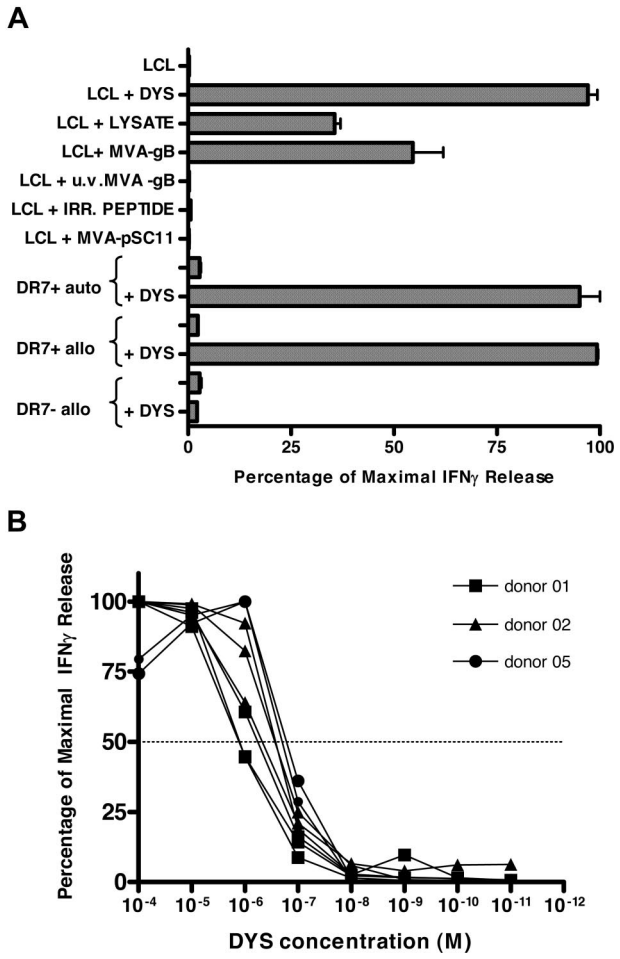

Figure 3. DYS-specific CD4+ T-cell clones recognize peptide derived from processed gB protein. (A) DYS-specific CD4+ T-cell clones were stimulated with *HLA-DRB*0701*–positive LCL pulsed with DYS peptide, irrelevant peptide, or CMV lysate, or following infection with MVA-gB, MVA-pSC11 (empty vector), or ultraviolet-irradiated MVA-gB. Antigen-specific responses were determined by IFN-γ ELISA and expressed as the percentage of maximal IFN-γ release from peptide-loaded targets. To confirm the HLA restriction of the DYS-specific T-cell response, autologous or allogeneic (HLA-DRB*0701–positive or HLA-DRB*0701–negative) LCL targets were pulsed with DYS peptide before incubation with DYS-specific T-cell clones. Recognition is expressed as the percentage of maximal IFN-γ release from DYS peptide–loaded autologous targets. (B) Functional avidity of DYS peptide–specific clones. LCLs were pulsed with variable concentrations of DYS peptide before incubation with DYS-specific T-cell clones. Results are expressed as the percentage of maximal IFN-γ release, and functional avidity was correlated with concentration of peptide required to produce 50% maximal response (broken line). Peptide titrations of six clones from 3 different donors are shown.

cells, T-cell receptor sequences were cloned and sequenced from T-cell clones derived from 4 donors. TCR usage of DYS-specific clones was extremely conserved within both the TCRα and TCRβ chains. All of the clones used the *TRBV6-5* gene segment, and a highly conserved Q-G amino acid motif was present in the non–germline-encoded hypervariable CDR3 region (Figure 5). An identical TCRβ chain sequence was shared by clones from 2 different donors, and a highly conserved length of the CDR3 loop was present in 11 of 12 sequences.

TCRα chain sequences could be cloned from 7 of the 12 T-cell clones and demonstrated restriction in variable region usage with only 2 different variable region segments being used (Figure 6). Clones from donors 01 and 05 used only the *TRAV25* gene segment, whereas clones from donor 02 used *TRAV25* or *TRAV9-2*. Two different transcripts were expressed by clones from donor 02, whereas clones from donor 01 and donor 05 used a single transcript. Junctional segment selection was also conserved with usage of only 3 different segments, and clones showed use of the same junctional region, despite having different variable gene segments.

In order to investigate whether the TCR sequence conservation of DYS-specific clones was subject to potential bias during T-cell cloning, we undertook flow cytometric analysis of TCRβ chain expression on DYS-specific cells isolated directly from peripheral blood. PBMCs from donor 01 were stimulated with DYS peptide and stained for surface expression of *TRBV6-5*. A total of 57% of the antigen-specific CD4+ cells expressed *TRBV6-5,* confirming conservation of TCR usage by this population (Figure 7A). *TRBV6-5* was the predominant TCR gene segment expressed within PBMCs from a further 2 donors, accounting for up to 88% of DYS-specific cells (Figure 7B,C).

Clonal focusing of cytotoxic cells generally occurs during maturation of chronic immune responses. The clonal expansion and immunodominance of the DYS-peptide specific response may have potentially developed over a number of years since primary CMV infection. Primary infection with CMV is almost always silent in immunocompetent individuals, and such subjects are thus difficult to identify. Despite this, we were able to study the DYS-specific response in an immunocompetent patient who developed symptomatic CMV primary infection (donor 11). The DYS-specific T-cell response represented 0.8% of the CD4+ T-cell pool within the first month after infection, and more than 40% of this population used the *TRBV6-5* gene segment, demonstrating that significant expansion and clonal restriction of this response can occur early after primary infection (Figure 7D).

Discussion

Immunodominance refers to the restricted antigenic specificity of T cells in an immune response to a complex antigen.[1] In addition, clonal selection within a peptide-specific immune response further limits the breadth of the T-cell repertoire, and these phenomena are commonly seen with CD8+ cytotoxic T-cell responses to murine or human viruses.[18-21] CD8+ T-cell immunity to the influenza matrix peptide is highly dependent on expression of the *TCRBV17* gene segment,[22] and structural analysis has indicated that this reflects optimal side chain interactions with the peptide-HLA complex.[23] CD8+ T cells specific for a peptide from EBV restricted by HLA-B8 show complete TCR conservation between individuals,[20,24] and less complete clonal conservation is seen in CD8+ T-cell recognition of epitopes from CMV.[15,25,26]

In contrast, CD4+ T-cell responses are characterized by much broader recognition of peptide epitopes[27,28] and, rather than undergoing clonal competition, CD4+ T cells of different specificities may act cooperatively.[29] It is not clear why the CD4+ T-cell response is less focused than that of the CD8 population, although HLA class II molecules have less stringent anchor positions than HLA class I and are able to bind and present a greater variety of peptide epitopes.

Cytotoxic CD4+ T cells have been identified recently as being of considerable importance in the clearance of viral infection[30] and bear many phenotypic similarities to their cytotoxic CD8+ counterparts. Antigen-presenting cell populations of DCs and B cells have constitutive expression of HLA class II proteins, whereas other cell types, such as endothelium, are induced to express HLA class II molecules by inflammatory mediators. These cell types are tropic for CMV infection, and thus become targets for CD4-mediated immune control. The

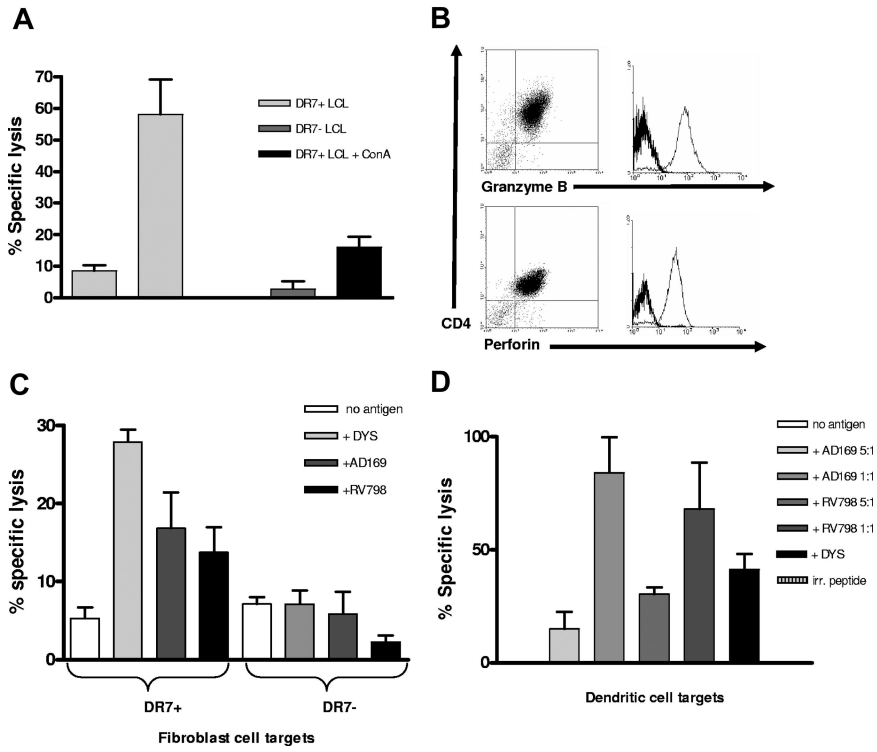

Figure 4. Cytotoxic activity of DYS specific T-cell clones. (A) HLA-DRB*0701–positive or HLA-DRB*0701–negative LCL targets were pulsed with DYS peptide and incubated with unmanipulated or ConA-treated, DYS-specific CD4$^+$ T-cell clones. Cytotoxicity was measured by chromium release assay. (B) Flow cytometric demonstration of intracellular perforin and granzyme B expression by DYS-specific CD4$^+$ T-cell clones. Fibroblasts (C) or DCs (D) infected with CMV strains AD169 or RV798 are killed by DYS-specific T-cell clones. Cytotoxicity was measured using the chromium release assay.

majority of HLA class II–restricted peptide presentation comes from uptake of exogenous proteins, and this pathway is unlikely to play an important role in the direct recognition of virally infected cells. However, recent work has shown that proteins from CMV and EBV can be presented directly by HLA class II molecules after viral infection.[9,31,32] This observation greatly increases the potential importance of cytotoxic CD4$^+$ T-cell populations in the control of lytic viral replication.

CD4$^+$ T cells with cytotoxic phenotype are commonly observed in cases of TCR$\alpha\beta^+$/CD4$^+$ T-LGL associated with cytopenia. Garrido et al recently studied TCR usage in 36 cases of LGL and found highly conserved TCR usage in 15 cases, all of which were

		TCRβ-Chain																							
Donor	Clone	TRBV						NDN							TRBJ										
01	1	TRBV6-5	C	A	S	S	Y	Q	G	A					N	I	Q	Y	F	G	A	G	T		TRBJ2-4
			tgt	gcc	agc	agt	tac	cag	ggc	gca					aac	att	cag	tac	ttc	ggc	gcc	ggg	acc		
	2	TRBV6-5	C	A	S	S	Y	Q	G	A					N	I	Q	Y	F	G	A	G	T		TRBJ2-4
			tgt	gcc	agc	agt	tac	cag	ggc	gca					aac	att	cag	tac	ttc	ggc	gcc	ggg	acg		
	3	TRBV6-5	C	A	S	S	Y	Q	G	A					N	I	Q	Y	F	G	A	G	T		TRBJ2-4
			tgt	gcc	agc	agt	tac	cag	ggc	gca					aac	att	cag	tac	ttc	ggc	gcc	ggg	acg		
	4	TRBV6-5	C	A	S	S	Y	Q	G	A					N	I	Q	Y	F	G	A	G	T		TRBJ2-4
			tgt	gcc	agc	agt	tac	cag	ggc	gca					aac	att	cag	tac	ttc	ggc	gcc	ggg	acg		
02	1	TRBV6-5	C	A	S	S	Y	Q	G	A	S	G			Q	Y	F	G	P	G	T				TRBJ2-3
			tgt	gcc	agc	agt	tac	cag	ggg	gcg	agt	ggg			cag	tat	ttt	ggc	cca	ggc	acc				
	2	TRBV6-5	C	A	S	S	Y	Q	G	A	S	G			Q	Y	F	G	P	G	T				TRBJ2-3
			tgt	gcc	agc	agt	tac	cag	ggg	gcg	agt	ggg			cag	tat	ttt	ggc	cca	ggc	acc				
	3	TRBV6-5	C	A	S			R	Q	Q	G	G			S	P	L	H	F	G	N	G	T		TRBJ1-6
			tgt	gcc	agc			aga	caa	cag	gga	gga			tca	ccc	ctc	cac	ttt	ggg	aat	ggg	acc		
03	1	TRBV6-5	C	A	S	S	Y	Q	G	A					N	I	Q	Y	F	G	A	G	T		TRBJ2-4
			tgt	gcc	agc	agt	tac	cag	ggc	gca					aac	att	cag	tac	ttc	ggc	gcc	ggg	acc		
	2	TRBV6-5	C	A	S	S	Y	Q	G	A					N	I	Q	Y	F	G	A	G	T		TRBJ2-4
			tgt	gcc	agc	agt	tac	cag	ggc	gca					aac	att	cag	tac	ttc	ggc	gcc	ggg	acc		
	3	TRBV6-5	C	A	S	S	Y	Y	A	R	G	T			N	S	P	L	H	F	G	N	G	T	TRBJ1-6
			tgt	gcc	agc	agt	tac	tac	gct	cgg	gga	acg			aat	tca	ccc	ctc	cac	ttt	ggg	aac	ggg	acc	
05	1	TRBV6-5	C	A	S	S		R	Q	G	R	L			G	Y	T	F	G	S	G	T			TRBJ1-2
			tgt	gcc	agc	agt		cga	cag	ggc	cgg	ctg			ggc	tac	acc	ttc	ggt	tcg	ggg	acc			
	2	TRBV6-5	C	A	S	S		R	Q	G	R	L			G	Y	T	F	G	S	G	T			TRBJ1-2
			tgt	gcc	agc	agt		cga	cag	ggc	cgg	ctg			ggc	tac	acc	ttc	ggt	tcg	ggg	acc			

Figure 5. TCRβ chain sequences of DYS-specific cells.

Donor	Clone	TCRα-Chain TRAV	N	TRAJ	
01	2	TRAV25	C A G tgt gca ggg	K S S aaa agt agc	N T G K L I F G Q G T aac aca ggc aaa cta atc ttt ggg caa ggg aca	TRAJ37
	4	TRAV25	C A G tgt gca ggg	K S S aaa agt agc	N T G K L I F G Q G T aac aca ggc aaa cta atc ttt ggg caa ggg aca	TRAJ37
02	1	TRAV9-2	C A L tgt gct ctg	R E agg gag	N Q G G K L I F G Q G T aac cag gga gga aag ctt atc ttc gga cag gga acg	TRAJ23
	2	TRAV9-2	C A L tgt gct ctg	R E agg gag	N Q G G K L I F G Q G T aac cag gga gga aag ctt atc ttc gga cag gga acg	TRAJ23
	3	TRAV25	C A G tgt gca ggg	F K A ttt aaa gct	N A G K S T F G D G T aat gca ggc aaa tca acc ttt ggg gat ggg act	TRAJ27
05	1	TRAV25	C A G tgt gcg ggg	S S G G tcc tca ggg ggc	Q G G K L I F G Q G T cag gga gga aag ctt atc ttc gga cag gga acg	TRAJ23
	2	TRAV25	C A G tgt gcg ggg	S S G G tcc tca ggg ggc	Q G G K L I F G Q G T cag gga gga aag ctt atc ttc gga cag gga acg	TRAJ23

Figure 6. TCRα chain sequences of DYS-specific cells.

associated with the HLA-DRB*0701 allele.[11] The authors speculated that this might result from antigenic simulation through a common peptide but did not identify the etiologic agent. We have focused on the CD4+ T-cell response to DYS peptide from cytomegalovirus glycoprotein B restricted through HLA-DRB*0701.[10] The magnitude of the DYS-specific CD4+ T-cell response averages 3.6% of the total CD4+ T-cell repertoire, and comparison with the response to whole viral lysate suggests that the DYS-specific immune response constitutes a very large proportion of the total CMV-specific CD4+ T-cell immune response in HLA-DRB*0701–positive donors. These findings are noteworthy for a number of reasons. First, this magnitude of CD4+ immunity to a single peptide is, to our knowledge, higher than any other peptide-specific immune response that has been reported to date. Moreover, the CMV-specific CD4+ cytotoxic response shows extreme immunodominance in HLA-DRB*0701–positive individuals and reveals that immunodominance within CD4+ T-cell responses can be at least as marked as that seen within the CD8+ population.

In order to investigate the possible factors that might determine immunodominance, we cloned DYS-specific T cells,

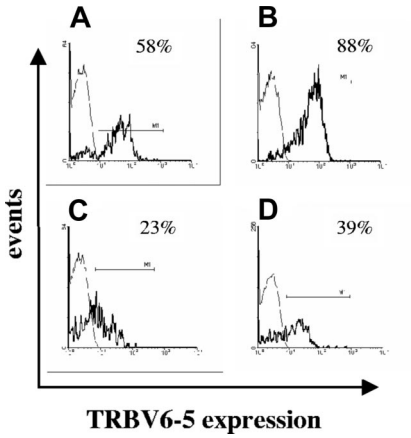

Figure 7. TRBV6-5 usage by DYS-specific CD4+ memory T cells. PBMCs from HLA-DRB*0701–positive CMV-seropositive subjects, donors 01 (A), 08 (B), 07 (C), and 11 (D) were stimulated with DYS peptide and stained for the expression of TRBV6-5 together with CD4 and intracellular IFN-γ. Histograms show TRBV6-5 expression by DYS-specific CD4+ cells (bold line) compared with an isotype control (narrow line). The percentage of CD4+IFN-γ+ cells expressing TRBV6-5 is shown.

and peptide titration analysis revealed avidity that was comparable with CD8+ cytotoxic clones. T-cell clones lysed target cells directly infected with vaccinia virus encoding the gB gene, demonstrating that gB epitopes can be presented from the cytosol of infected cells. Finally, we were able to infect target cells with CMV and demonstrate cytotoxicity mediated through DYS-restricted T-cell clones.

T-cell receptors from a number of DYS-specific T-cell clones were cloned and sequenced. These showed extreme conservation of T-cell–receptor sequence between different individuals, a finding not previously reported for peptide-specific CD4+ T cells.[33] The pattern of T-cell–receptor conservation within *TRA* and *TRB* genes is entirely analogous to that seen in the study of TCR usage of CD8+ cytotoxic T cells. In particular, the combination of unique *TRBV* gene-segment usage and CDR3 sequence and length together with strong, but less absolute, selection of *TRAV* and *TRAJ* segments is a highly characteristic pattern.[34] Structural analysis of T-cell receptor binding to HLA-peptide complexes has been performed for several CD8+ virus–specific T-cell clones and demonstrates a consistent pattern of TCR orientation over the MHC complex.[35] Crystallographic studies of TCRs isolated from cytotoxic CD4+ T cells are not yet available, but the T-cell receptor sequence conservation seen in the CD4+ cytotoxic T-cell response to the DYS peptide strongly suggests that TCR orientation will be conserved in this HLA class II–restricted T-cell response.

The biologic basis for this extreme immunodominance and clonal selection is unclear, but a number of possibilities require investigation. The first is that the T-cell receptor interaction with the DYS peptide is of unusually high affinity,[36] although peptide titration analyses do not provide convincing evidence for this. Equally, different CMV proteins may have differential access to direct presentation by HLA class II molecules following viral infection, and as gB accumulates in endosomes during viral assembly, peptides from this protein may gain selective access to an HLA class II pathway. It is tempting to speculate that the cytotoxic potential of this CD4+ T-cell response contributes directly to a wide degree of clonal selection observed within DYS-specific T cells. Experimental limitation of the number of antigen-presenting cells leads directly to selection of high-avidity T-cell clones due to competition of antigen at the cell surface. Cytotoxic T cells have the ability to lyse antigen-presenting cells,[37] and this may be one

factor that helps to control ongoing immune responses. Indeed, it is possible that the HLA class II restriction of $CD4^+$ cytotoxic T cells actually enhances their potential for clonal selection in comparison to $CD8^+$ cytotoxic cells as expression of HLA class II is focused on antigen-presenting cell populations. As such, more pronounced and rapid clonal competition may occur within $CD4^+$ cytotoxic T-cell responses than is widely believed.[8,38] Our observation that clonal focusing can occur very early after acute infection is in line with evidence from murine models in which immunodominance was established within the first few hours of an immune response.[39]

Of similar importance is the observation that the TCR usage is highly homologous to that reported in patients with $TCR\alpha\beta^+/CD4^+$ T-LGL associated with the HLA-DRB*0701 allele.[11] The TCRβ sequence uses the same *TCRVB* gene segment (albeit with different nomenclature) and retains the Q-G amino acid motif in the highly variable CDR3 region. It would be virtually impossible for such conservation to be observed by chance and, together with the absolute requirement for HLA-DRB*0701 expression in the LGL cases, identifies the CMV DYS peptide as the antigen that has driven the $CD4^+$ T-cell clonal expansion. This is the first identification of an infectious agent involved in the initiation or maintenance of LGL and suggests that CMV may also be involved in the pathogenesis of $TCR\alpha\beta^+/CD8^+$ or γδ LGL. Treatment with antiviral agents may thus be a reasonable therapeutic option in these cases.

In conclusion, we demonstrate that immunodominance and clonal restriction can occur to a very marked extent in a $CD4^+$ cytotoxic immune response. The clinical implications of such clonal restriction may be considerable and should be considered in the development of immunotherapeutic strategies designed to amplify $CD4^+$ cytotoxic immune responses. Such a potent and focused immune response may not be of benefit to the host. Indeed, HLA-DRB*0701–positive patients demonstrate an increased risk of CMV reactivation following organ transplantation,[40] suggesting that the DYS-specific response may contribute directly to immunopathology by an unknown mechanism. Excessive destruction of host antigen-presenting cells is one mechanism that deserves investigation.

Acknowledgment

This work was supported by the Medical Research Council.

Authorship

Contribution: L.C. and N.K. performed the laboratory research, N.K. and P.M. designed the research, R.K. and L.N. contributed vital new reagents, L.C. analyzed the data, and P.M. and L.C. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Prof Paul A.H. Moss, CRUK Institute for Cancer Studies, University of Birmingham, Vincent Drive, Edgbaston B15 2TT, United Kingdom; e-mail: p.moss@bham.ac.uk.

References

1. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu Rev Immunol. 1999;17:51-88.
2. Hopkins JI, Fiander AN, Evans AS, Delchambre M, Gheysen D, Borysiewicz LK. Cytotoxic T cell immunity to human cytomegalovirus glycoprotein B. J Med Virol. 1996;49: 124-131.
3. Lucin P, Pavic I, Polic B, Jonjic S, Koszinowski UH. Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. J Virol. 1992;66:1977-1984.
4. Gandhi MK, Khanna R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. Lancet Infect Dis. 2004;4:725-738.
5. Khan N, Hislop A, Gudgeon N, et al. Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. J Immunol. 2004;173:7481-7489.
6. Gamadia LE, Rentenaar RJ, van Lier RA ten Berge IJ. Properties of CD4(+) T cells in human cytomegalovirus infection. Hum Immunol. 2004; 65:486-492.
7. Jonjic S, Pavic I, Lucin P, Rukavina DKoszinowski UH. Efficacious control of cytomegalovirus infection after long-term depletion of CD8+ T lymphocytes. J Virol. 1990;64:5457-5464.
8. Bitmansour AD, Waldrop SL, Pitcher CJ, et al. Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus. J Immunol. 2001;167:1151-1163.
9. Hegde NR, Dunn C, Lewinsohn DM, Jarvis MA, Nelson JA, Johnson DC. Endogenous human cytomegalovirus gB is presented efficiently by MHC class II molecules to CD4+ CTL. J Exp Med. 2005;202:1109-1119.
10. Elkington R, Shoukry NH, Walker S, et al. Cross-reactive recognition of human and primate cytomegalovirus sequences by human CD4 cytotoxic T lymphocytes specific for glycoprotein B and H. Eur J Immunol. 2004;34:3216-3226.
11. Garrido P, Ruiz-Cabello F, Bárcena P, et al. Monoclonal TCR-Vβ13.1/CD4/NKa/CD8T-LGL lymphocytosis: evidence for an antigen-driven chronic T-cell stimulation origin. Blood. 2007;109: 4890-4898.
12. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401:708-712.
13. Faint JM, Annels NE, Curnow SJ, et al. Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotypic and migratory characteristics. J Immunol. 2001; 167:212-220.
14. Amyes E, Hatton C, Montamat-Sicotte D, et al. Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection. J Exp Med. 2003;198:903-911.
15. Wills MR, Carmichael AJ, Weekes MP, et al. Human virus-specific CD8+ CTL clones revert from CD45ROhigh to CD45RAhigh in vivo: CD45RAhighCD8+ T cells comprise both naive and memory cells. J Immunol. 1999;162:7080-7087.
16. Long HM, Haigh TA, Gudgeon NH, et al. CD4+ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines. J Virol. 2005;79:4896-4907.
17. Khanna R, Burrows SR, Steigerwald-Mullen PM, Moss DJ, Kurilla MG, Cooper L. Targeting Epstein-Barr virus nuclear antigen 1 (EBNA1) through the class II pathway restores immune recognition by EBNA1-specific cytotoxic T lymphocytes: evidence for HLA-DM-independent processing. Int Immunol. 1997;9:1537-1543.
18. Volkmer H, Bertholet C, Jonjic S, Wittek R, Koszinowski UH. Cytolytic T lymphocyte recognition of the murine cytomegalovirus nonstructural immediate-early protein pp89 expressed by recombinant vaccinia virus. J Exp Med. 1987;166:668-677.
19. Kedzierska K, La Gruta NL, Turner SJ, Doherty PC. Establishment and recall of CD8+ T-cell memory in a model of localized transient infection. Immunol Rev. 2006;211:133-145.
20. Argaet VP, Schmidt CW, Burrows SR, et al. Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. J Exp Med. 1994;180:2335-2340.
21. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med. 2005;202:673-685.
22. Moss PA, Moots R, Rosenberg W, et al. Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. Proc Natl Acad Sci U S A. 1991;88:8987-8890.
23. Stewart-Jones GB, McMichael AJ, Bell JI, Stuart DI, Jones EY. A structural basis for immunodominant human T cell receptor recognition. Nat Immunol. 2003;4:657-663.
24. Kjer-Nielsen L, Clements CS, Purcell AW, et al. A structural basis for the selection of dominant alphabeta T cell receptors in antiviral immunity. Immunity. 2003;18:53-64.
25. Trautmann L, Rimbert M, Echasserieau K, et al. Selection of T cell clones expressing high-affinity public TCRs within human cytomegalovirus-specific CD8 T cell responses. J Immunol. 2005;175: 6123-6132.
26. Khan N, Shariff N, Cobbold M, et al. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol. 2002;169:1984-1992.
27. Maini MK, Gudgeon N, Wedderburn LR, Rickinson AB, Beverley PC. Clonal expansions in acute EBV infection are detectable in the CD8 and not the CD4 subset and persist with a variable CD45 phenotype. J Immunol. 2000;165:5729-5737.
28. Scriba TJ, Purbhoo M, Day CL, et al. Ultrasensitive detection and phenotyping of CD4+ T cells

with optimized HLA class II tetramer staining. J Immunol. 2005;175:6334-6343.

29. Creusot RJ, Thomsen LL, Tite JP, Chain BMs. Local cooperation dominates over competition between CD4+ T cells of different antigen/MHC specificity. J Immunol. 2003;171:240-246.
30. Appay V, Zaunders JJ, Papagno L, et al. Characterization of CD4(+) CTLs ex vivo. J Immunol. 2002;168:5954-5958.
31. Munz C. Epstein-barr virus nuclear antigen 1: from immunologically invisible to a promising T cell target. J Exp Med. 2004;199:1301-1304.
32. Paludan C, Schmid D, Landthaler M, et al. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science. 2005; 307:593-596.
33. Wedderburn LR, O'Hehir RE, Hewitt CR, Lamb JR, Owen MJ. In vivo clonal dominance and limited T-cell receptor usage in human CD4+ T-cell recognition of house dust mite allergens. Proc Natl Acad Sci U S A. 1993;90:8214-8218.
34. Bowness P, Moss PA, Rowland-Jones S, Bell JI, McMichael AJ. Conservation of T cell receptor usage by HLA B27-restricted influenza-specific cytotoxic T lymphocytes suggests a general pattern for antigen-specific major histocompatibility complex class I-restricted responses. Eur J Immunol. 1993;23:1417-1421.
35. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. Annu Rev Immunol. 2006;24:419-466.
36. Smith AL, Wikstrom ME, Fazekas de St Groth B. Visualizing T cell competition for peptide/MHC complexes: a specific mechanism to minimize the effect of precursor frequency. Immunity. 2000;13: 783-794.
37. Hermans IF, Ritchie DS, Yang J, Roberts JM, Ronchese F. CD8+ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity. J Immunol. 2000;164:3095-3101.
38. van Leeuwen EM, Remmerswaal EB, Heemskerk MH, ten Berge IJ, van Lier RA. Strong selection of virus-specific cytotoxic CD4+ T cell clones during primary human cytomegalovirus infection. Blood. 2006;108:3121-3127.
39. Willis RA, Kappler JW, Marrack PC. CD8 T cell competition for dendritic cells in vivo is an early event in activation. Proc Natl Acad Sci U S A. 2006;103:12063-12068.
40. Kraat YJ, Christiaans MH, Nieman FH, van den Berg-Loonen PM, van Hooff JP, Bruggeman CA. Increased frequency of CMV infection in HLA-DR7 matched renal allograft recipients. Lancet. 1993;341:494-495.