A HCMV pp65 polypeptide promotes the expansion of CD4+ and CD8+ T cells across a wide range of HLA specificities

Provenzano, M; Sais, G; Bracci, L; Egli, A; Anselmi, M; Viehl, C T; Schaub, S; Hirsch, H H; Stroncek, D F; Marincola, F M; Spagnoli, G C
A HCMV pp65 polypeptide promotes the expansion of CD4+ and CD8+ T cells across a wide range of HLA specificities

Abstract

Human Cytomegalovirus (HCMV) can cause life threatening disease in infected hosts. Immunization with HLA-restricted immunodominant synthetic peptides and adoptive transfer of epitope specific T cells have been envisaged to generate or boost HCMV specific cellular immunity, thereby preventing HCMV infection or reactivation. However, induction or expansion of T cells effective against HCMV are limited by the need of utilizing peptides with defined HLA restrictions. We took advantage of a combination of seven predictive algorithms to identify immunogenic peptides of potential use in the prevention or treatment of HCMV infection or reactivation. Here we describe a pp65 derived peptide (pp65(340-355), RQYDPVAALFFFDIDL: RQY16-mer), characterized by peculiar features. First, RQY-16mer is able to stimulate HCMV pp65 specific responses in both CD4+ and CD8+ T cells, restricted by a wide range of HLA class I and class II determinants. Second, RQY-16mer is able to induce an unusually wide range of effector functions in CD4+ T cells, including proliferation, killing of autologous HCMV infected target cells and cytokine production. Third, and most importantly, the RQY-16mer is able to stimulate CD4+ and CD8+ T cell responses in pharmacologically immunosuppressed patients. These data suggest that a single reagent might qualify as synthetic immunogen for potentially large populations exposed to HCMV infection or reactivation.
A HCMV pp65 polypeptide promotes the expansion of CD4+ and CD8+ T cells across a wide range of HLA specificities

Maurizio Provenzano\textsuperscript{a,e,*}, Giovanni Sais\textsuperscript{a,e}, Laura Bracci\textsuperscript{a}, Adrian Egli\textsuperscript{b}, Maurizio Anselmi\textsuperscript{a}, Carsten T Viehl\textsuperscript{a}, Stefan Schaub\textsuperscript{c}, Hans H Hirsch\textsuperscript{b}, David F Stroncek\textsuperscript{d}, Francesco M Marincola\textsuperscript{d}, Giulio C Spagnoli\textsuperscript{a}.

\textsuperscript{a}Institute of Surgical Research and Hospital Management, University Hospital Basel, Switzerland
\textsuperscript{b}Transplantation Virology, Institute for Medical Microbiology, University of Basel, Switzerland
\textsuperscript{c}Department of Transplantation Immunology and Nephrology, University Hospital Basel, Switzerland
\textsuperscript{d}Department of Transfusion Medicine, Infectious Diseases and Immunogenetic Section, Clinical Center, NIH, Bethesda, USA
\textsuperscript{e}Both authors contributed equally to the paper

Address for correspondence:
Maurizio Provenzano, MD
Department of Urology
University Hospital of Zurich
Frauenklinikstrasse 10
CH-8091 Zurich, Switzerland
Phone: +41-44-2559297
Fax: +41-44-2556004
Email: Maurizio.provenzano@usz.ch
Abstract

Human Cytomegalovirus (HCMV) can cause life threatening disease in infected hosts. Immunization with HLA-restricted immunodominant synthetic peptides and adoptive transfer of epitope specific T cells have been envisaged to generate or boost HCMV specific cellular immunity, thereby preventing HCMV infection or reactivation. However, induction or expansion of T cells effective against HCMV are limited by the need of utilizing peptides with defined HLA restrictions. We took advantage of a combination of seven predictive algorithms to identify immunogenic peptides of potential use in the prevention or treatment of HCMV infection or reactivation. Here we describe a pp65 derived peptide (pp65<sub>340-355</sub>, RQYDPVAALFFFDIDL: RQY16-mer), characterized by peculiar features. First, RQY-16mer is able to stimulate HCMV pp65 specific responses in both CD4+ and CD8+ T cells, restricted by a wide range of HLA class I and class II determinants. Second, RQY-16mer is able to induce an unusually wide range of effector functions in CD4+ T cells, including proliferation, killing of autologous HCMV infected target cells and cytokine production. Third, and most importantly, the RQY-16mer is able to stimulate CD4+ and CD8+ T cell responses in pharmacologically immunosuppressed patients. These data suggest that a single reagent might qualify as synthetic immunogen for potentially large populations exposed to HCMV infection or reactivation.

Key words

Peptide cross-presentation
Immunotherapy
Vaccine
Introduction

Primary infection by Human Cytomegalovirus (HCMV) is usually mild or asymptomatic and it is effectively controlled by T cell-mediated immune response in healthy subjects[1]. However, congenital HCMV infection in newborns[2] and viral reactivation in pharmacologically immunosuppressed patients transplanted with solid organs (SOT)[3] or hematopoietic stem cells (HSCT)[4] are frequently associated with significant morbidity and, eventually, mortality, until the immune system is effectively competent or completely reconstituted[5]. Thus, women in childbearing age and transplant recipients may represent candidate target populations for HCMV active protection.

T cell-mediated immune response against immunogenic viral targets is of paramount importance due the potential capacity of epitope-specific cytotoxic T lymphocytes (CTLs) to control viral fitness. The administration of MHC-restricted immunodominant synthetic peptides to generate CTL immunity preventing the reactivation of HCMV infection represents a promising approach already tested in clinical trials[6]. Interestingly, vaccination of HSCT donors expressing specific HLA determinants might also be important for the generation of HCMV specific T cells of potential use in adoptive immunotherapy. In this case, healthy virus carriers might represent an important reservoir of antigen specific CD4+ and CD8+ T cells.

Although much emphasis has been placed on the role of MHC class I restricted CD8+ T cells in the recognition of HCMV infected cells, there is increasing evidence that CD4+ T cells also have a crucial role in the control of HCMV infection[7].

The cytotoxic potential of CD4+ T cells specific for viral antigens has been repeatedly documented[8,9]. Furthermore, transfer of HCMV specific CD4+ T cells into HSCT has also been found to promote the expansion of HCMV specific CD8+ CTLs[10] likely due to the helper function exerted by antigen expanded CD4+ T cell populations[11].
The concomitant use of MHC class I and II restricted peptides would be highly desirable to achieve effective immune responses[12]. However, limitations inherent in MHC diversity and epitope hierarchies within HLA presentation[13] suggest that this approach would require the administration of a panel of immunogenic HLA-binding peptides particularly in case of unknown typing or when rare HLA specificities should restrict immune responses. Ideally, more than one immunogenic reagent should be used[14,15] for each HLA determinant of interest.

Indeed, a large number of immunodominant HLA class I and II restricted HCMV derived peptides have been identified[16]. However, only a minority of them has been used in clinical procedures due to the necessity of tailoring immunogens according to specific HLA typing[17].

To address these issues, we attempted the identification of highly immunogenic sequences within HCMV pp65 possibly encompassing both class I and class II restricted epitopes using a combination of predictive algorithms. Here we report the functional characterization of a highly immunogenic pp65 derived peptide (RQYDPVAALFFFDIDL; thereafter referred as RQY-16mer peptide) capable of inducing class I and class II restricted cytotoxic and lymphoproliferative responses across a wide range of HLA specificities.
Materials and methods

Peptide selection, design and synthesis

Peptides derived from the immunodominant HCMV 65 kd matrix phosphoprotein (pp65; Human herpesvirus 5 laboratory strain AD169) were used in this study. Six major algorithms, PAProC (http://www.paproc.de/), MAPPP (http://www.mpiib-berlin.mpg.de/MAPPP/), NetChop (http://www.cbs.dtu.dk/services/NetChop/), Bimas (http://www-bimas.cit.nih.gov/molbio/), SYFPEITHI (http://www.syfpeithi.de) and IEDB (http://www.immuneepitope.org/home.do), were utilized to select candidate immunodominant epitopes within pp65. Furthermore, when indicated, NetMHCIIpan (http://www.cbs.dtu.dk/services/NetMHCpan/) algorithm was also used. Peptides were synthesized by Princeton Biomolecules (Langhorne, PA) with purity ranging from 90 to 100% as analyzed by HPLC, dissolved in 100% DMSO and stored at -70°C until use.

Characterization of immunogenic sequences and algorithm predictions

MAPPP, PAProc and NetChop algorithms were initially used to screen pp65 sequence. Nine peptides, pp6549-70, pp65113-127, pp65128-144, pp65186-208, pp65293-305, pp65319-330, pp65340-355, pp65343-515 and pp65340-355 displayed the highest score of potential immunogenicity, thus extending previous results[18]. These sequences were further analyzed for proteasome cleavage sites, TAP transport and MHC binding as evaluated by IEDB, Bimas and SYFPEITHI algorithms. Based on these sets of data, we selected pp65 sequences nesting high numbers of 9mer-peptides restricted by the most frequently represented HLA class I specificities included in the algorithms. Pp65340-355 (RQY-16mer) turned out to be the sequence containing the highest numbers of potentially immunogenic HLA class I restricted 9mer-peptides. In particular, 6/8 9mers within RQY-16mer encompassed HLA-A1, A2, A24, A30,
A32 and A68 epitope-restrictions (n=6) and HLA-B7, B15, B18, B27, B35, B40, B44, B51, B53, B57 and B58 epitope-restrictions (n=6) for a total of 17 valuable HLA class I associations out of the 41 covered by the programs (42%).

**Donor selection, cord blood collection, patient accrual, HCMV serology and HLA genotyping.**

Fourteen HCMV-seropositive and two HCMV-seronegative donors were age, race and sex randomly selected and enrolled in the study upon informed consent. Five umbilical cord blood samples were collected at University Hospital in Basel after either vaginal or cesarean delivery. Ten patients receiving kidney transplant and post-transplantation care were accrued at the Department of Transplantation Immunology and Nephrology, University Hospital in Basel according to a protocol approved by the internal review board (299/06). All patients were treated with an immunosuppressive regimen consisting of mycophenolate, cyclosporine and prednisone, and one underwent prophylactic antiviral treatment, as detailed below. The presence of anti HCMV antibodies in the serum (anti-pp65 IgG titer) was analyzed with passive latex agglutination (CMVSCAN kit, Becton Dickinson Microbiology System, Cockeysville, MD or AxSymTM assay, Abbott, Sligo, Ireland [www.abbott.com](http://www.abbott.com)).

Quantitative real time PCR (qrt-PCR) assays detecting HCMV pp65 DNA in CD14+ cells of healthy donors were performed by using 5’-GTCAGCGTTCGTGTTTCCCA-3’ direct primer, 5’-GGGACACAACACCCTAAAGC-3’ reverse primer and 5’FAM-CCCGCACAACCAACCCTTTCATG-3’TAMRA fluorogenic probe [19]. Regarding transplanted patients, HCMV-replication was quantified after DNA extraction from EDTA-anticoagulated whole blood (MagnaPure TM, Hoffman-LaRoche, Basel, Switzerland [www.roche.com](http://www.roche.com)) by using 5’-TTTTTTCTAGGCGCCTTCCGA-3’ and 5’-ACACTGCGGCTTTGTATTCTTTATC-3’ primers and 5’FAM-
AGGCGAAGCCGGCGACGA-3’TAMRA probe at the laboratory of Transplantation Virology, Institute for Medical Microbiology, University of Basel, Switzerland, by taking advantage of previously published amplification protocols[20].

A complete, high resolution, HLA genotyping (HLA-A*, HLA-B* and HLA-DRB1*) was performed by PCR with allele-specific sequencing primers (PCR SSP Protrans, Ketsch am Rhein, Germany) according to the manufacturer's specifications.

**T lymphocytes collection, separation and in vitro expansion**

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation. T lymphocyte subpopulations (CD8+, CD4+) were purified by magnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany) according to producers’ protocols. Cells were then harvested in RPMI medium supplemented with 100μg/ml Kanamycin, 10mM Hapes, 1mM sodium pyruvate, 1mM Glutamax and nonessential amino acids (all from GIBCO Paisley, Scotland), thereafter referred to as complete medium supplemented with 5% human serum (Blutspendezentrum Universitätsspital Basel Switzerland). Both, CD8+ and CD4+ purified T cells were subsequently plated in complete medium with 5% human serum in 24-well plates at a final concentration of 1x10⁶ cells/ml and were co-cultured (37°C, 5% CO₂ atmosphere) with irradiated (750 sec in a gamma ray irradiator equipped with a ¹³⁷Cs radiation source emitting 100 rad/min) autologous mature dendritic cells (mDCs) (2x10⁵/ml; see below for DCs generation) previously pulsed for 2h with peptides at a final concentration of 10μg/ml either for priming or for re-stimulation rounds. Recombinant human (rh) IL-2 (Hoffmann-LaRoche, Basel, Switzerland) was added to the cultures at 1ng/ml, 1ng/ml and 5ng/ml, on days 3, 7 and 10, respectively, and cells were restimulated with specific peptide in the presence of irradiated mDCs, as detailed above, on
day 7 of culture. In the indicated experiments (see below) control cultures were performed as specified above but in the absence of antigenic peptides.

**Dendritic cells generation and CMV infection**

For DCs generation, isolated CD14+ (Miltenyi Biotech, Bergisch Gladbach, Germany) were cultured for 5 to 7 days in RPMI complete medium supplemented with 10% fetal calf serum (FCS) (GIBCO Paisley, Scotland), 0.004% β-mercaptoethanol, rhIL-4 (1000UI/ml) and recombinant human GM-CSF (50ng/ml) to generate immature DCs (iDCs). Maturation of iDCs was induced by exposure to lipopolysaccaride (*Abortus Aequi*, Sigma-Aldrich, St. Louis, MO) at a concentration of 1μg/ml.

Immature DCs were infected with cell-free endotheliotrophic (HUVEC) HCMV VR1814 strain at a MOI of 10 and incubated in complete medium supplemented with 10% FCS for 24h in round-bottom 96-well plates at 37°C in a 5% CO₂ atmosphere[21]. As control, iDCs were mock-infected with the clarified medium of uninfected HUVEC cultures. Infection grade was measured by both immunofluorescent detection of pp72 and qrt-PCR analysis of pp65 gene expression, as described above (see “Donor selection, cord blood collection, patient accrual, HCMV serology and HLA genotyping”). Immunofluorescence analysis was performed on paraformaldehyde-fixed DCs using allophycocyanine-conjugated anti-CD1a (BD Pharmingen), or on acetone: methanol (1:1)-fixed iDCs using a goat anti-IE72 mAb followed by Alexa Fluor-555-conjugated chicken anti-rabbit antibodies (Molecular Probes). Fluorescence was visualized using a 100X Plan Neofluar oil immersion objective (NA 1.3) mounted on a Zeiss Axiovert 100 confocal microscope.
Generation of EBV transformed cell lines

EBV transformed B cell lines were generated from each donor by infecting $1 \times 10^6$ freshly isolated PBMCs with Epstein-Barr virus containing supernatants from B95.8 cell line. Five $\mu g/ml$ Cyclosporin A (Novartis, Basel, CH) was added to prevent T cell response and infected cells were cultured in complete medium with 10% FCS in the presence of rhIL-6 until complete expansion. Cells were then harvested and used for experimental procedures or aliquoted and stored in liquid nitrogen for further use.

Antigenic stimulation and qrt-PCR analysis

Peptide-induced cytokine gene expression was investigated as follows. On d14 of culture (see above), CD4+ and CD8+ T cells expanded in vitro in the presence or absence of antigenic peptide(s) were plated in U-bottom 96-well plates with irradiated autologous mDCs at 10:1 ratio ($2 \times 10^5$ CD8+ or CD4+ and $2 \times 10^4$ mDCs per well). At d15, after an overnight resting, cells were either peptide-stimulated ($1 \mu g/ml$) or left unstimulated. Three hours after cells were harvested for total cellular RNA extraction (RNeasy® Mini Kit Protocol, Qiagen, Basel, Switzerland) and cDNA synthesis (Invitrogen, Carlsbad, CA). Cytokine mRNA transcript amplification was performed as previously described[22] by an ABI prism™ 7500 FAST sequence detection system using TaqMan® Universal PCR Master Mix Reagents Kit (Applied Biosystems, Rotkreuz, Switzerland) and sets of primers and probes from cytokine genes (IFN-γ, TNF-α, IL-2, IL-10) already extensively utilized[23]. CD8+, CD4+, CD14+, and β-actin were used as endogenous reference genes[23]. Normalized data were subsequently evaluated as relative quantification. The $2^{ΔΔCt}$ method [$ΔΔC_T = (C_T, \text{cytokine} – C_T, \beta\text{-actin})_{\text{induction}} – (C_T, \text{cytokine} – C_T, \beta\text{-actin})_{\text{baseline}}$, where $C_T$ is the mean cycle times of the triplicate well readings] was utilized to compute the fold change of cytokine gene expression after peptide induction relative to baseline (unstimulated cells), normalized to endogenous
reference genes[24]. When indicated, absolute numbers of cytokine encoding gene copies were calculated as previously described[22].

**Cytokine intracellular staining and T cell phenotype**

Peptide expanded CD4+ T lymphocytes (5x10⁵) were rested overnight in a 14 ml polypropylene tube (Becton Dickinson, Franklin Lakes, NJ) and then stimulated with peptide-pulsed (10μg/ml) mDCs at a final concentration of 5x10⁴ per each tube. One hour after cell activation, 10μg/ml of Brefeldin A (Sigma, Saint Louis, MI) was added. After a 5h additional incubation, cells were transferred to 5mL round-bottom tubes (Becton Dickinson, Franklin Lakes, NJ) and cell incubation was stopped by washing cells in 2ml cold phosphate-buffered saline (PBS) for 5min. Pellets were re-suspended in 1ml PBS containing 1mM EDTA and 0.5% FCS and cells were extra-cellularly stained with 10μl of monoclonal antibodies (mAb) recognizing CD3, CD4 and CD8 (BD, Bioscience, San Jose, CA) for 15min at 4°C in the dark. Cell fixation was performed with 2ml of BD FACS Lysis Solution (BD, Bioscience, San Jose, CA) according to the producers’ instructions. Cellular permeabilization was performed by re-suspending cells in 500μl of FACS Permeabilizing Solution 2 (BD, Bioscience, San Jose, CA) at a 1:10 dilution in DEPC water at room temperature (RT) for 10min. For intracellular staining, cells were stained with either 10μl of human anti IFN-γ-FITC, TNF-α-FITC, IL-2-FITC mAbs or mouse IgG1 isotype-FITC (BD, Bioscience, San Jose, CA) and incubated for 30min at 4°C in the dark. Samples were analyzed on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose, CA).

**3H-Thymidine incorporation assays**

Isolated CD4+ T cells from freshly separated PBMCs were cultured in complete medium with 5% human serum in flat-bottom 96-well plates (Becton Dickinson, Le Pont de
Claix, France) at 2x10^5 cells/well and stimulated with peptide-pulsed mDCs. On d5, ^3^H-thymidine (Amersham, Little Chalfont, UK) was added at 1μCi per well. After a 18h incubation, cells were harvested and tracer incorporation was measured by beta counting. Data were expressed as ^3^H-thymidine incorporation in counts per minutes (cpm).

**Caspase-3 production by dendritic cell**

Caspase-3 release was carried out by using PhiPhiLux® (Oncoimmunin Inc, Gaithersburg, MD) as follows. Infected iDCs were suspended in complete medium with 10% FCS at 1x10^6/ml. Cells were then incubated at 37°C, for 1h in the presence of 3M specific dye from CyToxiLux OncoImmunin (CTO™; Molecular Probes, Eugene, Oregon) and peptides (1μg/μl). The cells were then washed once and re-suspended in complete medium with 10% FCS at 1x10^6/ml. For each condition, 2x10^6 of 2-week peptide stimulated CD4+ effector-cells were co-cultured with 2x10^5 CTO stained target cells (infected or control iDCs) in round-bottom 96-well plates (Becton Dickinson, Le Pont de Claix, France) for 2h at 37°C. The supernatant was then removed and the cells were incubated in 75μl/well of the indicated caspase-3 substrate (10M, OncoImmunin, Gaithersburg, Maryland) for 30min at 37°C and washed twice with PBS[25]. An extra staining for CD1a was performed to identify iDCs. Cells were then analyzed by flow cytometry (FACScalibur; Becton Dickinson, San Josè, CA) using Cell Quest software (Becton Dickinson) and gated for CD1a expression.

**Cytotoxicity assays**

Cytotoxic activity of peptide expanded CD8+ T cells was tested on d14 by 4h chromium release assays using, as targets, autologous EBV transformed B cells previously labelled with ^5^1Cr (50μCi of ^5^1Cr for 1h at 37°C) and pulsed for 2h with cognate or control peptides at a concentration of 2.5μg/ml. Specific lysis of target cells from triplicate wells was
calculated according to the standard formula: 100 × [(cpm experimental release - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release)]. Results are reported as mean of delta specific lysis (ΔSL; experimental positive release – experimental negative release)[26].

**T lymphocytes multimer staining**

HLA-A*0201 and HLA-A*2402 MCH Pro5™ PE-labeled pentamers for pp65_{340-348} (RQY-9mer) and pp65_{341-349} (QYD-9mer) (Proimmune, Oxford, UK) were used for surface staining of cells under investigation. Cells were first stained with 1μl MHC PE-labeled pentamer for 15min at 4°C in the dark. Subsequently, they were washed once and stained with 5μl of CD8-FITC (BD, Bioscience, San Jose, CA) for 30min at 4°C in the dark. After staining, samples were analyzed on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose, CA).

**Glutaraldehyde fixation**

Immature DCs were fixed as described by Shimonkevitz et al[27]. Briefly, cells were re-suspended in PBS and fixed by addition of glutaraldehyde (final concentration 0.05%) for 30sec at room temperature. The reaction was stopped by adding L-glycine (final concentration 0.2M) for 45sec and by three washes with PBS.

For the measurement of antigen-specific T cell activation, 5×10⁴ fixed or unfixed autologous iDCs were co-cultured in U-bottom 96-well plates with 2×10⁵ antigen specific in vitro expanded T cells for 3h in the presence of the indicated peptides. T cell reactivation was evaluated by IFN-γ gene expression.
Statistical analysis

Statistical analysis was performed by using Prism 4 (GraphPad) software. Data were reported as mean ± standard deviation or as median and ranges where appropriate. Categorical markers were analyzed by Pearson’s chi-square test. A two-tailed paired T test was used to calculate p-values. Due to variability related to heterogeneous HLA restriction, unpaired T-tests and F tests were used to compare variance when appropriate. Differences were considered significant at p<0.05, CI 95%.
Results

Both CD4+ and CD8+ T cell respond to RQY-16mer peptide.

In order to define the overall immunogenicity of RQY-16mer, we evaluated its capability to elicit CD4+ and/or CD8+ immune responses in T cells. CD4+ and CD8+ T cells from either HCMV-seropositive or seronegative donors (Table 1A) were 2-week in vitro stimulated using RQY-16mer peptide-loaded or untreated autologous mDCs. A 3h assay of RQY-16mer mediated induction of IFN-γ, TNF-α, IL-2, and IL-10 cytokine gene expression was then performed on expanded cells. A cut-off level of responsiveness (=1.9 folds) was calculated based on the responses to 3h stimulation of 2-week cultures from seropositive donors conducted in the absence of antigenic peptide.

RQY-16mer induced the activation of T cells pre-cultured in the presence of the peptide from all HCMV-seropositive donors, as detectable by a significant increase in proinflammatory cytokine gene expression as compared to unstimulated controls, irrespective of their HLA specificities (Figure 1A). In particular, IFN-γ gene expression was increased 6.27±2.96 folds in RQY-16mer stimulated CD4+ T cells, as compared to control cultures. TNF-α and IL-2 gene expression were also induced accordingly (6.83±4.67 folds and 8.141±0.85 folds, respectively). In contrast, no concomitant induction of IL-10 gene expression was detectable (0.83±0.44 folds; IFN-γ versus IL-10 gene expression p=0.001; TNF-α versus IL-10 gene expression p=0.02; IL-2 versus IL-10 gene expression p=0.0001).

Most intriguingly, expression of IFN-γ, TNF-α and IL-2 genes was also induced by RQY-16mer stimulation in purified CD8+ T cells putatively responding only to shorter HLA class I restricted peptides. Indeed, the expression of these genes was increased 5.11±2.94 folds, 6.99±2.45 folds and 5.62±0.95 folds, respectively in RQY-16mer stimulated CD8+ T cells from HCMV-seropositive donors, in comparison to unstimulated controls. Again, no induction
of IL-10 gene expression was observed (1.28±0.61 folds; IFN-γ versus IL-10 gene expression p=0.02; TNF-α versus IL-10 gene expression p=0.004; IL-2 versus IL-10 gene expression p=0.009, Fig. 1A).

Notably, cytokine gene expression in HCMV-seronegative donors was not significantly increased following RQY-16mer stimulation of CD4+ or CD8+ T cells, in comparison with unstimulated cells, and its variations did not reach the calculated cut-off level of 1.9folds.

Importantly, a highly significant correlation between the extent of the expression of IFN-γ and TNF-α genes in CD4+ or CD8+ T cells upon RQY-16mer peptide induction and the amount of pp65 DNA transcripts in CD14+ monocytes from the same donors was also observed (CD4+/IFN-γ: r=0.797, p=0.0004; CD4+/TNF-α: r=0.710, p=0.003; CD8+/IFN-γ: r=0.734, p=0.002; CD8+/TNF-α: r=0.696, p=0.004; Fig. 1B,C,D,E).

To further support the notion of an antigen specific responsiveness to RQY-16mer, unrelated to unspecific mitogenic effects, 5 cord blood specimens expressing a variety of HLA class I and II molecules (Table 1B) were tested for ex vivo IFN-γ gene expression upon peptide stimulation. RQY-16mer failed to induce significant increases in IFN-γ gene expression in these samples. In contrast, positive control phytoemagglutinin (PHA) induced a strong cytokine gene expression (p=0.0001; Fig. 1F). Three specimens were from HLA-A*0201 donors. As expectable in cord blood T cells, control virus derived HLA-A*0201 restricted peptides selected among latent (CMV pp65495-503[28], BKV LTag579-587[26], EBV LMP1159-167[12]) and non-latent viruses (Flu M158-66[29]) were also unable to induce cytokine gene expression.

CD8+ T cell reactivity to nonamer (9mer)-peptides nested within RQY-16mer peptide

Cytokine gene expression data indicated that RQY-16mer peptide was able to efficiently stimulate CD8+ T cells, in spite of its relatively large size. We explored in more detail the
ability of each of the eight 9mer-peptides tiled at one amino acid pace within RQY-16mer peptide to recall epitope-specific immune responses from CD8+ T cells previously expanded upon RQY-16mer induction.

Representative results from three donors (D9, D10 and D11), expressing HLA class I alleles accounting for >60% of the alleles expressed in our study cohort and detectable with high frequency in different populations are reported (Fig. 2A). At least four 9mer-peptides were found to be able to stimulate significant IFN-γ gene transcription in cells from these donors. Interestingly, the 9mer peptides inducing IFN-γ gene expression in cells from specific donors were frequently predictable based on the IEDB algorithm. For instance, according to the IEDB MHC binding score (IC₅₀), peptide RQY-9mer could be restricted by several alleles including i.e. HLA-A*0201, A*3001, A*3201 HLA-B*0702, B*1501, B*2701, B*4001. Indeed, among the donors tested in the experiments, as reported in Fig.2, this peptide induced significant IFN-γ gene expression in cells from the two donors (D9 and D10) bearing one or more of these specificities, but its immunogenic activity was barely detectable in the donor (D11) lacking the expression of these alleles (Fig. 2A).

Table 2 cumulatively reports predicted HLA restricting determinants and specific scores of 9mer peptides encompassed by RQY-16mer. Numbers of donors expressing defined HLA alleles and showing evidence of specific response are also displayed. Notably, considering the redundancy of the restriction of the presentation of some of the peptides under investigation, we are unable to unequivocally attribute responsiveness to restriction by individual allelic products. Nevertheless, it is of interest that at least 6/8 peptides nested within the RQY-16mer sequence were able to induce specific responses. Furthermore, 2 peptides (RQY- and ALF-9mers) fulfilled immunodominance criteria, as defined by Nastke et al. [30], in that they were able to stimulate specific responses in at least 6/8 donors (75%) expressing HLA-A*0201. The previously described immunodominant QYD-9mer[31] was also found to be able to stimulate
specific responses in 3/3 HLA-A*2401 donors, thereby confirming the integrity of our experimental approach.

These findings were supported by cytotoxicity assays performed by using RQY-16mer peptide expanded CTLs from the three representative donors under investigation as effectors and autologous EBV transformed B cell loaded with specific HLA class I restricted matching peptides as targets. Significant levels of specific lysis at 10:1 E/T ratio ($\Delta$SL$_{10:1}$) could be observed by pulsing target cells with 9mer-peptides capable of inducing IFN-\(\gamma\) gene expression (Fig 2B).

Comparative analysis of CD8+ T cell re-activation by RQY-16mer and RQY- or QYD-9mers induction.

To further characterize the CD8+ T cell stimulating capacity of RQY-16mer, multimeric reagents inclusive of HLA-A*0201 or HLA-A*2402 and immunodominant RQY- or QYD-9mers, respectively, were generated. These tools were able to ex vivo stain sizeable percentages of CD8+ T cells from HCMV-seropositive donors expressing specific HLA alleles. T cells from two of these donors (D9 and D10, Fig. 2C,F) were co-cultured with autologous mDCs in the presence of RQY-16mer or RQY- or QYD-9mers peptides. Multimer-stainings and specific cytotoxic activities were then evaluated. Predictably, RQY- or QYD-9mers were highly effective in expanding specific CD8+ T cells endowed with cytotoxic capacity (Fig. 2D,G). Most interestingly, RQY-16mer was also effective in expanding CD8+ T cells with similar phenotypic and functional features (Fig. 2E,H).
Stimulation of both CD4+ and CD8+ T cell responses by RQY-16mer requires active processing by peptide-pulsed DCs

We hypothesized that RQY-16mer could be fully internalized into DCs, processed through an exogenous pathway and cross presented in the context of matching HLA class I determinants. To test this hypothesis, iDCs from three HCMV-seropositive donors bearing different HLA antigens were glutaraldehyde (Gla)-fixed and used to stimulate T cell responses in the presence of RQY-16mer or selected 9mer-peptides. As control, live iDCs were used. We found that the ability of in vitro expanded CD4+ or CD8+ T cells to respond to RQY-16mer peptide stimulation by IFN-γ gene expression was significantly jeopardized when fixed iDCs were used as antigen presenting cells, as compared to live cells (CD4+/IFN-γ: 1.65±0.21 versus 5.08±1.58 fold increase, p=0.048; CD8+/IFN-γ: 1.34±0.25 versus 6.28±1.47 fold increase, p=0.046; Fig. 3A). In contrast, fixed iDCs fully retained their capability to activate CD8+ T cells in the presence of 9mer-peptides, comparably to their unfixed counterparts (Fig. 3B)[32].

Cytokine production by RQY-16mer specific CD4+ T cells.

Due to the active role possibly played by CD4+ T cells in controlling viral infections and considering the high immunogenicity of the RQY-16mer, the activity of specific CD4+ T cells was also tested at protein level. Intracellular cytokine staining (ICS) was performed in CD4+ T cells cultured for 2-weeks in the presence or absence of antigenic peptide and reactivated for 6h by RQY-16mer loaded autologous mDCs. Seven HCMV-seropositive donors were selected to represent 90% the HLA-DRB1* specificities detectable in our group of donors (Table 1A). The two HCMV-seronegative donors were also included as negative controls.

Cut-off values (0.18%) were calculated based on the stainings observed in cultures conducted for two weeks in the presence of mDCs and IL-2 but without antigenic stimulation.
Percentages of IFN-γ, TNF-α and IL-2 positive cells in 6h assays performed in the presence of antigenic peptide significantly exceeding cut-off values were observed in antigen pre-stimulated cultures from each HCMV-seropositive donor tested irrespective of their specific HLA DRB1* association (IFN-γ: 4.76±10.18%, median 0.84%, range 0.24-27.8%; TNF-α: 5.69±13.02, median 0.95%, range 0.22-35.2%; IL-2: 3.11±7.14, median 0.39%, range 0.23-19.3% **Fig. 4A**). In contrast, percentages of ICS cytokine positive cells in 6 hours assays performed in the absence of antigenic peptide, were equal to or lower than cut-off values (0.08±0.04% for IL-2, 0.18±0.03% for IFN-γ and 0.08±0.04% for TNF-α). Notably, RQY-16mer stimulation of cells from HCMV-seronegative donors did not result in the production of cytokines (<0.05% in all cases). It is of note that analysis of RQY-16mer by NetMHCIIpan algorithm revealed the existence within this peptide of a number of core sequences capable of binding the DRB1* allelic products expressed by the donors under investigation, as reported in figure 4B.

**Functional features of RQY-16mer expanded CD4+ T cells**

To confirm the ability of RQY-16mer peptide to activate CD4+ T cells and to demonstrate their possible direct role in the control of CMV infection, we investigated their antigen specific proliferation and cytotoxic activity.

Freshly obtained CD4+ T cells from HCMV-seropositive donors (n=4) accounting for a high percentage of the HLA-DRB1* alleles expressed in our cohort (75%) were co-cultured with autologous mDCs in the presence or absence of the specific peptide for 7 days. A 0.5 μg/ml concentration of RQY-16mer sufficed to stimulate a significant proliferation of specific CD4+ T cells in all donors tested, thus confirming its ability to reactivate and expand immune responses from HCMV-seropositive individual irrespective of their HLA-DRB1* specificities. It is remarkable that core sequences within RQY-16mer capable of binding each of these class
II determinants were revealed by analysis with NetMHCIIpan algorithm. In contrast no response was induced upon stimulation of cells from a control HCMV-seronegative donor DRB1*07,14 (p=0.4) (Fig. 5A).

The cytotoxic capacity of RQY-16mer peptide stimulated CD4+ T cells was then evaluated by caspase release assays. Immature DCs were infected with HCMV as previously described[33]. CD4+ T cells from HCMV-seropositive donors bearing different HLA class II antigens (n=3) were expanded in vitro for two weeks by RQY-16mer peptide stimulation. They were then incubated for 4h with HCMV-infected iDCs at 5:1 E/T ratio. Cultures were then harvested and stained for intracellular caspase-3 release and for surface expression of CD1a as marker for DC identification.

Caspase-3 production in the absence of expanded T cells was negligible in HCMV-infected iDCs (positive cells=0.02±0.01%, mean fluorescence intensity –MFI– =3, left histogram, Fig 5B) and comparable to that of untreated iDCs (data not shown). Co-culture in the presence of RQY-16mer peptide expanded CD4+ T cells induced caspase-3 production in mock-infected autologous iDC targets, likely due to unspecific stimulation of cytotoxic activity by the IL-2 used in the T cell expansion protocol, and, possibly, at least in part, to their expression of endogenous HCMV genes, including pp65 [34] (positive cells=19.72+7.56%, MFI=57, mid histogram, Fig 5B). Superinfection of target cells with HCMV significantly increased their sensitivity to the cytotoxic activity of RQY-16mer stimulated CD4+ T, resulting in a 60% MFI increase upon caspase-3 staining (positive cells=59.55+4.01%, MFI=280, right histogram, Fig 5B). Figure 5C reports cumulative data obtained in three independent experiments (mock-infected iDCs fluorescence index –FI– = 3.33±1.52 versus HCMV-infected iDCs FI=77.26±7.39, p=0.0073; Fig. 5C).
IFN-γ an IL-2 protein production upon RQY-16mer peptide stimulation in kidney transplanted patients

HCMV infection is frequently reactivated in pharmacologically immunosuppressed transplanted patients. Thus, the capability of specific antigenic peptides to stimulate immune responsiveness in these conditions is of utmost clinical relevance. We tested the ability of RQY-16mer of reactivating a peptide specific immune response in isolated T cells from kidney transplanted patients (n=10; Table 3A). All patients were treated with an immunosuppressive regimen consisting of mycophenolate, cyclosporine and prednisone and tested 183±102 (range 83-374) days after transplantation. One seronegative patient (P3) transplanted with a kidney from a seropositive donor and thereby at high risk of HCMV infection underwent a prophylactic regimen (VALgcv) for the first three months post-transplant.

T cells from these patients were cultured in the presence of antigenic peptide for two weeks and then tested by ICS as detailed in “materials and methods”. IFN-γ and IL-2 protein production upon 6h RQY-16mer stimulation was significantly increased in comparison with unstimulated cells in CD4+ T lymphocytes from, 4/8 (P1,2,7,10) and 5/8 (P1,2,7,8,10) HCMV-seropositive patients, respectively. However, no responses were observed in either patient P3 undergoing primary infection under antiviral prophylactic treatment or in the HCMV-seronegative patient P5 (Table 3A). Responsiveness to class I restricted epitopes encompassed by RQY-16mer was also investigated in CD8+ T cells from 4 patients. Interestingly, in three of them, P4, P6 and P9, showing no CD4+ T cell responsiveness, 9mer peptides nested within RQY-16mer were able to elicit IFN-γ production in CD8+ T cells. In P10 CD8+ T cell responsiveness to nested 9mers matched the vigorous response of CD4+ lymphocytes to RQY-16mer (Table 3B).
Discussion

A number of different approaches are currently being investigated to prevent HCMV infection or its reactivation. They include active immunization with live attenuated virus, subunit vaccines, recombinant viral vectors, neutralizing immunoglobulins, synthetic peptides or DNA vaccines[35]. Furthermore, adoptive transfer of HCMV-specific T cells expanded by using specific and unspecific HLA matching peptides has also been utilized[36].

In this study we addressed the identification of HCMV pp65 derived antigenic peptides of potential use across a wide range of HLA specificities. Given the decisive role of helper T cells in the optimal expansion of CTL, and, in particular, in the anti HCMV specific immune response, the characterization of both class I and class II restricted epitopes was envisaged. Clearly, single peptides gathering the ability to stimulate HLA class I and II restricted T cells beyond the limits of foretold MHC-peptide binding would represent reagents of potentially high clinical relevance.

Algorithms are commonly used to predict HLA-peptide affinity and they usually provide preliminary insights for the identification of novel antigenic epitopes[26]. Binding to HLA gene products represents a fundamental prerequisite of peptide immunogenicity[37-39]. However studies based on linear regression analysis[40,41] have demonstrated that the immunogenicity of a number of peptides predicted to avidly bind HLA restricted molecules is not always validated by in vitro or in vivo studies.

In this work we demonstrate that concurrent use of different epitope prediction algorithms is not only highly informative regarding the likelihood of individual peptides to be processed and presented within specific HLA restrictions but it is also able to predict with low margin of error the immunogenic potential of specific sequences.

The novel HCMV derived immunogenic peptide described here is characterized by a potentially high clinical relevance due to a number of important features.
First, RQY-16mer is able to stimulate HCMV pp65 specific responses in both CD4+ and CD8+ T cells, restricted by a wide range of HLA class I and class II determinants. This responsiveness is not related to intrinsic stimulatory activity because it does not extend to cord blood lymphocytes. Thus, a single reagent might qualify as synthetic immunogen for potentially large populations exposed to HCMV infection or reactivation. A large number of pp65 derived class I restricted antigenic peptides have been described[16] and key-target class II restricted peptides have also been identified[42]. However the number of peptides capable of simultaneously stimulating CD4+ and CD8+ T cells is very limited[43]. Notably, the latter reagents usually induce CTL responses restricted by one HLA class I determinant only. Although they have provided proof of principle of the possibility of activating CD8+ T cells by using “long” peptides, they hardly qualify for a wider clinical use.

Interestingly, RQY-16mer displays a relatively hydrophobic pattern and its capability to stimulate either CD4+ or CD8+ T cell responsiveness requires processing by live antigen presenting cells. These data suggest that its promiscuity could be ascribed to internalization into iDCs, peptide trimming by immunoproteasome activity and processing of shorter sequences swapped into different HLA class I and class II clefts to better fit multiple associations, as predicted by algorithm binding scores for RQY-16mer relevant peptide-cores. The sequence might thus retain the power of inducing specific immune responses restricted by a wide range of HLA allelic products.

Intriguingly, RQY-16mer has previously been studied by other groups[30,43], but its high immunogenicity was not fully disclosed. It is worth noting that in these studies either total PBMC were used as responder cells[30,44] or T cell stimulation was achieved by using TNF-α matured DC or transfected B cells as APC[43,45]. Dendritic cells matured through TLR-4 triggering used in our study are characterized by a significantly higher APC capacity. In particular, they produce IL-12, while TNF-α matured DCs fail to do so[46].
A second important feature of the RQY-16mer is represented by its capability to induce an unusually wide range of effector functions in CD4+ T cells. As expectable, specific CD4+ T cells are capable of producing IFN-γ and TNF-α and of proliferating in response to RQY-16mer. Most interestingly, however, they are also able to elicit a significant cytotoxic activity against virus infected autologous target cells. CD4+ HCMV specific cytotoxic T lymphocytes have been typically detected during early phases of HCMV infection[47]. However, their elicitation upon in vitro stimulation by antigenic peptides represents a relatively infrequent event[43].

Third, and most importantly, the RQY-16mer is able to stimulate CD4+ and CD8+ T cell responses in pharmacologically immunosuppressed patients. These data suggest that this reagent could be advantageously utilized to prevent or treat HCMV infection or reactivation in a population of patients at high risk of developing disease.

Finally, we are confident that the concurrent use of different predictive algorithms, exemplified in this study, could be of particular relevance for the identification of immunogenic sequences within candidate viral and tumor antigens, irrespective of discrete MHC associations [48-51]. Universal peptides eventually encapsulated into virosome formulations[52,53] or encoded by viral vectors [52,54] might represent key-reagents for the generation of powerful vaccine preparations aimed at strengthening cellular immune responses. Data bases inclusive of their sequences might help unraveling specific motives associated with the ability to promiscuously activate T cells across a wide variety of MHC class I and class II restricting determinants.
Acknowledgments

This work was partially supported by a grant from Swiss National Funds for Scientific Research to Giulio C Spagnoli. Cell-free endotheliotrophic (HUVEC) HCMV VR1814 strain and the goat anti-IE72 mAb were provided by G. Gerna (Policlinico San Matteo, Pavia, Italy). Recombinant human IL-4 and IL-6 were a gift of A. Lanzavecchia (Institute for Research in Biomedicine, Bellinzona, Switzerland). Recombinant human GM-CSF was a gift from the Laboratorio Pablo Cassara', Buenos Aires, Argentina.
References


Figure legends

Figure 1. Responsiveness of CD4+ and CD8+ T cells to immunostimulation by RQY-16mer peptide irrespective of HLA typing.

(A) CD4+ and CD8+ T cells from 14 seropositive healthy donors were cultured for two weeks in the presence of RQY-16mer peptide, as detailed in the “materials and methods” section. Peptide-induced expression of the indicated cytokine genes was then assessed following a three hours incubation in the presence or absence of RQY-16mer. Data are reported as cytokine gene relative quantification (2^{-ΔΔCt}) in RQY-16mer triggered cells, as compared to cells incubated in the absence of peptide. Interquartile ± s.d. are reported for each single cytokine gene expressed by either CD4+ or CD8+ T cells upon RQY-16mer induction. Negative cut-off (dotted line) was set at 1.9 fold increase based on average variation of cytokine gene expression in T cells cultured in the absence of antigenic peptide for two weeks and then challenged for three hours in the presence or absence of RQY-16mer peptide. RQY-16mer stimulation of T cells from seronegative donors (n=2) failed to induce increases in cytokine gene expression exceeding cut-off values (see panels B-E, empty circles).

(B through E) Specific RQY-16mer T cell reactivation as an hallmark of HCMV latency. Numbers of IFN-γ and TNF-α gene transcript copies induced by RQY-16mer stimulation in either CD4+ or CD8+ cultured T cells were correlated to those of pp65 DNA copies detectable in autologous fresh CD14+ cells for each of the 14 HCMV-seropositive (full circles) and 2 HCMV-seronegative (empty circles) donors under investigation. Categorical markers were analyzed by Pearson’s chi-square test. A two-tailed paired T-test was used to define p-values (B: CD4+/IFN-γ: p=0.0004; C: CD8+/IFN-γ: p=0.002; D: CD4+/TNF-α: p=0.003; E: CD8+/TNF-α: p=0.004).

(F) Antigen specific responsiveness to RQY-16mer is not related to unspecific stimulatory effects. IFN-γ gene expression was studied in cells from 5 cord blood specimens
upon ex vivo RQY-16mer peptide stimulation (median 1.28 folds, range 0.33-1.85) or, in HLA-A*0201 cord blood specimens (n=3) following stimulation with different HLA-A*0201 restricted virus derived antigens (HCMV pp65495-503, BKV large T antigen 579-587, EBV LMP-2426-434, influenza matrix58-66; median 1.01, range 0.32-1.57) as compared to mitogenic PHA stimulation (median 14.52 folds, range 9.85-15.14; p=0.0001). Data are reported as IFN-γ gene expression relative quantification ($2^{-\Delta\Delta C_T}$) as compared to cells from the same donors preincubated in the absence of stimuli.

**Figure 2.** Peptide-specific immune stimulation induced by 9mer-peptides nested within RQY-16mer in CD8+ T cells following in vitro expansion driven by RQY-16mer.

(A) CD8+ T cells from representative HCMV seropositive healthy donors (D9-D11, see Table 1) expressing the indicated HLA class I specificities were cultured in the presence of RQY-16mer, as detailed in “materials and methods”. Cells were then stimulated for three hours in the presence of 9mer-peptides encompassed within RQY-16mer sequence, and cytokine gene expression was assessed by qrt-PCR. Data are reported as percentage of IFN-γ gene expression, as compared to positive control PHA. Black bars identify stimulatory 9mer-peptides inducing cytokine gene expression >2% of PHA triggered levels. The HLA-A*0201 restricted pp65495-503 (NLV) peptide was used as control.

(B) Cytotoxic activity of RQY-16mer stimulated CD8+ T cells against nested 9mer-peptides. CD8+ T cells from the same experiments depicted in A were tested as effector cells in $^{51}$Cr release assays by using, as targets, autologous EBV transformed B cells upon pulsing with the indicated peptides. Data are reported as mean of delta specific lysis ($\Delta SL$) ± s.d at 10:1 E/T ratio.

(C through H) Frequencies of HCMV epitope specific cells ex vivo or following peptide driven in vitro (i.v.) T cell expansion. Total T cells (C-E) or purified CD8+ T cells (F-H) from
two HCMV-seropositive donors (D10 and D9, respectively) expressing appropriate HLA specificities were stained ex vivo with RQY-9mer/HLA-A*0201- (C) and QYD-9mer/HLA-A*2402-pentamers (F). Cells were considered positive if their mean fluorescence intensity (MFI) exceeded by at least tenfold that of multimer negative CD8+ cells. The same cell preparations were then stimulated for 2 weeks in the presence of RQY-9mer (D), QYD-9mer (G), or RQY-16mer (E,H) and staining with RQY-9mer/HLA-A*0201- and QYD-9mer/HLA-A*2402-pentamers was repeated on cultured cells. Cells expanded in the presence of specific 9mer-peptides or RQY-16mer were also used as effector cells in cytotoxicity assays utilizing, as targets autologous EBV transformed B cells upon pulsing with specific (squares) or a control (gp100280-288, HLA-A*0201 restricted melanoma associated epitope, diamonds) peptide. Data are reported as percentage specific lysis at the indicated E/T ratios. Standard deviations, never exceeding 10% of the reported values, were omitted.

**Figure 3.** Glutaraldehyde (Gla)-fixation of dendritic cells jeopardizes the induction of RQY-16mer peptide specific immune responses.

(A) IFN-γ gene expression by cultured CD4+ and CD8+ T cells is significantly (p<0.05) impaired if RQY-16mer peptide is presented by autologous Gla-fixed (white bars) iDCs, as compared to RQY-16mer treated live iDCs (black bars). Data are reported as IFN-γ gene expression as related to the response in the absence of antigenic peptide. A two-tailed paired T-test was used to define p-values (p=0.05; CI 95%).

(B) Gla-fixation of iDCs (white squares) does not impair responsiveness of CD8+ T cells to HLA class I restricted 9mer-peptides, as compared to reactivity upon peptide presentation by live iDCs (black squares). Instead, a significant increment of IFN-γ gene expression is observed in CD8+ T cells co-cultured with live iDCs in the presence of RQY-16mer peptide, as compared to Gla-fixed cells (514% for the first donor and 549% for the second donor).
Figure 4. RQY-16mer is a promiscuous epitope stimulating cytokine production by CD4+ T cells across a wide range of HLA class II restrictions.

(A) Percentages of CD4+ T cells producing proinflammatory cytokines (IFN-γ: circles; TNF-α: triangles; IL-2: diamonds) upon RQY-16mer induction were assessed by ICS in cells from 7 donors previously cultured for 2 weeks in the presence of the peptide, as detailed in “materials and methods”. Each symbol represents the average of two independent experiments performed by using cells from the same donor. Cut-off limit was set at 0.18% (dotted line) based on RQY-16mer peptide-induced cytokine production by CD4+ T cells expanded for 2 weeks in the presence of mDC and IL-2, but in the absence of peptide. Quadrants were set based on an IgG1 isotype control (see below). Median percentages of responding cells are indicated by bars.

(B) Representative ICS of RQY-16mer stimulated cytokine production from HCMV-seropositive donors expressing 90% of the HLA-DRB1* alleles represented in our group. Data reported in the quadrants refer to percentages of CD4+ T cells showing evidence of the production of the indicated cytokines induced by RQY-16mer in CD4+ T cells from different donors expanded for 2 weeks in the presence of the peptide. For each donor, HLA-DRB1* tissue typing data and predicted netMHCIIpan algorithm associations of core sequences embedded within RQY-16mer with defined allelic products are also reported (characters in bold, right column).

Figure 5. Functional features of RQY-16mer induced CD4+ T cells. (A) Proliferation of CD4+ T cells upon RQY-16mer stimulation. Freshly isolated PBMCs from 4 HCMV seropositive donors and one seronegative donor were cultured for 1 week in the presence (black bars) or absence (white bars) of RQY-16mer, as detailed in material and methods. Proliferation was measured by ³H-Thymidine incorporation. Results are reported as mean of
cpm ± s.d. of triplicate wells. Two-tailed paired T-test (stimulation vs non stimulation) was used to define p-values (p=0.05; CI 95%). Significant increases in RQY-16mer induced proliferation were observed in seropositive donors (DRB1*04,14 p=0.04; DRB1*01,13 p=0.05; DRB1*04,12 p=0.03; DRB1*03,15 p=0.004), but not in the seronegative donor (DRB1*07,14, p=0.4).

(B) Caspase-3 production by HCMV infected iDCs co-cultured with RQY-16mer expanded CD4+ T cells. Following RQY-16mer driven expansion (see “materials and methods”), CD4+ T cells were co-cultured at 10:1 E/T ratio with autologous iDCs infected at a MOI of 10 with HCMV VR1814 or mock-infected. Caspase-3 production, as detected in CD1a+ DCs was used as read-out. The histogram shows a significant increase of caspase-3 production in either mock-infected (middle red peak) or HCMV infected iDCs (right peak), upon co-culture with RQY-16mer expanded CD4+ T cells, as compared to that detectable in negative control iDCs in the absence of effectors (left peak). A 60% increase of caspase-3 production, as detectable by specific MFI, in HCMV infected iDCs as compared to mock-infected counterparts was observed in the presence of RQY-16mer expanded CD4+ T cells.

(C) Caspase-3 production by HCMV infected versus mock-infected iDCs following co-culture with CD4+ T cells expanded in the presence of RQY-16mer, as observed in three independent experiments by using cells from different HCMV seropositive donors. The fluorescence index was calculated by the following formula: (MFI experimental sample – MFI background)/(MFI background) %. Statistical significance (p=0.0073) was analyzed by two-tailed paired T-test.
Table 1: HLA typing and HCMV serology of donors and cord blood specimens.

<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA-A*</th>
<th>HLA-B*</th>
<th>HLA-DRB1*</th>
<th>HCMV serological testing (i)</th>
<th>HCMV molecular testing (ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>0201,0201</td>
<td>4901,5101</td>
<td>14,16</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D2</td>
<td>0101,0201</td>
<td>0801,5101</td>
<td>03,15</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D3</td>
<td>0201,0301</td>
<td>15,4001</td>
<td>1302,1302</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D4</td>
<td>0101,0201</td>
<td>0501,4402</td>
<td>07,11</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D5</td>
<td>0201,0301</td>
<td>2701,4402</td>
<td>04,16</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D6</td>
<td>1101,2402</td>
<td>0702,5701</td>
<td>07,15</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D7</td>
<td>0212,0301</td>
<td>0709,1801</td>
<td>1301,15</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D8</td>
<td>0101,0301</td>
<td>1401,5701</td>
<td>04,07</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D9</td>
<td>2402,3001</td>
<td>0702,0801</td>
<td>03,15</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D10</td>
<td>0201,0201</td>
<td>0702,1501</td>
<td>04,12</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D11</td>
<td>1101,2402</td>
<td>35,53</td>
<td>0101,1302</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D12</td>
<td>0201,3201</td>
<td>0801,5801</td>
<td>03,03</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D13</td>
<td>02,6801</td>
<td>5101,55</td>
<td>04,14</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D14</td>
<td>0201,0201</td>
<td>35,55</td>
<td>11,14</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>D15</td>
<td>0201,2301</td>
<td>5101,5301</td>
<td>07,14</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>D16</td>
<td>0201,0301</td>
<td>1302,1501</td>
<td>1301,15</td>
<td>neg</td>
<td>neg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cord blood</th>
<th>HLA-A*</th>
<th>HLA-B*</th>
<th>HLA-DRB1*</th>
<th>HCMV serological testing (i)</th>
<th>HCMV molecular testing (ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1</td>
<td>0101,0201</td>
<td>0702,4001</td>
<td>13,15</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>CB2</td>
<td>2402,1101</td>
<td>15,4001</td>
<td>07,11</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>CB3</td>
<td>0201,0301</td>
<td>1506,1801</td>
<td>14,16</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>CB4</td>
<td>0201,0201</td>
<td>4001,4601</td>
<td>07,14</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>CB5</td>
<td>0101,0201</td>
<td>0702,5101</td>
<td>01,03</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

(i) specific anti-pp65 IgG titer. Pos = agglutination >1:8 dilution
(ii) qrt-PCR amplification of pp65 DNA in CD14+ cells.
na = not available
Table 2: Algorithm predictions and responsiveness to 9mer-peptides nested within RQY-16mer.

<table>
<thead>
<tr>
<th>HLA class I alleles (i)</th>
<th>9-mer peptides restricted by HLA specificities listed in column 1 (ii)</th>
<th>IEDB score (iii)</th>
<th>MHC affinity IC50[nM] (iv)</th>
<th>number of responding donors out of those bearing the allele (v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A*0101 (n=3)</td>
<td>QYDPVAALF</td>
<td>-1.26</td>
<td>4812.7</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>RQYDPVAAL</td>
<td>-0.36</td>
<td>264.6</td>
<td>6/8 (75%)</td>
</tr>
<tr>
<td></td>
<td>VAAALFFFDI</td>
<td>-1.74</td>
<td>683.1</td>
<td>5/8</td>
</tr>
<tr>
<td></td>
<td>ALFFFDIDL</td>
<td>-0.13</td>
<td>99.4</td>
<td>6/8 (75%)</td>
</tr>
<tr>
<td>HLA-A*0201 (n=8)</td>
<td>QYDPVAALF</td>
<td>0.17</td>
<td>178.7</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>RQYDPVAAL</td>
<td>0.6</td>
<td>29.3</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>VAAALFFFDI</td>
<td>-0.33</td>
<td>572.9</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>ALFFFDIDL</td>
<td>1.04</td>
<td>6.7</td>
<td>1/1</td>
</tr>
<tr>
<td>HLA-A*2402 (n=3)</td>
<td>QYDPVAALF</td>
<td>-1.09</td>
<td>1426.9</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>YDPVAAALFF</td>
<td>0.12</td>
<td>88.9</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>YDPVAAALFF</td>
<td>-0.98</td>
<td>3044.4</td>
<td>1/2</td>
</tr>
<tr>
<td>HLA-A*3001 (n=1)</td>
<td>QYDPVAAL</td>
<td>0.6</td>
<td>29.3</td>
<td>1/1</td>
</tr>
<tr>
<td>HLA-A*3201 (n=1)</td>
<td>QYDPVAALF</td>
<td>0.6</td>
<td>29.3</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>VAAALFFFDI</td>
<td>-0.83</td>
<td>1920</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>VAAALFFFDI</td>
<td>1.01</td>
<td>110.1</td>
<td>0/1</td>
</tr>
<tr>
<td>HLA-A*6801 (n=1)</td>
<td>DPVAAALFF</td>
<td>-0.26</td>
<td>285.9</td>
<td>1/1</td>
</tr>
<tr>
<td>HLA-B*0702 (n=3)</td>
<td>RQYDPVAAL</td>
<td>-1.15</td>
<td>1640.8</td>
<td>1/3</td>
</tr>
<tr>
<td>HLA-B*1501 (n=2)</td>
<td>RQYDPVAAL</td>
<td>0.12</td>
<td>88.9</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>YDPVAAALFF</td>
<td>-0.98</td>
<td>3044.4</td>
<td>1/2</td>
</tr>
<tr>
<td>HLA-B*1801 (n=1)</td>
<td>YDPVAAALFF</td>
<td>0.12</td>
<td>88.9</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>DPVAAALFF</td>
<td>-0.78</td>
<td>1920</td>
<td>0/1</td>
</tr>
<tr>
<td>HLA-B*2701 (n=1)</td>
<td>RQYDPVAAL</td>
<td>-0.91</td>
<td>941.7</td>
<td>0/1</td>
</tr>
<tr>
<td>HLA-B*3501 (n=2)</td>
<td>YDPVAAALFF</td>
<td>-0.62</td>
<td>1312.1</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>DPVAAALFF</td>
<td>0.45</td>
<td>55.4</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>ALFFFDIDL</td>
<td>-3.44</td>
<td>3926.3</td>
<td>1/2</td>
</tr>
<tr>
<td>HLA-B*4001 (n=1)</td>
<td>RQYDPVAAL</td>
<td>-0.74</td>
<td>635.6</td>
<td>0/1</td>
</tr>
<tr>
<td>HLA-B*4402 (n=2)</td>
<td>DPVAAALFF</td>
<td>-1.38</td>
<td>3733.4</td>
<td>1/2</td>
</tr>
<tr>
<td>HLA-B*5101 (n=3)</td>
<td>DPVAAALFF</td>
<td>-0.99</td>
<td>1513</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>VAAALFFFDI</td>
<td>-1.65</td>
<td>558.9</td>
<td>1/3</td>
</tr>
<tr>
<td>HLA-B*5301 (n=1)</td>
<td>DPVAAALFF</td>
<td>0.6</td>
<td>29.3</td>
<td>1/1</td>
</tr>
<tr>
<td>HLA-B*5701 (n=2)</td>
<td>VAAALFFFDI</td>
<td>-2.05</td>
<td>1407.9</td>
<td>1/2</td>
</tr>
<tr>
<td>HLA-B*5801 (n=1)</td>
<td>VAAALFFFDI</td>
<td>-1.82</td>
<td>822.6</td>
<td>1/1</td>
</tr>
</tbody>
</table>

(i) HLA class I alleles listed in column 1 are those carried by HCMV seropositive donors tested. In parenthesis = number of donors bearing the allele
(ii) Prediction based on IEDB score and MHC affinity
(iii) The IEDB score combines proteasome cleavage and peptide processing, TAP transport and MHC binding
(iv) units of IC50[nM]: MHC high affinity (<50nM); MHC intermediate affinity (<500nM); MHC low affinity (<5000nM).
Cut-off for selecting HLA-allele specificities = 5000nM.
(v) IFN-γ gene expression >2fold the baseline (no peptide induction). For responding peptides potentially restricted by more than one HLA allele for individual donors, HLA restriction reported in column 5 has been assigned according to the highest predicted MHC affinity.
In bold = immunodominant peptides (75% of positive responses in 6 or more individuals). HLA-A*2402/QYD association was used as internal positive control.
Table 3: Effect of RQY-16mer peptide stimulation in pharmacologically immunosuppressed kidney transplanted patients.

<table>
<thead>
<tr>
<th>Code</th>
<th>HLA-A*</th>
<th>HLA-B*</th>
<th>HLA-DRB1*</th>
<th>Recipient type</th>
<th>Replication post transplant</th>
<th>IFN-γ</th>
<th>IL-2</th>
<th>Full Peptide Specific Induction upon RQY-16mer (iv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1101,3101</td>
<td>35,5101</td>
<td>11,14</td>
<td>D-/R+ reactivation</td>
<td>none</td>
<td>-</td>
<td>154</td>
<td>++</td>
</tr>
<tr>
<td>P2</td>
<td>0101,3001</td>
<td>0801,4202</td>
<td>17,18</td>
<td>D+/R+ reactivation</td>
<td>none</td>
<td>-</td>
<td>254</td>
<td>++</td>
</tr>
<tr>
<td>P3</td>
<td>0301,2402</td>
<td>0702,0702</td>
<td>4,15</td>
<td>D+/R- primary (325cp/ml)</td>
<td>Valganciclovir</td>
<td>85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P4</td>
<td>0101,0101</td>
<td>0801,5701</td>
<td>07,17</td>
<td>D+/R+ reactivation (488cp/ml)</td>
<td>-</td>
<td>320</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P5</td>
<td>0101,2301</td>
<td>4401,52</td>
<td>07,08</td>
<td>D-/R- none</td>
<td>none</td>
<td>-</td>
<td>203</td>
<td>-</td>
</tr>
<tr>
<td>P6</td>
<td>02,2402</td>
<td>46,56</td>
<td>09,14</td>
<td>D+/R+ reactivation</td>
<td>none</td>
<td>-</td>
<td>162</td>
<td>-</td>
</tr>
<tr>
<td>P7</td>
<td>1101,1201</td>
<td>0501,52</td>
<td>01,15</td>
<td>D-/R+ none</td>
<td>none</td>
<td>-</td>
<td>91</td>
<td>+++</td>
</tr>
<tr>
<td>P8</td>
<td>1101,1101</td>
<td>0801,55</td>
<td>12,14</td>
<td>D-/R+ reactivation (1045cp/ml)</td>
<td>-</td>
<td>83</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>P9</td>
<td>0201,1101</td>
<td>35,53</td>
<td>01,15</td>
<td>D-/R+ none</td>
<td>none</td>
<td>-</td>
<td>112</td>
<td>-</td>
</tr>
<tr>
<td>P10</td>
<td>2402,6801</td>
<td>4402,62</td>
<td>04,17</td>
<td>D+/R+ none</td>
<td>none</td>
<td>-</td>
<td>374</td>
<td>+++</td>
</tr>
</tbody>
</table>

(i) EDTA anti-coagulated whole blood.
(ii) 450mg daily above unstimulated cells (CI 95%; p<0.05).
(iii) Limit of detection 300cp/ml /3 months (+) = 2fold; (++) > 2 < 3fold; (+++) > 3fold.

(iv) IFN-γ protein production (ICS) fold increase above unstimulated cells (CI 95%; p<0.05). (+) = 2fold; (++) > 2 < 3fold; (+++) > 3fold.
Figure 3

(A) IFN-γ gene expression fold increase for CD4+ T cells and CD8+ T cells.

(B) IFN-γ gene expression fold increase upon DC antigen presentation for different peptides.

Peptide concentrations:
- RQY-9mer, ALF-9mer, RQY-16mer
- QYD-9mer, VAA-9mer, RQY-16mer

HLA-A*0201
- RQY-9mer: -16%
- ALF-9mer: 11%
- RQY-16mer: 549%

HLA-A*2402
- QYD-9mer: 42%
- VAA-9mer: 514%

HLA-B*0702
- RQY-16mer: 514%
Figure 4

(A) Scatter plot showing percentage of CD4+ T cells producing proinflammatory cytokines: IL-2, TNF-α, and IFN-γ.

(B) Flow cytometry plots for CD4+ T cells stained with IgG1 isotype, IFN-γ, TNF-α, and IL-2, with different HLA class II alleles listed.

DRB1*04,16: RQYDPVAALFF-
DRB1*07,15: YDPVAALFF-AALFFFDIDL-
DRB1*03,15: RQYDPVAALFF-
DRB1*01,13: YDPVAALFF-QYDPVAALF-
DRB1*04,14: RQYDPVAALF-
A

HCMV-seropositive donors

DRB*07,14
DRB*04,14
DRB*01,13
DRB*04,12
DRB*03,15

no peptide
RQY-16mer

cpm

B

CD1a+ iDCs

Caspase-3 MFI

C

HCMV infected iDCs
mock-infected iDCs

caspase-3 fluorescence index