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Adoptive immunotherapy for viral infections after allogeneic stem cell transplantation

Zandvliet, M.L.

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Adoptive immunotherapy for viral infections after allogeneic stem cell transplantation

Maarten L. Zandvliet

Adoptive immunotherapy for viral infections after allogeneic stem cell transplantation

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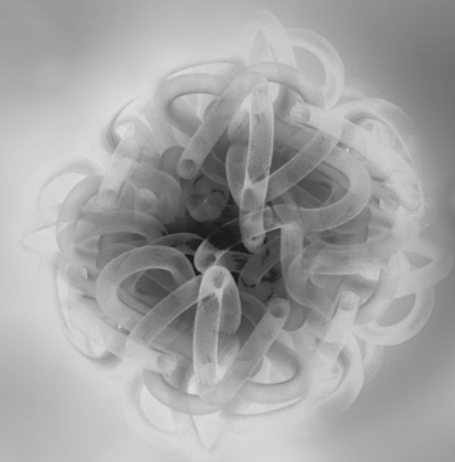
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1

General introduction and aim of the thesis

Adapted from:
Cellular immunotherapy for viral infections after allogeneic stem cell transplantation

Maarten L. Zandvliet
J.H. Frederik Falkenburg
Jaap Oostendorp
Henk-Jan Guchelaar
Pauline Meij

Submitted

General introduction

Antiviral cellular immunity

In immunocompetent individuals, viral infections are controlled by virus-specific antibodies and by virus-specific T cells. Virus-specific antibodies are produced by plasma cells and capture circulating viral particles, thereby preventing viral infection of cells. In the absence of sufficient humoral antiviral protection, cellular infection can be followed by intracellular viral replication to produce new viral particles, which may eventually result in viral disease. Virally infected cells are efficiently lysed by virus-specific T cells. These T cells express T cell receptors (TCRs) that recognize specific peptides derived from viral proteins, which are presented in major histocompatibility complex (MHC) molecules on the surface of infected cells. Generally, the TCR of an individual T cell is specific for a particular combination of an antigenic peptide and MHC molecule. Within the T cell compartment, CD8+ and CD4+ T cells can be distinguished that recognize peptides in the context of MHC class I or MHC class II molecules, respectively. During a primary viral infection, naive virus-specific T cells are activated and develop into functional effector T cells, which can proliferate and control the initial viral infection. Subsequently, apoptosis of effector T cells results in contraction of the virus-specific T cell response, during which part of the effector T cells differentiate into antigen-experienced memory T cells. The virus-specific memory T cells persist in low frequencies to provide long-term protection against subsequent infections with the same virus [1,2]. Based on the expression of cell surface markers, different naive and memory T cell subsets have been characterized [2,3]. Naive T cells express CD45RA, CC-chemokine receptor 7 (CCR7), lymph-node homing receptor CD62 ligand (CD62L), and the co-stimulatory receptors CD27 and CD28. After primary infection, differentiated virus-specific memory T cells express CD45RO at the cell surface. During subsequent infections, the expression of phenotypic markers CCR7, CD62L, CD27, and CD28 may be progressively lost, reflecting the differentiation state of the virus-specific memory T cells [2,3]. Upon recognition of viral antigen, virus-specific memory T cells mount an immune response by vigorous proliferation, the secretion of inflammatory cytokines like interferon-gamma (IFNγ), tumor-necrosis factor alpha (TNFα) and interleukin-2 (IL-2), and direct cytotoxicity towards the target cell by degranulation with granzyme B and perforin [2,4]. CD8+ T cells predominantly have the capacity to kill infected target cells. CD4+ T cells have a more pronounced helper function in the immune response and support the production of specific antibodies and the development of CD8+ T cells by production of inflammatory cytokines, although they can also exert direct cytotoxicity [5,6].

Not all viruses are completely cleared from the host after acute infection. Through evolutionary interaction of viral pathogenesis with the host cellular immune response, some viruses have acquired properties to establish persistent infections. Persistence can result in chronic infections, with active virus continuously replicating in host cells for a prolonged period of time. Alternatively, the viral genome may persist in a latent form in host cells without active replication. From this state of latency, the virus can reactivate to start lytic replication again.

The function of antiviral cellular immunity can be hampered by genetic immunodeficiency disorders. Furthermore, antiviral cellular immunity can be suppressed by immunosuppressive agents, which may be required for prevention of graft rejection in recipients of solid organ transplantation or allogeneic stem cell transplantation, or for treatment of auto-immune disease. In immunocompromised patients, the lack of functional antiviral cellular immunity may allow primary viral infections and reactivation of latent viral infections to cause serious morbidity and mortality.

Allogeneic stem cell transplantation

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment modality for a variety of hematologic malignancies and inherited hematopoietic disorders [7,8]. In patients with hematologic malignancies, chemotherapy is first used to reduce the tumor load. Prior to transplantation, conditioning regimens which may include high-dose chemotherapy, irradiation, and immune suppression aim to further eradicate malignant cells, and to reduce and suppress the hematopoietic system of the patient to allow engraftment of a donor-derived hematopoietic system. Traditionally, myeloablative conditioning regimens have been used that induce considerable toxicity, limiting the procedure to younger patients. To provide the curative potential of alloSCT to patients of higher age or with concomitant disease, reduced intensity conditioning regimens have been developed [9]. Subsequently, donor hematopoietic stem cells are transferred, which have the ability to proliferate and differentiate into mature blood cells and reconstitute the patient hematopoietic system with donor-derived blood cells. Hematopoietic stem cells for alloSCT may be directly harvested from donor bone marrow, but are usually acquired from donor peripheral blood after mobilization from the bone marrow by administration of granulocyte colony stimulating factor (G-CSF) [10,11].

In patients receiving alloSCT, the transfer of donor T cells in the stem cell graft can result in beneficial as well as detrimental clinical effects. Transferred virus-specific donor T cells may provide immediate protection from viral disease. Although patient and donor are preferentially matched for MHC molecules, donor-derived immunity may be directed

against patient cells. Genetically polymorphic proteins can differ between donor and patient and may contain immunogenic polymorphic epitopes, which have been defined as minor histocompatibility antigens. Immunogenic polymorphic peptides derived from patient proteins can be presented in MHC class I or II molecules and can be recognized by donor T cells. Donor-derived immunity directed against residual malignant cells of the patient is referred to as the graft-versus-leukemia effect, and can result in durable eradication of hematopoietic malignancies. Donor-derived T cell responses directed against the recipient's healthy tissue can lead to potentially lethal graft-versus-host disease (GvHD), characterized by lesions of the skin, gut, and liver. To decrease the incidence and severity of GvHD, depletion of T cells from the stem cell graft before transplantation or in vivo after transplantation may be performed [12-14]. However, the concomitant absence of virus-specific donor T cells will increase the risk of viral complications, and the decrease in graft-versus-leukemia activity will increase the risk of persistence of residual malignant cells [15-18]. After engraftment of the donor hematopoietic system, the administration of donor T cells may therefore be considered to prevent or treat viral infections or malignant relapses.

Reconstitution of cellular immunity after alloSCT

Following engraftment, the allogeneic stem cells give rise to a new hematopoietic system of donor origin, although small numbers of patient-derived hematopoietic cells may survive the intensive conditioning regimens. Multiple factors influence the rate and diversity of reconstitution of the immune repertoire, such as patient age, stem cell source, conditioning therapy, and T cell depletion of the graft [19]. Recovery of innate immunity by appearance of NK cells, monocytes, and granulocytes occurs rapidly in the first year after transplantation. However, reconstitution of adaptive immunity by T cells and B cells can be considerably delayed when performing depletion of the graft, and is often incomplete [19-21]. Both patient-derived mature T cells that have survived the conditioning regimen and donor-derived mature T cells that have been transferred with the stem cell graft can be a source for T cell reconstitution due to homeostatic expansion. The mature T cells from patient or donor origin may also comprise virus-specific memory T cells, which can readily provide protection from viral disease after alloSCT. Subsequently, de novo generation of naive T cells derived from the transplanted donor stem cells and produced in the recipient thymus can start to be a new source of T cell reconstitution [19-21]. In the absence of residual patient-derived or transferred donor-derived virus-specific memory T cells, patients will therefore be exposed to a prolonged period of high risk for viral complications.

Viral infections after alloSCT

As explained above, the delayed reconstitution of virus-specific T cells is associated with a high incidence of viral infections after alloSCT. The major viral pathogens causing serious morbidity and mortality after alloSCT are the common viruses cytomegalovirus, Epstein-Barr virus, and human adenovirus. Furthermore, varicella-zoster virus can reactivate in alloSCT recipients to produce herpes zoster, which presents with localized cutaneous eruptions that can be accompanied by neuralgic pain [22-24]. Other herpesviruses and the human polyomaviruses may also reactivate from latency, leading to overt disease [25-29]. We will focus on cytomegalovirus, Epstein-Barr virus, and human adenovirus, due to their significant impact on mortality after alloSCT.

Cytomegalovirus (CMV) is a beta-herpesvirus, and the largest known human herpesvirus with a genome of about 230 kb. The virus has double-stranded linear DNA enveloped by a tegument matrix, which is surrounded by a lipid bilayer that contains viral glycoproteins [30]. After initial infection, CMV establishes lifelong latency in endothelial cells and cells of the myeloid lineage. Depending on the region, 40-90% of the human population is seropositive for CMV [31,32]. Replication of CMV is restrained by CMV-specific T cells, comprising on average 10% of both CD8+ and CD4+ memory T cells in peripheral blood of healthy CMV seropositive adults [32]. Extensive characterization of CMV-specific T cell responses has demonstrated that peptides derived from many different CMV proteins were recognized. A large number of immunogenic CMV peptides derived from the immunodominant tegument protein pp65 and immediate-early protein IE1 has been defined [5,33-35]. In alloSCT recipients, the disrupted control of CMV can result in viral reactivation presenting with pneumonitis and gastrointestinal disease, and less frequently hepatitis, retinitis, and encephalitis [31,36]. In the absence of adequate numbers of CMV-specific T cells, CMV reactivation can progress to serious CMV disease, which is associated with a high rate of mortality [31,37]. The availability of the antiviral agents ganciclovir, foscarnet, and cidofovir has contributed to a significant reduction of CMV-related morbidity and mortality following alloSCT. However, administration of these drugs is limited by hematological and renal toxicity, and subsequent viral reactivations and refractory disease are commonly observed [37,38]. Reconstitution of the CMV-specific T cell repertoire directed against immunodominant CMV proteins has been demonstrated to confer sustained protection from CMV disease after alloSCT [39-41].

Epstein-Barr virus (EBV) is a human gamma-herpesvirus, composed of double-stranded DNA of approximately 172 kb, enveloped by a nucleocapsid and protein tegument, surrounded by glycoproteins [42]. EBV has established lifelong latent persistence in B cells in more than 90% of the human population. Replication of EBV in infected B cells is

adequately controlled by EBV-specific T cells in healthy individuals, although symptomatic EBV mononucleosis may occur upon primary infection [42]. EBV-specific T cells have been shown to recognize epitopes derived from a number of proteins expressed during latency, as well as proteins expressed during lytic infection [43-45]. A unique set of genes provides EBV with the potential to deregulate replication of infected cells, which may eventually lead to cancer formation [46-48]. Due to lack of viral control in immunocompromised patients after alloSCT, reactivation of EBV may result in the malignant transformation of infected B cells, which can progress to post-transplant lymphoproliferative disease (PTLD). In the absence of sufficient EBV-specific T cells, EBV-associated malignancies can cause serious disease and mortality [49,50]. The risk of EBV-PTLD is particularly increased when the balance between circulating B cells and T cells is disturbed. Therefore, the incidence of EBV-PTLD is dependent on the regimen of conditioning and graft manipulation, and differs greatly between treatment schedules. Treatment of EBV-PTLD with monoclonal antibodies directed against CD20 expressed on mature B cells is not always effective. Furthermore, drawbacks of this treatment are long-term depletion of all B cells, and the risk of emergence of CD20-negative malignant cells [51].

Human adenovirus (HAdV) is a double-stranded DNA virus composed of a protein capsid, containing 240 hexon and 12 penton components, and a nucleoprotein core that contains the DNA viral genome and internal proteins [52]. There are currently 51 HAdV serotypes described, which are grouped into six subgroups (A-F) [53]. DNA homology within subgroups ranges from 48% for group A to 99% for group C, but DNA homology between subgroups is less than 20% [54]. Symptomatic HAdV infections are most common in children, with a peak incidence between the ages of 6 months and 5 years, which mainly affect the respiratory, ocular, skin, and gastrointestinal systems [52]. Following initial HAdV infection, HAdV DNA has been detected in peripheral blood monocytes, lung, and upper airways, and may also reside in cells of the gastrointestinal tract [55-57]. However, reactivation of latent HAdV has not been described, and HAdV disease may result from de novo infections as well. Low frequencies of HAdV-specific T cells have been detected in healthy individuals, which were predominantly CD4+ T cells directed against the abundant hexon protein [58-60]. Only recently, a number of CD8+ and CD4+ epitopes derived from the HAdV hexon protein have been identified [59-64]. Since these epitopes are largely conserved, specific T cells were cross-reactive towards HAdV serotypes from different HAdV subgroups, and may therefore provide protection against a wide range of HAdV serotypes [62-64]. In absence of immune control in particular in pediatric recipients of alloSCT, HAdV infections can progress to disseminated HAdV disease, which is associated with high mortality [52,65]. The efficacy of antiviral agents such as ribavirin and cidofovir

for treatment of HAdV infection has not been proven in clinical trials yet. It has been demonstrated that reconstitution of HAdV-specific T cells is essential to control HAdV infection after alloSCT [66-68]. However, epitope specificity of these responses has not been characterized. Furthermore, mainly CD4+ T cells and no CD8+ T cells specific for HAdV were detected. Since HAdV-specific CD4+ T cells are capable to directly lyse HAdV infected target cells, the relative contribution of HAdV-specific CD4+ and CD8+ T cells in protection from HAdV disease after alloSCT remains to be elucidated [6,69].

Generation of virus-specific T cell lines

Since the reconstitution of T cells specific for CMV, EBV, and HAdV after alloSCT has been shown to be associated with sustained protection from viral disease, the adoptive transfer of donor-derived virus-specific T cells is an attractive strategy for prophylaxis or treatment of viral disease in alloSCT recipients. Infusion of unselected donor T cells may be an effective method to provide virus-specific T cell reconstitution. However, this approach is limited by the risk of simultaneous infusion of alloreactive T cells, which may result in the development of GvHD. Therefore, much effort has been put in the development of strategies to select virus-specific T cells from donor peripheral blood, to provide effective reconstitution of virus-specific T cells after alloSCT with a minimal risk of development of GvHD.

Peripheral blood can be stimulated with viral antigens in vitro, resulting in enrichment for virus-specific T cells due to preferential expansion of the activated cells during subsequent culture. This approach is feasible, and has been described in many studies for the enrichment of T cells specific for CMV, EBV, or HAdV antigens [70-74]. However, repetitive antigenic stimulations and long culture periods with IL-2 are required to generate T cell lines containing high frequencies of virus-specific T cells. In mice, in vitro restimulation and culture of virus-specific T cells negatively affected their efficacy after adoptive transfer [75]. Likewise, some human studies demonstrated that in vitro expanded CMV-specific, but also HIV-specific and melanoma-specific T cells did not persist long-term in vivo [76-78]. In vivo efficacy and survival is likely to be hampered by abrupt withdrawal from IL-2, or exhaustion, and functional changes developing during prolonged culture [79]. Furthermore, culture periods of several weeks may preclude treatment of patients with rapidly progressive viral disease.

Several strategies have been developed for the direct isolation of virus-specific T cells from peripheral blood for the generation of highly specific T cell lines without the need for long-term culture in vitro. Isolated virus-specific T cells can be cultured for a short period to allow expansion and quality controls before infusion, or can be directly infused. Peptide-

MHC class I multimers with high affinity for a specific TCR can be used for isolation of epitope-specific CD8+ T cell populations (Figure 1, method 1) [80,81]. However, to provide T cell therapy for every patient at risk of developing viral disease and to generate T cell lines against multiple epitopes, this method requires knowledge of defined epitopes restricted by prevalent MHC class I molecules, and production of a large number of peptide-MHC multimers. Furthermore, virus-specific CD4+ T cells can not be isolated using this method due to the lack of functional peptide-MHC class II multimers.

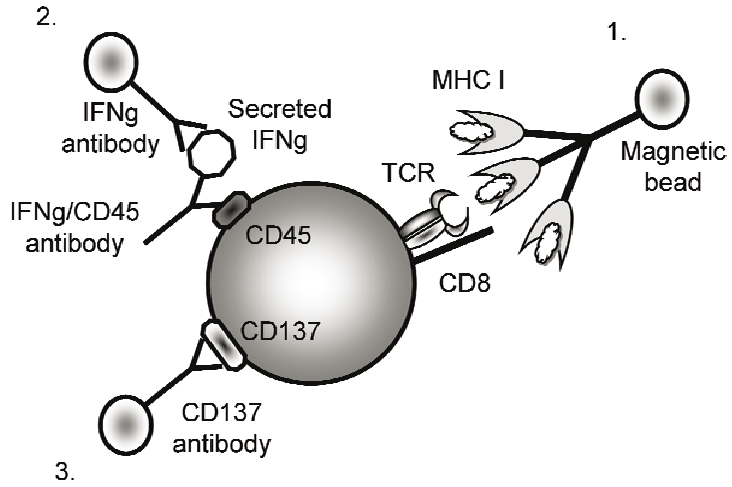


Figure 1. Selection of virus-specific T cells for adoptive immunotherapy. Virus-specific T cells can be labeled with magnetic beads using several methods. 1) Multimeric complexes composed of MHC molecules containing a specific viral peptide can bind to the TCR of virus-specific T cells. 2) After stimulation of virus-specific T cells, the secreted cytokine IFNγ can be captured at the cell membrane by bispecific IFNγ/CD45-antibodies, after which a secondary IFNγ-antibody attached to a magnetic bead can bind. 3) After stimulation of virus-specific T cells the CD137 molecule is expressed at the cell surface, after which a CD137-antibody attached to a magnetic bead can bind. Subsequently, the virus-specific T cells labeled with magnetic beads can be isolated by rinsing all the cells over a magnetic column.

Alternatively, peripheral blood can be stimulated with viral antigen, after which the activated virus-specific T cells can be isolated based on an activation-induced effect, such as the secretion of a cytokine, or the expression of an activation marker at the cell surface. IFNγ-producing T cells can be isolated using the IFNγ capture assay. The secreted IFNγ is captured by an antibody at the cell membrane, after which a secondary IFNγ-specific antibody attached to a magnetic bead can be used to isolate the labeled cells by magnetic separation (Figure 1, method 2) [82]. Since most virus-specific T cells produce IFNγ upon activation, the IFNγ capture assay has been successfully applied for isolation of T cells specific for CMV, EBV, or HAdV following antigen-specific activation [83-85]. In these

studies, the IFNγ-secreting cells were isolated after overnight incubation with viral antigen. However, the kinetics of the IFNγ response by human virus-specific T cells upon activation has not been studied in detail. It is unknown whether T cell populations specific for different viruses show similar kinetics of IFNγ production upon activation, which would allow simultaneous IFNγ-based isolation of T cells specific for multiple viruses. Although the protocols used resulted in virus-specific T cell lines after culture, which might be due to both enrichment and preferential expansion, the efficiency of isolation was not addressed and may have been suboptimal. Significant retention of specific T cells in the unselected cell fraction can limit the potential of this isolation method. Furthermore, the IFNγ-based isolation procedure is technically demanding, which may have hampered a wider clinical application. Therefore, a variety of molecules that are expressed at the surface of virus-specific T cells upon antigen-specific activation has been evaluated for use as isolation marker.

The co-stimulatory molecule CD40L (CD154) has been shown to be specifically expressed by virus-specific CD4+ T cells and by part of virus-specific CD8+ T cells upon activation. Since CD40L is only transiently expressed at the cell surface and is rapidly internalized after interaction with CD40 on the antigen-presenting cell (APC), labeling of antigen-specific T cells with a CD40L-antibody can be performed during stimulation, or by blocking the interaction with CD40 [86,87]. Another isolation marker may be the co-stimulatory molecule CD137, which has shown to be specifically upregulated following activation of CD8+ T cells specific for CMV and EBV [88-90]. The surface expression of CD137 allowed a simple procedure of labeling with a CD137-specific antibody and magnetic beads for isolation of the activated T cells, as is shown in Figure 1 (method 3). The expression of CD137 by activated CD8+ T cells was demonstrated to peak 24 hours after stimulation [88,90,91]. Slow and prolonged expression of CD137 may provide a wide timeframe for efficient isolation, which can contribute to the robustness of the isolation method. However, the kinetics of CD137 expression was analyzed on cultured T cells, while the expression of CD137 by uncultured virus-specific T cells in donor peripheral blood is unknown. Furthermore, conflicting results have been reported on the activation-induced expression of CD137 by virus-specific CD4+ T cells [88,89].

These different techniques for isolation of virus-specific T cells have been routinely used for research purposes. The reagents for IFNγ-based and peptide-MHC-based labeling of cells with magnetic beads and the closed system of magnetic separation have become available for clinical application, while reagents for CD137-based isolation have not been produced for clinical purposes yet.

Viral antigens

Since many immunodominant epitopes derived from CMV, EBV, or HAdV proteins have been characterized, T cells recognizing these defined epitopes can be isolated using peptide-MHC multimers, or using IFNγ-based or CD137-based isolation after stimulation with the corresponding minimal synthetic peptides. However, in healthy individuals, polyclonal virus-specific CD8+ and CD4+ T cell responses are directed against a broad repertoire of epitopes from multiple viral proteins [32,33,43-45,59,64]. Furthermore, restoration of antiviral protection in immunodeficient individuals has been shown to be associated with an enlarged repertoire of virus-specific T cells [92]. Therefore, the adoptive transfer of a coordinated CD8+ and CD4+ T cell response against multiple viral epitopes may be most effective for prophylaxis or treatment of viral disease in alloSCT recipients.

The use of full-length viral proteins for stimulation of virus-specific T cells in donor peripheral blood circumvents the need of knowing the exact epitopes, and can provide coordinated CD8+ and CD4+ T cell lines specific for multiple viral epitopes, irrespective of the HLA type of the patient. However, the simultaneous activation of both CD8+ and CD4+ virus-specific T cells in peripheral blood has been shown to be difficult. Viral antigen endogenously synthesized by transduction of specific cDNA into antigen-presenting cells was predominantly presented in MHC class I, while exogenously added viral proteins or lysates resulted in selected presentation of viral peptides in MHC class II [93-97]. Interestingly, pools of 15-mer peptides overlapping with 11 amino acids have been described to simultaneously induce activation of both CD8+ and CD4+ T cells specific for viral epitopes, and may be used for the generation of combined CD8+ and CD4+ T cell lines [73,98,99]. Synthetic protein-spanning peptide pools can be produced under good manufacturing practice (GMP) conditions more easily than recombinant proteins or vectors for genetic modification, and may therefore allow feasible and cost-effective production of virus-specific T cell lines for adoptive immunotherapy.

The processing of exogenous antigen followed by presentation in MHC class II has been extensively described, and the exogenously added long peptides may also directly bind in MHC class II molecules at the cell surface [100]. However, the mechanisms that result in presentation of exogenously added long peptides in MHC class I molecules, which usually present peptides of 8-11 amino acids in length, are not clear. Various models have been proposed for the presentation of exogenous long peptides in MHC class I, which may explain the activation of specific CD8+ T cells following stimulation of peripheral blood with long peptides. Elution of endogenously synthesized and processed peptides from surface MHC I molecules has demonstrated the presentation of different length variants of minimal MHC I binding motifs extended at the N- or C-terminus, indicating that some long peptide

variants can directly bind in MHC class I [101-105]. Long peptides that bind in MHC I molecules have been shown to adapt a centrally bulged conformation, or to protrude from one side of the peptide binding groove [106-110]. Furthermore, exogenous processing of long peptides by serum peptidases or membrane-bound peptidases may allow extracellular binding of trimmed peptides to cell surface MHC I molecules [111-114]. Alternatively, following uptake of exogenous long peptides, transport over endosomal membranes to the cytosol may result in the classical route of presentation which is dependent on proteasomal degradation and the transporter associated with antigen processing (TAP) [115]. Internalized peptides may also enter endosomal compartments which have fused with the endoplasmic reticulum (ER), and therefore contain the machinery for peptide loading of MHC I molecules [116]. Finally, entrance of MHC class I molecules in the recycling endocytic MHC II pathway has been demonstrated to allow exchange with exogenous peptides, which can be edited by endosomal peptidases [117-120]. Although the mechanisms that result in MHC presentation following stimulation of peripheral blood with exogenously added long peptides remain to be elucidated, the use of synthetic protein-spanning peptide pools may be a very effective strategy for simultaneous activation and isolation of virus-specific CD8+ and CD4+ T cells for adoptive immunotherapy.

Clinical results of antiviral adoptive immunotherapy

The first cases of alloSCT recipients with life-threatening EBV-PTLD and HAdV disease that were successfully treated by the adoptive transfer of donor-derived T cells were described in 1994 [121,122]. In these patients, sustained viral clearance was observed within weeks after administration of approximately $1x10^6$ unselected donor T cells per kilogram body weight. Although these and subsequent reports showed that the adoptive transfer of unselected donor T cells can be very effective for treatment of viral disease after alloSCT, the infusion of donor T cells that can cause potentially lethal GvHD is a major limitation of this approach. Acute or chronic GvHD has been shown to develop in 50-60% of alloSCT recipients after infusion of $5x10^6$ unselected donor T cells per kilogram body weight, and can even result in mortality in 10% of patients [123,124]. Therefore, virus-specific T cell lines have been generated to provide effective antiviral immune reconstitution with a minimal risk of GvHD. These virus-specific T cell products have been used for prophylaxis or treatment of viral disease in a number of small clinical trials.

In several clinical studies, CMV-specific T cells were infused for prophylaxis of CMV disease approximately 1 month after alloSCT [78,95,97,125-127]. Transient increases in frequencies of CMV-specific T cells were demonstrated within weeks after administration. In one study, the infusion of CMV-specific CD4+ T cell clones appeared to promote the

development of CMV-specific CD8+ T cells in vivo [95]. Although CMV-related morbidity and mortality appeared to be reduced compared to historical controls, these non-randomized trials can not provide evidence on protection from CMV disease. In other studies, CMV-specific T cells were infused pre-emptively after detection of a CMV DNA plasma load [94,128,129]. Reconstitution of CMV-specific T cells was detected in vivo, even after administration of low numbers of CMV-specific T cells with a median of $9x10^3$ T cells per kilogram body weight [129]. It was likely that the CMV-specific T cells expanding in vivo were derived from the T cell infusions, since the TCR CDR3 sequence of the CMV-specific T cells was shown to be identical. After adoptive transfer of the CMV-specific T cells, the CMV DNA load was cleared in all patients. In one clinical trial, CMV-specific T cells were administered to patients without detectable CMV-specific T cells and experiencing CMV reactivation that was refractory to standard pharmacotherapy [93]. Despite cessation of antiviral pharmacotherapy, the CMV load dropped significantly after administration of CMV-specific T cells, and sustained clearance of CMV occurred in 5 of 7 patients. Importantly, no adverse events related to infusion of CMV-specific T cells were reported in these clinical trials.

In the first clinical study on adoptive transfer of donor-derived EBV-specific T cells after alloSCT for prophylaxis of EBV-PTLD, the EBV-specific T cells were labeled with a marker gene [130,131]. Using this marker gene, persistence of the transferred T cells was demonstrated up to 9 years after infusion [132,133]. Furthermore, the marked EBV-specific T cells were shown to expand in vivo during EBV reactivation [130,133]. In another study, pre-emptive administration of EBV-specific T cells was shown to decrease the EBV DNA load in 8 of 9 patients, although no sustained viral clearance was observed and 1 of the patients progressed to fatal EBV-positive lymphoma [134]. Treatment of early stage EBV-PTLD by infusion of EBV-specific T cells was shown to result in complete and stable remissions, while treatment of late stage EBV-PTLD was not successful [133,135]. To provide EBV-specific T cells for treatment of EBV-PTLD in solid organ transplant recipients, banked EBV-specific T cell lines were used to select the best HLA-matched product [136]. The response rate of 52% appeared promising, since these patients had failed conventional therapy. Despite the partial HLA-mismatch, the infused EBV-specific T cells persisted up to 194 days in vivo, and antibodies against a mismatched HLA antigen were only detected in 1 patient. Even in the partially mismatched setting, no adverse events related to the EBV-specific T cell infusions were described.

Only a very limited number of alloSCT recipients has been treated with HAdV-specific T cells yet. In one published study, donor peripheral blood was stimulated with HAdV lysate, and IFNγ-producing cells were isolated using the IFNγ capture assay [137]. After isolation,

the HAdV-specific T cells were administered to 9 pediatric patients after alloSCT with a detectable HAdV DNA load which did not respond to pharmacotherapy. In 5 of 6 evaluable patients, increased frequencies of HAdV-specific T cells were detected after infusion, which was accompanied by a decrease in HAdV load. In 1 of the patients, pre-existing GvHD of the skin increased 10 days after administration of HAdV-specific T cells. Furthermore, HAdV-specific T cell lines are being generated by culture of donor PBMC with 5 conserved HAdV hexon 30-mer peptides at the department of Pediatrics of the Leiden University Medical Center [62]. Administration of these HAdV-specific T cell lines to pediatric alloSCT recipients for treatment of HAdV disease is under investigation.

Recently, the generation of T cell lines specific for antigens derived from different viruses has been proposed to provide prophylaxis or treatment for multiple viruses after alloSCT. In two studies, combined T cell lines specific for CMV, EBV, and HAdV were administered to alloSCT recipients for prophylaxis of viral reactivation and disease [96,138]. Increased frequencies of virus-specific T cells were demonstrated after adoptive transfer, which may have prevented the development of viral disease.

From these clinical trials it can be concluded that the adoptive transfer of donor-derived virus-specific T cells appeared to be safe and did not result in GvHD. Even high numbers of virus-specific T cells, up to $1x10^9$ T cells per square meter of body surface area, were well tolerated [125]. Furthermore, the T cell infusions resulted in increased frequencies of circulating virus-specific T cells, which were demonstrated to be able to expand and persist long-term in vivo. Since the trials were non-randomized, no conclusions can be drawn on efficacy of treatment. However, in most studies the adoptive transfer of virus-specific T cells was associated with a decrease in viral DNA plasma load or with sustained viral clearance, indicating that adoptive immunotherapy may be effective. The methods used to generate virus-specific T cell products were very variable with regard to viral antigens used, isolation steps, culture period, and cell numbers infused. Furthermore, the patient populations were very variable in conditioning, transplantation regimens, viral infections, and the time of adoptive transfer. Therefore, the optimal strategy for adoptive transfer of donor-derived virus-specific T cells and its clinical benefit remain to be elucidated.

Quality assurance and regulatory aspects of adoptive immunotherapy

Before adoptive transfer of virus-specific T cells can be implemented in clinical studies, the quality of the final cellular product has to be assured. Analogous to the production of conventional medicinal products, the starting material and reagents should be released for clinical use, and the production process and environment should be strictly defined. Donor peripheral blood cells can be reliably obtained according to the standard procedures used

for blood donation and processing. Clinical grade culture media and devices can be obtained, and appropriate cleanroom facilities are available in a number of hospitals across Europe. Clinical grade viral antigens and isolation reagents are currently being developed. The small scale production of viral lysate or recombinant proteins can not easily be performed under GMP conditions, and poses the risk of introducing intact viral particles and animal-derived components. The production of synthetic peptides for use as viral antigen in vitro can be more easily performed under GMP conditions. The reagents for IFNγ-based and peptide-MHC-based labeling of cells with magnetic beads and the closed system of magnetic separation are available for clinical application. The CD137-specific monoclonal antibodies have not been produced for clinical purposes yet.

The release criteria of the final T cell product should be designed for each product for clinical use to ensure maximal safety, efficacy, and feasibility. The absence of microbiological contamination should be analyzed in cultured samples of the starting material and of the final T cell product. Furthermore, the correct donor origin of the starting peripheral blood cells can be confirmed by DNA profiling. The composition of the final product can be analyzed by flow cytometry, and should confirm the presence of mainly viable T cells. When using a well defined viral antigen, the total frequency of virus-specific T cells in the cellular product can be properly analyzed by antigen-specific restimulation followed by functional analysis. In addition, the frequency of T cell populations specific for defined viral epitopes can be analyzed using peptide-MHC multimeric complexes. Confirmation of high frequencies of virus-specific T cells that can be achieved by isolation methods (>50% of cells) will indicate that the production process has been adequately performed, and is crucial to justify release. The concomitant low absolute numbers of T cells with unknown specificity will assure a minimal risk of the development of GvHD. In addition to these criteria for the composition and specificity of the cellular product, the functional response of the virus-specific T cells can be confirmed towards patient and donor cells loaded with viral antigen, and should be absent towards unloaded control patient and donor cells. Possible functional read-out systems include the measurement of cytokine production or the analysis of cytotoxic activity. The absence of residual alloreactive potential can be further confirmed by the analysis of functional responses during co-culture of the virus-specific T cell product with a panel of peripheral blood cells from allogeneic third party donors and from the patient. When all standard operation procedures for the process of production as well as for the analysis of the cellular product have been properly followed and documented, the virus-specific T cell lines can be released for administration to the patient.

In parallel to the developments in detection, isolation and culture of virus-specific T cells for clinical application, the regulatory framework for cellular therapies has also evolved. With the adoption of the European Directive 2003/63/EC, somatic cellular products were for the first time defined as medicinal products when their biological characteristics have been substantially altered as a result of their manipulation to obtain a therapeutic, diagnostic or preventive effect. By the end of 2008, with the adoption of the Advanced Therapy Medicinal Product (ATMP) regulation, this definition was further specified to products that are substantial manipulated and/or not intended to be used for the same essential functions in the recipient as in the donor. According to the ATMP regulation, isolation and expansion of virus-specific T cells should be seen as substantial manipulation. This means that the production and application of these cells should comply with all the relevant regulations for investigational medicinal products for human use, including GMP and good clinical practice (GCP) guidelines. Although the ATMP regulation is primarily adopted to aid the marketing authorization of these advanced products, it will also influence the early development of AMTP from an academic perspective. The ATMP regulation states that "advanced therapy medicinal products which are prepared on a non-routine basis according to specific quality standards, and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient, should be excluded from the scope of this regulation, whilst at the same time ensuring that relevant community rules related to quality and safety are not undermined". Hopefully, the adoption of the ATMP regulation will not hamper the early development of cellular therapies from an academic perspective, but aid to reach high quality standards for maximal safety and efficacy in clinical application.

Aim of the thesis

Extended knowledge of antiviral T cell responses in vivo and the advanced methods for isolation, culture, and analysis of virus-specific T cells in vitro may contribute to improved strategies for adoptive immunotherapy with virus-specific T cells. In this thesis, we investigated how the immunological techniques that are currently available can be exploited to detect and select virus-specific T cells in peripheral blood. To support the rationale for antiviral T cell therapy, the role of virus-specific T cells in protection from viral disease after alloSCT was studied. Furthermore, strategies were developed for the efficient isolation of virus-specific T cells from peripheral blood for adoptive transfer. The studies aimed to develop methods for the rapid generation of clinical grade T cell lines with high specificity against multiple viral antigens, which may be used to establish the clinical benefit of adoptive immunotherapy for viral infections after alloSCT.

In **chapter 2**, we performed a detailed analysis of the kinetics of IFNγ production by CMV pp65-specific CD8+ T cells upon stimulation to determine the optimal time point for IFNγ-based isolation. The effect of the strength of antigenic stimulation on TCR downregulation, the kinetics of IFNγ production, and expansion was investigated. Based on these findings, we defined optimal conditions for IFNγ-based isolation of CMV pp65 peptide-specific CD8+ T cells for clinical application.

In **chapter 3**, we aimed to isolate coordinated CD8+ and CD4+ T cells specific for a broad repertoire of CMV pp65 and IE1 epitopes from peripheral blood, since these cells may be more effective for adoptive immunotherapy as compared to CD8+ T cells specific for a single CMV epitope. The efficiency of activation and kinetics of IFNγ production by CD8+ and CD4+ CMV-specific T cells was analyzed after stimulation of peripheral blood with different CMV antigens. The results were translated into a procedure for the simultaneous isolation of CD8+ and CD4+ T cells specific for multiple CMV pp65 and IE1 epitopes. Subsequently, the specificity, phenotype and functionality of the combined CD8+ and CD4+ CMV-specific T cell lines were analyzed.

Since the relative contribution of HAdV-specific CD8+ and CD4+ in protection from HAdV disease after alloSCT is not clear, and the epitope specificity of HAdV-specific T cell responses after alloSCT has not been characterized, we studied the development of T cell responses against HAdV hexon epitopes in pediatric and adult alloSCT recipients in **chapter 4**. Subsequently, a clinical grade method was developed for rapid generation of CD8+ and CD4+ T cell lines with high and defined specificity for HAdV hexon epitopes for adoptive immunotherapy. The repertoire of HAdV hexon epitopes recognized by HAdV-

specific T cell lines was investigated to identify novel HAdV hexon epitopes, and recognition of HAdV-infected target cells was analyzed.

In **chapter 5**, we aimed to define a single and feasible method for isolation of CD8+ and CD4+ T cells recognizing epitopes derived from different viral proteins from peripheral blood. To ensure that the method was applicable to a wide variety of virus-specific T cells that may differ in phenotypic and functional properties, we focused on T cells specific for the persistent viruses CMV and EBV, as well as T cells specific for HAdV and influenza (FLU), which are not repetitively activated in vivo after initial viral clearance The kinetics of IFNγ production and CD137 expression were analyzed, to allow simultaneous IFNγ-based or CD137-based isolation of CD8+ and CD4+ T cells specific for multiple viruses. Based on these findings, an efficient and widely applicable strategy for generation of multivirus-specific T cell lines was developed.

To activate virus-specific CD8+ and CD4+ T cells for the purpose of detection or isolation, synthetic pools of overlapping long peptides spanning viral proteins are routinely used. Furthermore, synthetic long peptides have been used for vaccination to induce or boost antigen-specific CD8+ T cells in vivo. However, the mechanisms that result in presentation of exogenously added long peptides in MHC class I molecules, resulting in activation of peptide-specific CD8+ T cells, are not clear. In **chapter 6**, the efficiency of CD8+ T cell activation by several extended variants of minimal MHC class I binding peptides was analyzed. Subsequently, we investigated whether the CD8+ T cell activation resulted from direct binding of long peptides in MHC class I or from peptide processing followed by MHC I binding of the minimal peptides.

In **chapter 7**, clinical results are presented of a phase I/II study for treatment of refractory CMV reactivation after alloSCT by the adoptive transfer of donor- or patient-derived CMV pp65-specific CD8+ T cells. Refractory CMV reactivation was defined as high CMV DNA load in peripheral blood for more than 2 weeks under antiviral pharmacotherapy or early relapse after antiviral pharmacotherapy. CMV pp65-specific CD8+ T cell lines were generated from peripheral blood by IFNγ-based isolation following CMV pp65 peptide stimulation. Patient follow-up and immune monitoring provided information on the relation between adoptive transfer of CMV-specific CD8+ T cells and the occurrence of adverse events, detection of CMV pp65-specific CD8+ T cells, and decrease of CMV DNA load.

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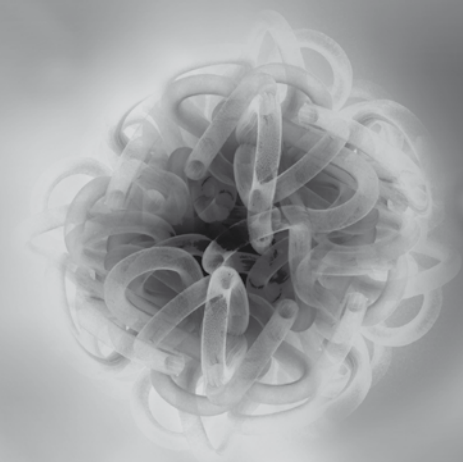
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2

Detailed analysis of IFNγ response upon activation permits efficient isolation of cytomegalovirus-specific CD8+ T cells for adoptive immunotherapy

Maarten L. Zandvliet
J.H. Frederik Falkenburg
Inge Jedema
Roel Willemze
Henk-Jan Guchelaar
Pauline Meij

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Abstract

Adoptive transfer of donor-derived cytomegalovirus (CMV)-specific T cells may provide long-lived protection from CMV disease after allogeneic stem cell transplantation. Isolation of IFNγ-secreting cells following CMV peptide stimulation can be performed by IFNγ capture assay to generate highly specific T cell lines without the need for extensive culture, which may hamper their in vivo efficacy. To exploit the full potential of this approach, we analyzed the IFNγ response of CMV-specific CD8+ T cells in detail. Kinetic studies showed that TCR downregulation coincided with the induction of IFNγ production upon activation, which rapidly declined thereafter despite the continued presence of specific peptide. By varying the strength of stimulation we observed that overstimulation can result in profound TCR downregulation, more rapid decline of IFNγ production and reduced expansion. Based on these findings, we defined optimal conditions for IFNγ-based isolation of CMV-specific CD8+ T cells with maximal potential for clinical application. These data stress the importance of analyses of the kinetics of cytokine production for isolation of T cells specific for other infectious or malignant antigens to exploit the full potential of cytokine capture isolation of antigen-specific T cells.

Introduction

Reactivation of cytomegalovirus (CMV) can cause serious morbidity and mortality during the prolonged period of immune deficiency following allogeneic stem cell transplantation (alloSCT). Viral replication can occur in the absence of adequate numbers of CMV-specific T cells, which are eradicated or impaired by the conditioning regimen and immune suppression [1,2]. Reconstitution of the CMV-specific T cell repertoire directed against immunodominant proteins pp65 and IE-1 in the first year after transplantation has been demonstrated to confer sustained protection from CMV disease [3-8]. This is also illustrated by increased CMV-related morbidity in patients receiving a T cell depleted graft or a graft from a CMV-seronegative donor [6-10]. Treatment of CMV reactivation by pharmacological agents is limited by toxicity and not sufficient for long-term anti-viral protection in the absence of CMV-specific T cells [11-13]. Several clinical trials have shown that adoptive transfer of donor-derived CMV-specific T cells can be a safe and effective strategy to control CMV reactivation [14-23]. However, the generation of CMV-specific T cell lines has to be markedly improved with regard to specificity, reproducibility, and culture conditions, before the clinical benefit of this treatment can be confirmed, resulting in broad application.

In most studies CMV-specific T cell lines were generated by preferential expansion, including repetitive antigenic stimulations and long culture with high dose IL-2. In mice, in vitro restimulation and culture of CMV-specific T cells negatively affected their efficacy following adoptive transfer [24]. Likewise, some human studies demonstrated that in vitro expanded CMV-specific, but also HIV- and melanoma-specific CD8+ T cells did not persist long-term in vivo [14,22,25,26]. In vivo efficacy and survival is likely to be hampered by abrupt withdrawal from IL-2, or exhaustion, and functional changes developing during prolonged culture [27]. Moreover, the high concentrations of antigen which are used for activation and restimulation may not be physiological, and may reduce T cell functionality [28-31]. Alternatively, the lack of in vivo survival may be due to selection of effector memory T cells, while only T cells derived from CD62L-positive central memory T cells have been reported to establish persistent CMV-specific T cell memory in macaques [32].

Direct isolation of antigen-specific T cells allows the generation of highly specific T cell lines without the need for extensive culture. Since the presence of CMV-specific memory T cells that produce IFNγ upon activation has been found to be associated with protection from CMV reactivation after alloSCT, isolation of this population may be a good strategy for generating effective CMV-specific T cell lines [4,5,7,8]. It has been demonstrated that adoptive transfer of IFNγ-producing memory T cells can provide long-lived functional memory T cell reconstitution [33]. Enrichment for IFNγ-producing T cells by the IFNγ

capture assay is the only isolation method currently available which can be performed under GMP regulations. Only antigen-specific T cells that secrete IFNγ during the isolation procedure can be isolated using the IFNγ capture assay. Murine virus-specific T cells have shown to constantly produce IFNγ between 1 and 24 hours after activation in the continued presence of antigen [34-36]. Production of IFNγ by human virus-specific T cells is commonly detected by cumulative techniques like intracellular staining and ELISA after 4 to 24 hours of stimulation. However, the real-time kinetics of the IFNγ response of human virus-specific T cells have not been studied in detail. To date, virus-specific T cells were isolated from peripheral blood mononuclear cells by the IFNγ capture assay after overnight stimulation with high concentrations of viral antigen [21,37-40]. Although these protocols resulted in specific T cell lines after culture, which can be due to both enrichment and preferential expansion, the efficiency of isolation was not addressed and may have been suboptimal. Significant retention of specific T cells in the unselected cell fraction can limit the potential of this isolation method.

Another strategy for adoptive transfer of antigen-specific T cells is T cell receptor (TCR) gene transfer, which has shown promising results in the first clinical study [41]. Isolation of virus-specific T cells offers the possibility to redirect their specificity to malignant cells by TCR gene transfer. Activation of the endogenous TCR due to latent presence of viral antigen in vivo may result in enhanced survival of TCR-transferred T cells. Furthermore, introduction of a tumor-reactive TCR into restricted virus-specific T cell populations will minimize the risk of autoimmunity [42].

In this study, we performed detailed analysis of the IFNγ response by CMV-specific CD8+ T cells and defined optimal conditions for activation and isolation of CMV-specific CD8+ T cells based on IFNγ secretion. Kinetic studies showed that the rapid induction of IFNγ secretion by CMV-specific CD8+ T cells upon activation was followed by a strong decrease already after several hours. By varying the strength of antigenic stimulation we illustrated that overstimulation can result in extensive TCR downregulation, more rapid decline of IFNγ production and reduced expansion. In contrast, a more physiological strength of stimulation permitted efficient isolation of CMV-specific T cells while preserving maximal in vivo potential for clinical application.

Materials and methods

Donor cells and stimulation

Peripheral blood was obtained from CMV-seropositive healthy individuals after informed consent, and mononuclear cells (PBMC) were cryopreserved after Ficoll-Isopaque

separation. After thawing, cells were resuspended in culture medium, consisting of Iscove's modified Dulbecco's medium (IMDM, Lonza, Basel, Switzerland) supplemented with 10% pooled human serum, 100 U/ml penicillin/streptomycin (Lonza), and 3 mM L-glutamine (Lonza). Cells were cultured at $10x10^6$ cells/ml at 37°C and 5% CO_2, and stimulated with 10^{-4} to 10^{-11} M CMV pp65-derived peptide YSEHPTFTSQY (YSE/HLA-A*0101), NLVPMVATV (NLV/HLA-A*0201), TPRVTGGGAM (TPR/HLA-B*0702), RPHERNGFTVL (RPH/HLA-B*0702), or IPSINVHHY (IPS/HLA-B*3501).

Flow cytometric analyses

Cells were stained with FITC-labeled CD3, CD14, CD27 (BD Biosciences, San Jose, CA, USA), CD45RO (Caltag, Burlingame, CA, USA), CD62L (Bender MedSystems, Vienna, Austria), PE-labeled CD28, CD56 (BD), CD45RA (Beckman Coulter, Fullerton, CA, USA), CCR7 (R&D Systems, Minneapolis, MN, USA), PerCP-labeled CD3, CD8 (BD), APC-labeled CD4 (Beckman Coulter), and CD19 (BD) monoclonal antibodies (mAbs). PE- and APC-labeled CMV peptide-MHC tetramers were produced as described previously [43]. Fluorescence was analyzed using a FACSCalibur and Cellquest software (BD).

Analysis of cytokine production

For cumulative measurement of intracellular cytokines, 10 μg/ml brefeldin A (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added at the start of peptide stimulation. For real-time measurement of intracellular cytokines, 10 μg/ml BFA was added only during the last hour of peptide stimulation. After harvesting, cells were washed in phosphate-buffered saline (PBS) and stained with PE-labeled tetramers for 15 minutes at 37°C. Subsequently, mAbs for cell-surface staining were added and incubated for 30 minutes at 4°C. Cells were washed in PBS and fixed with 1% paraformaldehyde for 8 minutes at 4°C. For permeabilization, samples were washed in PBS with 0.1% saponin (Sigma-Aldrich) and incubated for 30 minutes at 4°C. Cells were stained with APC-labeled IFNγ mAb or PE-labeled TNFα mAb (BD) for 30 minutes at 4°C, washed, and fluorescence was analyzed by flow cytometry. To analyze production of multiple cytokines by CMV-specific T cells, supernatant was harvested after peptide stimulation and analyzed by the human Th1/Th2/Th17 multi-analyte profiler ELISArrayTM kit (SA Biosciences, Frederick, MD, USA), performed according to the manufacturer's instructions.

Detection, isolation, and culture of IFNγ-secreting cells

Cells were harvested, thoroughly washed in PBS, and IFNγ-secreting cells were stained by the IFNγ capture assay (Miltenyi Biotec, Bergisch Gladbach, Germany) performed according

to the manufacturer's instructions. Briefly, cells were labeled with IFNγ-catch reagent and cultured for 45 minutes at 37°C. Cells were counterstained with PE-labeled IFNγ mAb. For subsequent isolation, the IFNγ-PE-labeled cells were bound to anti-PE microbeads, and isolated using the midi-MACS system (Miltenyi Biotec). For analysis of IFNγ-secreting cells and composition of the isolated fractions, samples were stained with APC-labeled tetramers for 15 minutes at 4 °C. Subsequently, mAbs for cell-surface staining were added and incubated for 30 minutes at 4°C. Low dose propidium iodide (0.2 μg/ml) was added upon flow cytometric analysis of the isolated fractions to exclude dead cells. The isolated IFNγ-enriched and IFNγ-depleted cell fractions were both cultured at $1x10^6$ cells/ml in culture medium, containing 50 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) with $10x10^6$ cells/ml 30 Gy-irradiated feeder cells derived from the IFNγ-depleted fraction. Cultures were supplemented with fresh medium and 50 IU/ml IL-2 every 3-4 days.

Proliferation assay

PBMC were thoroughly washed with PBS, resuspended at $10x10^6$ cells/ml, and labeled with 3.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37 °C. The reaction was stopped by the addition of an equal volume of fetal calf serum (Lonza). After overnight incubation in culture medium at 37 °C, the cells were washed and incubated in culture medium at $10x10^6$ cells/ml with 10^{-4} to 10^{-9} M CMV peptide and 50 IU/ml IL-2. After 6 days of incubation at 37°C, samples were washed in PBS and stained with APC-labeled tetramers for 15 minutes at 37°C. Subsequently, mAbs for cell-surface staining were added and incubated for 30 minutes at 4°C. Cells were washed and fluorescence was analyzed by flow cytometry.

Results

Kinetics of IFNγ response by CMV-specific CD8+ T cells upon peptide stimulation

The fraction of CD8+ T cells recognizing immunodominant CMV pp65 epitopes which produces IFNγ upon antigen-specific activation was determined in PBMC from healthy CMV-seropositive donors. To measure the induction of the IFNγ response at the single cell level, cumulative intracellular IFNγ staining was performed at several time points following stimulation with a saturating concentration (10^{-6} M) of CMV peptide. T cells recognizing this epitope were identified by peptide-MHC tetramer after stimulation to analyze both IFNγ production and TCR downregulation of the specific cells. As shown in Figure 1A, tetramer-positive CD8+ T cells produced IFNγ upon peptide stimulation within 6 hours. The mean fluorescence intensity (MFI) of tetramer-staining decreased 92% after 6 hours, indicating

extensive TCR downregulation that showed similar kinetics to IFNγ production. Expression of CD8, CD3, and TCRab by IFNγ-producing T cells decreased to similar levels, indicating downregulation of the complete TCR complex.

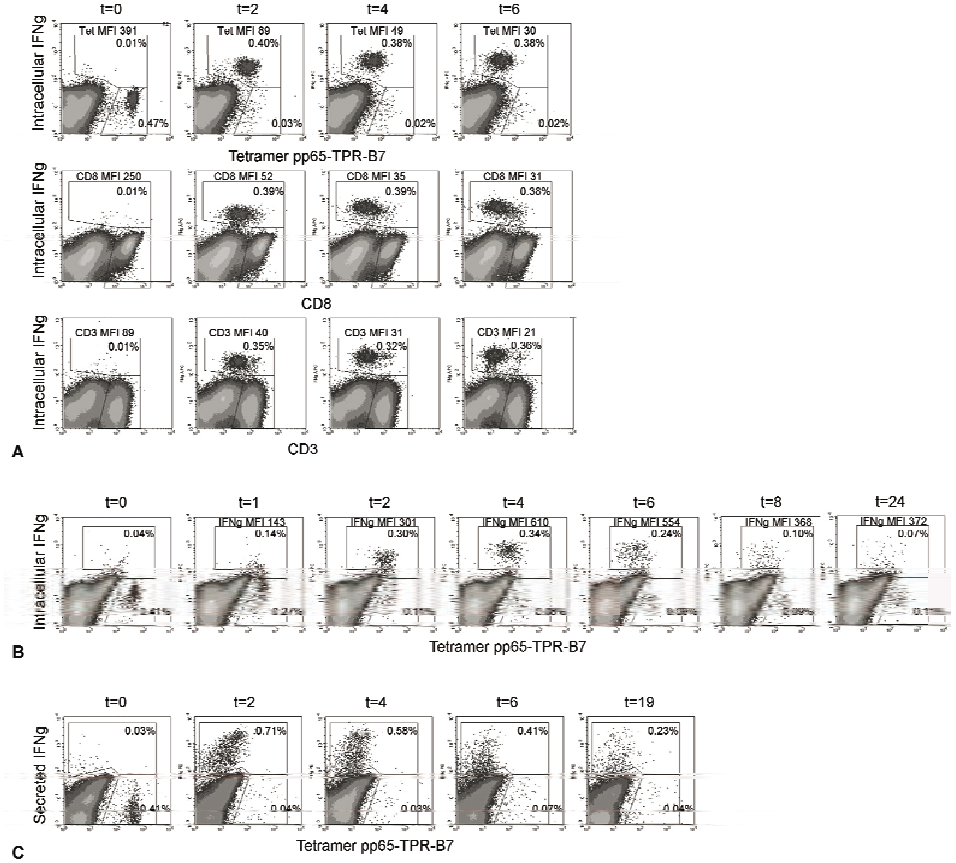


Figure 1. Kinetics of IFNγ response by CMV-specific CD8+ T cells upon stimulation with 10^{-6} M CMV pp65 TPR peptide. **A)** Cumulative intracellular IFNγ staining of PBMC after 0-6 hours of peptide stimulation in the presence of BFA. The MFI of tetramer, CD8, and CD3 staining of peptide-specific CD8+ T cells is depicted. **B)** Real-time intracellular IFNγ staining of PBMC after 0-24 hours of peptide stimulation with addition of BFA only during the last hour of incubation. The MFI of intracellular IFNγ staining of peptide-specific CD8+ T cells is depicted. **C)** IFNγ capture assay staining of PBMC after 0-19 hours of peptide stimulation. Samples were stained with pp65-TPR-B7 tetramer after stimulation.

To investigate the kinetics of the IFNγ response, real-time production of IFNγ was measured at several time points after stimulation by addition of BFA only during the last hour of peptide incubation. Figure 1B demonstrates that IFNγ production by peptide-specific T cells started already in the first hour after stimulation, and reached a maximum between 2 and 4 hours when most tetramer-positive cells produced IFNγ. Thereafter, the production of IFNγ rapidly declined both in the percentage of tetramer-positive cells actively producing IFNγ and the amount of IFNγ produced per cell (decrease in IFNγ MFI). To confirm that kinetics of intracellular IFNγ production correlated with kinetics of IFNγ secretion, detection of IFNγ by capture assay was performed at the same time points after stimulation, reflecting IFNγ secretion in a fixed timeframe of 45 minutes. Figure 1C shows that the kinetics of IFNγ secretion were consistent with intracellular IFNγ production, with peak IFNγ secretion between 2 and 4 hours of stimulation, followed by a rapid decrease. Peptide-specific T cells could not be identified among IFNγ-labeled cells by staining with peptide-MHC tetramer due to downregulation upon activation. The IFNγ-labeled cells contained some CD14+, and few CD4+, CD19+, and CD56+ cells (data not shown), indicating non-specific labeling or indirect labeling with IFNγ secreted by peptide-specific CD8+ T cells, since intracellular staining had shown that only the peptide-specific T cells actively produced IFNγ (Figure 1A).
These experiments demonstrate that stimulation with this high peptide concentration resulted in rapid induction of IFNγ secretion by nearly all peptide-specific T cells, and rapid and vigorous TCR downregulation of at least 90%. Thereafter, the production of IFNγ strongly declined.

Effect of epitope density on TCR downregulation and IFNγ production

To study the relation between the strength of stimulation, TCR downregulation and IFNγ production, the epitope density was varied by stimulation with different peptide concentrations.
As shown in Figure 2A, stimulation of donor PBMC with CMV peptide for 4 hours resulted in both a decrease of tetramer staining and increase of IFNγ production starting at a peptide concentration of 10^{-11} M, gradually progressing up to plateau levels at peptide concentrations above 10^{-7} M. The direct correlation between TCR downregulation and IFNγ production (R^2=0.994) is further illustrated in Figure 2B.

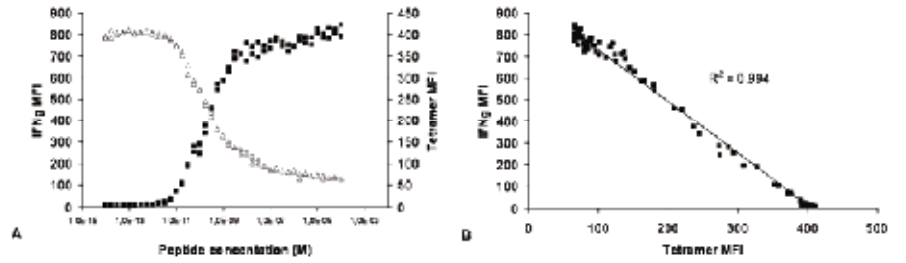


Figure 2. Effect of epitope density on TCR downregulation and IFNγ production. Cumulative intracellular IFNγ staining of PBMC was performed after 4 hours of stimulation with 10^{-4} to 10^{-14} M CMV pp65 TPR peptide. **A)** IFNγ MFI of CMV pp65 TPR-specific cells (black squares) is plotted against the left Y-axis, and tetramer MFI (white triangles) is plotted against the right Y-axis. **B)** Tetramer MFI of CMV pp65 TPR-specific cells is plotted against IFNγ MFI. Samples were stained with pp65-TPR-B7 tetramer after stimulation.

To study the effect of epitope density on the kinetics of IFNγ production, real-time measurement of IFNγ production was performed following stimulation with different peptide concentrations. Figure 3A and 3B show that after stimulation with 10^{-7} M HLA-B7 restricted CMV TPR peptide a maximal percentage of IFNγ-producing cells was induced after 4 hours, which decreased thereafter. The kinetics of IFNγ production were similar for peptide concentrations below 10^{-7} M with maximal IFNγ production after 4 hours of stimulation, however, the percentage of cells producing IFNγ and the amount of IFNγ produced per cell (IFNγ MFI) was lower. Stimulation with peptide concentrations higher than 10^{-7} M induced a more rapid peak of IFNγ production, which also declined rapidly thereafter, resulting in only marginal IFNγ production after 8 hours with 10^{-5} M peptide stimulation (Figure 3B). Furthermore, as shown in Figure 3A, peptide concentrations higher than 10^{-7} M induced more extensive TCR downregulation (4 hr tetramer MFI 10^{-5} M=62 vs 10^{-7} M=150). Stimulation with HLA-A1 or HLA-B35 restricted CMV YSE or IPS peptides, which are less immunodominant epitopes, showed a slightly slower induction of IFNγ production, and higher peptide concentrations of 10^{-5} M (YSE) to 10^{-6} M (IPS) were required for maximal IFNγ production (Figure 3C).

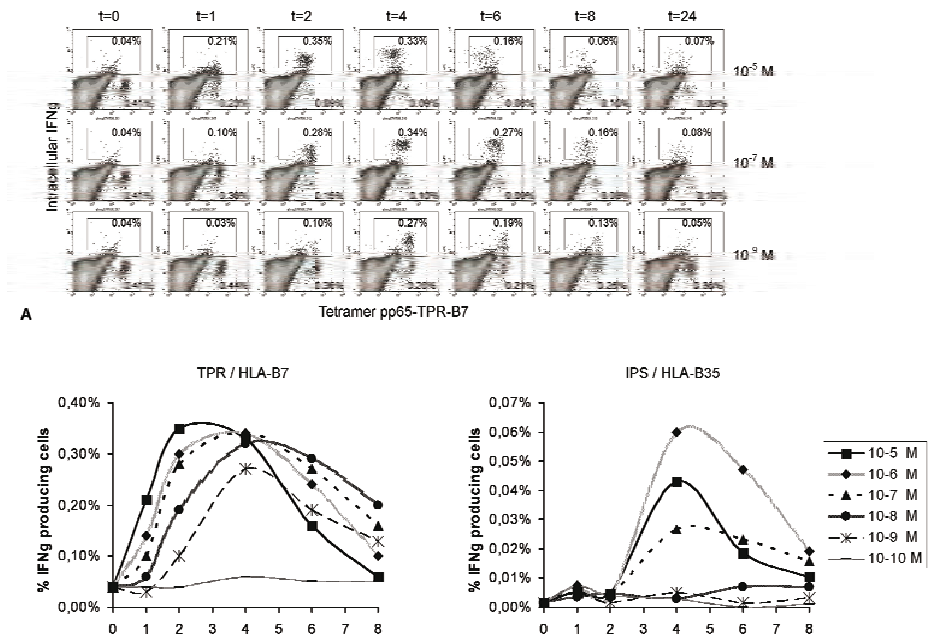


Figure 3. Effect of epitope density on the kinetics of IFNγ production by CMV-specific CD8+ T cells. Real-time intracellular IFNγ staining of PBMC after 0-24 hours of stimulation with CMV peptide with addition of BFA only during the last hour of incubation. Samples were stained with peptide-MHC tetramer after stimulation. **A)** Dot plots are shown for stimulation with HLA-B7 restricted TPR peptide using concentrations of 10^{-5}, 10^{-7} and 10^{-9} M. **B)** Percentage of IFNγ-producing cells after stimulation with 10^{-5} to 10^{-10} M HLA-B7 restricted TPR peptide over time. **C)** Percentage of IFNγ-producing cells after stimulation with 10^{-5} to 10^{-10} M HLA-B35 restricted IPS peptide over time.

To investigate whether the different peptide concentrations required for maximal IFNγ production resulted from differences in peptide-MHC affinity between selected epitopes, we determined the efficiency of stabilizing peptide-MHC monomers by the different peptides by the addition of different concentrations of CMV peptide to a fixed concentration of heavy chain and beta-2-microglobulin. All selected CMV pp65 peptides were shown to stabilize monomers at low peptide concentrations (1:1 ratio of peptide:heavy chain), indicating high affinity for the relevant MHC complex. The highest yield of peptide-MHC monomers at low peptide concentrations was observed for IPS/HLA-B35, followed by NLV/HLA-A2, indicating that these peptides had highest peptide-MHC binding affinity. These data indicate that the differences in peptide concentration required for maximal IFNγ production were not due to differences in peptide-MHC binding affinity, but more likely resulted from differences in avidity between T cell populations.

These data show that the kinetics and amount of IFNγ produced by activated peptide-specific cells were determined by the peptide concentration. A maximal IFNγ response was observed after stimulation with 10^{-7} to 10^{-5} M peptide, depending on the epitope. More vigorous triggering of TCRs by higher peptide concentrations resulted in increased TCR downregulation and a more brief IFNγ response.

Effect of epitope density on CMV-specific CD8+ T cell expansion

Overstimulation of CMV-specific T cells may not only cause a more rapid decline and reduced secretion of IFNγ, but also affect proliferation. To determine the effect of epitope density on CMV-specific CD8+ T cell expansion, CFSE-labeled PBMC were stimulated with different concentrations of CMV peptide and cultured with 50 IU/ml IL-2. As shown in Figure 4A, maximal 70-fold expansion of peptide-specific T cells was observed after stimulation for 6 days with 10^{-8} to 10^{-7} M HLA-B7 restricted TPR peptide. Increasing peptide concentrations above this optimum reduced expansion to only 12-fold after incubation with 10^{-4} M peptide. Dilution of CFSE by tetramer-positive T cells indicated that limited expansion was not due to a lack of proliferation, but numbers of dividing cells were lower (data not shown). Stimulation with less immunodominant HLA-A1 or HLA-B35 restricted YSE or IPS peptides resulted in maximal expansion of peptide-specific T cells after stimulation with higher peptide concentrations of 10^{-5} M (YSE) and 10^{-7} to 10^{-6} M (IPS), but similarly reduced expansion was observed when increasing peptide concentrations above the optimum (Figure 4B). These data show that overstimulation of CMV-specific T cells by a non-physiological level of TCR triggering impairs expansion.

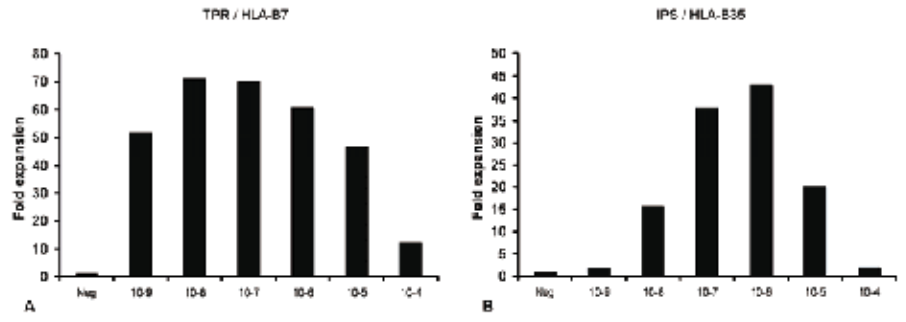


Figure 4. Effect of epitope density on CMV-specific CD8+ T cell expansion. The fold increase in the absolute number of **A)** CMV pp65 TPR peptide-specific CD8+ T cells and **B)** CMV pp65 IPS-specific CD8+ T cells is depicted after 6 days of stimulation of CFSE-labeled PBMC without peptide or with peptide concentrations of 10^{-9} M to 10^{-4} M in the presence of 50 IU/ml IL-2. Samples were stained with CMV peptide-MHC tetramer at day 6 after stimulation.

Enrichment by IFNγ capture assay and subsequent expansion of CMV-specific CD8+ T cells

These findings were translated into a procedure for optimal activation, isolation, and expansion of peptide-specific CD8+ T cells, allowing efficient generation CMV-specific T cell lines. To define optimal activation conditions, IFNγ-based enrichment was performed after stimulation with 10^{-7}, 10^{-6} and 10^{-5} M peptide for 4 hours or overnight. Since not all specific T cells produced IFNγ using lower peptide concentrations, these were not included. As depicted for a representative experiment in Figure 5A, IFNγ capture labeling at the peak of the IFNγ response after 4 hours resulted in a higher percentage and amount of IFNγ secretion by peptide-specific T cells compared to overnight stimulation. Tetramer-staining revealed modest TCR downregulation after 4 hours of stimulation, and confirmed that all peptide-specific cells were activated and secreted IFNγ. As shown in Table 1, the percentage of cells isolated from donor PBMC in the IFNγ-enriched fraction was higher after 4 hours of stimulation compared to overnight stimulation, and was highest using 10^{-7} M peptide. Directly after enrichment, the recovery of CMV peptide-specific T cells was assessed by CD8 and IFNγ capture staining, because tetramer staining was not possible due to TCR downregulation. Due to indirect IFNγ labeling of other cells, the percentage of virus-specific CD8+IFNγ+ cells was approximately 2-fold lower after 4 hours of stimulation compared to overnight stimulation. Therefore, comparing the absolute numbers of isolated CD8+IFNγ+ cells with tetramer-positive T cells in donor PBMC indicated that the recovery of peptide-specific CD8+ T cells from donor PBMC in the enriched fraction was only modestly higher after 4 hours of stimulation compared to overnight stimulation (Table 1). Preferential expansion of the specific cells resulted in IFNγ-enriched fractions with similar frequencies of 73-79% tetramer-positive CD8+ T cells for the different conditions after 9-11 days culture. The yield of peptide-specific cells after culture was higher using 4 hours of stimulation, as shown in Figure 5B and Table 1 by the fold increase in absolute number of specific cells compared with donor PBMC. In line with the highest recovery of peptide-specific cells after enrichment, a mean 62-fold yield of peptide-specific cells was observed after culture using 4 hour stimulation with 10^{-7} M peptide. In contrast to the IFNγ-depleted fractions after 4 hours of stimulation, the IFNγ-depleted fractions after overnight stimulation contained significant residual tetramer-positive cells (day 6-7: 0.3% vs 4.0%) and higher absolute numbers of tetramer-positive cells compared with IFNγ-enriched fractions, confirming the difference in isolation efficiency (Figure 5A and 5B). Although not statistically significant, amongst others due to variable frequencies of peptide-specific T cells in donor PBMC (0.14-1.14%), the trend of optimal isolation efficiency and expansion after 4 hours of 10^{-7} M peptide stimulation is consistent with the previous results.

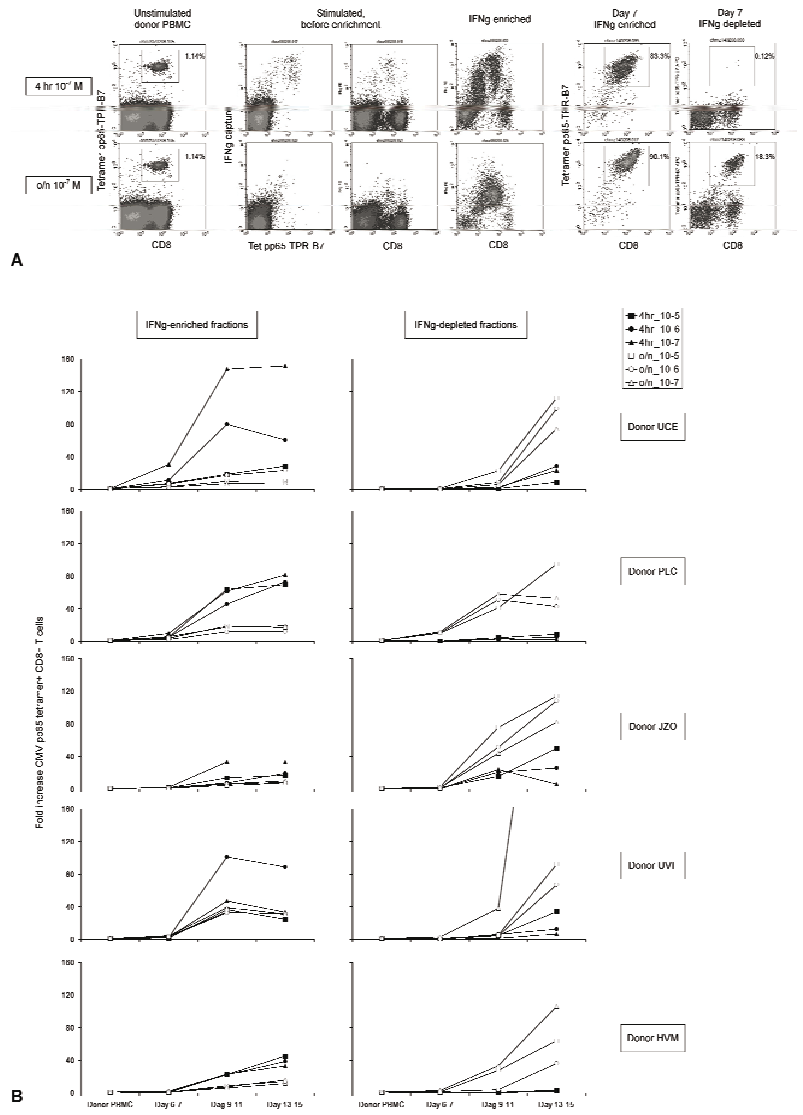


Figure 5. Enrichment by IFNγ capture assay and subsequent expansion of CMV-specific CD8+ T cells. **A)** PBMC from donor PLC were stimulated for 4 hours (upper panel) or overnight (lower panel) with 10^{-7} M CMV pp65 TPR peptide. IFNγ staining by IFNγ capture assay before enrichment, and of the IFNγ-enriched fraction are shown. Subsequently, CMV pp65 TPR-specific CD8+ T cells were identified in both the IFNγ-enriched and IFNγ-depleted fractions after 7 days of culture. **B)** PBMC from 5 donors were stimulated for 4 hours or overnight with 10^{-5}, 10^{-6}, or 10^{-7} M CMV pp65 peptide, and both IFNγ-enriched (left panels) and IFNγ-depleted (right panels) fractions were cultured. The fold increase in absolute number of tetramer-positive CD8+ T cells compared with starting PBMC is shown during culture.

Table 1. Enrichment by IFNγ Capture Assay and Subsequent Expansion of CMV-specific CD8+ T Cells.

	After Enrichment			After 9 to 11 d Culture	
Stimulation	% Isolated From Donor PBMC	% $CD8^+IFNg+$	% Recovery*	% Tetramer +	Fold Increase†
4 h 10^{-5} M	0.92 (0.31-1.48)	16 (2-25)	32 (5-58)	73 (31-86)	31 (14-64)
4 h 10^{-6} M	1.21 (0.36-1.67)	15 (3-25)	45 (7-107)	77 (42-90)	51 (8-101)
4 h 10^{-7} M	1.56 (0.33-3.20)	15 (3-22)	46 (7-89)	79 (41-93)	62 (23-147)
o/n 10^{-5} M	0.43 (0.33-0.53)	30 (2-50)	26 (7-60)	74 (55-86)	14 (4-33)
o/n 10^{-6} M	0.35 (0.24-0.54)	32 (2-49)	23 (3-40)	78 (57-96)	15 (6-32)
o/n 10^{-7} M	0.41 (0.19-0.58)	33 (1-56)	23 (3-45)	79 (58-94)	15 (6-39)

Numbers represent the mean value of 5 experiments using PBMC from different CMV-seropositive donors, with the range reported in parentheses.
*Absolute number of $CD8^+IFNg+$ cells directly after isolation in the IFNg-enriched fraction divided by absolute number of CMV tetramer-positive $CD8^+$ T cells in donor PBMC starting material.
†Absolute number of CMV tetramer-positive $CD8^+$ T cells after culture divided by absolute number of CMV tetramer-positive $CD8^+$ T cells in donor PBMC starting material.
CMV indicates cytomegalovirus; IFNg, interferon-gamma; PBMC, peripheral blood mononuclear cells.

To determine which peptide-specific memory T cell subsets were isolated, phenotypic cell surface markers were analyzed on specific T cells in donor PBMC and T cell lines generated with the different activation protocols. As shown for a representative experiment in Figure 6, CMV tetramer-positive CD8+ T cells in donor PBMC expressed either CD45RO (25-63%) or CD45RA (22-70%), 5-61% expressed both CD27 and CD28, and 4-48% expressed both CCR7 and CD62L. Following activation and isolation, at day 7 all cultured CMV-specific T cells expressed CD45RO, CD28, CCR7 and CD62L, and low levels of CD27. During further culture, peptide-specific cells remained CD45RO+, but lost some CD28 expression and most expression of CD27, CCR7 and CD62L. No differences in phenotype of tetramer-positive T cells were observed after culture between the different isolation protocols. These data do not indicate that the different activation protocols select for different subsets of T cells, but show that the phenotype of the cells changed during in vitro activation and culture.

We next determined whether CMV-specific T cells isolated on basis of IFNγ production were multifunctional, since this may correlate with anti-viral protection. Analysis by ELISA of supernatant obtained after stimulation with CMV peptide showed production of IFNγ and TNFα by isolated CMV-specific T cells, while no IL-2 could be detected. As shown by intracellular staining in Figure 7, single CMV-specific T cells in generated T cell lines produced both IFNγ and TNFα upon peptide stimulation. Furthermore, intracellular staining showed production of perforin and granzyme B (data not shown). These experiments demonstrated that the functional profile of isolated CMV-specific T cells was comparable to CMV-specific T cells in donor PBMC, which also produced IFNγ and TNFα, but no IL-2.

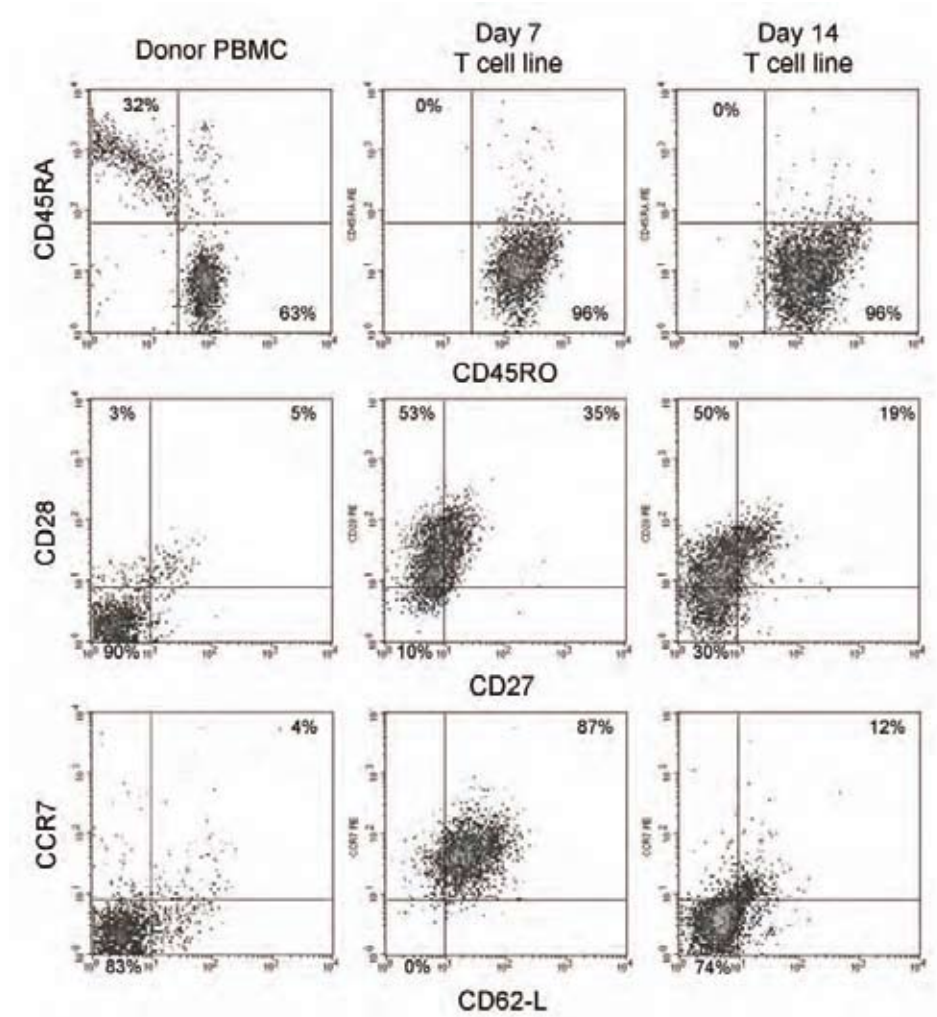


Figure 6. Phenotype of CMV pp65 TPR-specific CD8+ T cells in donor PBMC (left panels), in IFNγ-enriched cell lines after 7 days of culture (middle panels), and after 14 days of culture (right panels). Dot plots are gated on pp65-TPR-B7 tetramer-positive CD8+ cells.

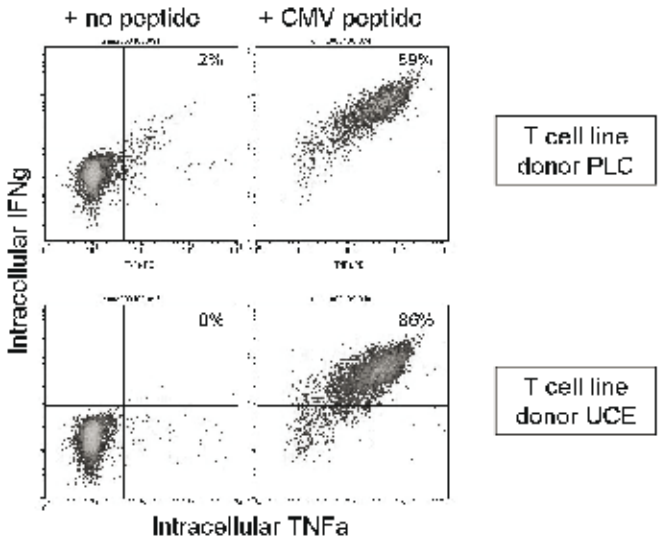


Figure 7. Intracellular staining of IFNγ and TNFα of T cell lines derived from donor PLC (top) and donor UCE (bottom) containing CMV pp65 TPR- and RPH-specific T cells isolated on basis of IFNγ secretion without restimulation (left) and following CMV peptide restimulation (right).

Discussion

Based on the detailed analysis of the IFNγ response, we developed an optimal method for IFNγ-based isolation of CMV peptide-specific CD8+ T cells. Efficient isolation allows adoptive immunotherapy with the complete population of CMV peptide-specific CD8+ T cells, while suboptimal isolation may result in the absence of functional subpopulations. Since the presence of T cells with multiple effector functions has been described to correlate with anti-viral protection [44], isolated CMV-specific T cells were shown to produce both IFNγ and TNFα upon stimulation, which was comparable to CMV-specific T cells in donor PBMC. The isolated CMV-specific T cells may be directly infused or cultured for a short period, allowing the generation of highly specific cell lines containing on average 62-fold the number of CMV peptide-specific CD8+ T cells in donor PBMC. Large-scale isolation from $1x10^9$ PBMC with a frequency of 0.2% peptide-specific CD8+ T cells therefore permits the generation of $1.2x10^8$ specific T cells after only 9-11 days. This approach allows infusion of high numbers of CMV-specific T cells, which may be readily effective and detectable after administration.

The rapid production of IFNγ by CMV-specific CD8+ T cells in the first hours after activation was consistent with the common detection of IFNγ production by human virus-specific T cells by cumulative techniques after 4 to 24 hours of stimulation. However, the rapid decline thereafter was in sharp contrast with the prolonged IFNγ production in the presence of antigen reported for murine virus-specific T cells [34-36]. Although these observations may reflect a general difference in IFNγ response kinetics between human and murine virus-specific T cells, the rapidity of the induction of IFNγ production by virus-specific T cells has been reported to be associated with immunodominance [45]. We therefore speculate that persistent and frequent presentation of human immunodominant CMV epitopes in vivo not only drives differentiation of specific T cells, but may also shape the kinetics of the IFNγ response to limit the extent of inflammation during minor reactivations. Likewise, the vigorous TCR downregulation, more rapid decline of IFNγ production and reduced expansion as observed following high epitope density stimulation may serve to dampen the immune response to highly abundant antigens [28-31].

Whereas minimal amounts of antigen have been reported to be required for cytolytic activity, robust IFNγ secretion needed stimulation with higher levels of antigen, accompanied by TCR downregulation of at least 20-50% [46,47]. Downregulation of surface TCR after activation of T cells by TCR triggering has been described to be predominantly caused by increased intracellular retention and degradation of the complete TCR complex after constitutive endocytosis, although increased internalization may contribute as well [48,49]. Our data confirm that triggering of TCRs resulting in downregulation is associated with IFNγ production. Moreover, a direct correlation between TCR downregulation and the amount of IFNγ production was observed, with maximal IFNγ production induced by 10^{-5} to 10^{-7} M peptide stimulation. The differences in peptide concentration required for maximal IFNγ production or expansion were not due to differences in peptide-MHC affinity, since we did not observe a relation with peptide-MHC binding affinity of selected CMV pp65 epitopes [unpublished data], but more likely result from differences in avidity between T cell populations. Stimulation with higher peptide concentrations resulted in increased TCR downregulation, a more brief IFNγ response and reduced expansion. Since proliferation by specific T cells was observed, the reduced expansion was likely to result from activation-induced cell death (AICD), and not anergy. Death of peptide-specific T cells resulting from contact between specific T cells presenting CMV peptide themselves is unlikely to occur due to the low frequency of specific cells among total PBMC.

Our data indicate that nearly all tetramer-positive cells produced IFNγ and could be isolated using the IFNγ capture assay following activation. This may be due to the selection of immunodominant CMV epitopes, for which high frequencies of specific T cells with

predominantly a late differentiation phenotype circulate in healthy donors, although in some donors we observed up to 50% of CMV-specific T cells showing an early differentiation phenotype. Phenotypic changes of CMV-specific T cells in vitro have been described before, among which functional upregulation of CCR7 upon antigenic stimulation, and the downregulation of CD28, CCR7 and CD62L during prolonged culture [32,50,51], which is consistent with our findings. Since central memory T cells were described to retain the capacity to reacquire their phenotypic and functional properties following adoptive transfer [32], these surface molecules may not be valid markers for memory T cell subsets during in vitro activation and culture. However, the upregulated expression of CD27, CD28, CCR7 and CD62L may result in increased survival and efficacy after adoptive transfer due to increased co-stimulatory interaction and homing to lymph nodes, which can be a reason to administer T cells within 9 days of culture.

This study provides an isolation method enabling efficient and reproducible generation of high numbers of CMV-specific CD8+ T cells for adoptive transfer without the need for extensive culture. The applicability of this strategy may be further extended by the simultaneous isolation of antigen-specific CD8+ and CD4+ T cells without regard to patient HLA following activation with long synthetic peptides or recombinant protein. Since optimal peptide concentrations differed between epitopes and kinetics of IFNγ production may be variable, we suggest that similar analyses should be performed for isolation of T cells specific for other infectious or malignant antigens to exploit the full potential of cytokine capture isolation of antigen-specific T cells.

Acknowledgements

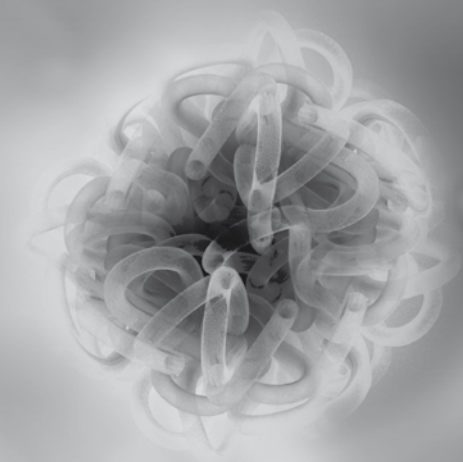
We thank M.G.D. Kester for the production of fluorescently-labeled CMV peptide-MHC tetramers.

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3

Coordinated isolation of CD8+ and CD4+ T cells recognizing a broad repertoire of cytomegalovirus pp65 and IE1 epitopes for highly specific adoptive immunotherapy

Maarten L. Zandvliet
Ellis van Liempt
Inge Jedema
Louise A. Veltrop-Duits
Roel Willemze
Henk-Jan Guchelaar
J.H. Frederik Falkenburg
Pauline Meij

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Abstract

Background

Adoptive transfer of CMV-specific memory T cells can be used for treatment of CMV reactivation after allogeneic stem cell transplantation. Since coordinated CD8+ and CD4+ T cells specific for a broad repertoire of CMV epitopes may be most effective for adoptive immunotherapy, the aim of this study was to isolate these cells from peripheral blood of CMV seropositive donors, irrespective of their HLA type.

Methods

Activation of CMV-specific CD8+ and CD4+ T cells was compared after stimulation of donor peripheral blood with minimal epitope peptides, pools of overlapping 15-mer peptides, or full-length protein. Furthermore, the kinetics of IFNγ production after stimulation was analyzed to determine the optimal time-point for IFNγ-based isolation of CMV-specific T cells. The specificity, phenotype and functionality of generated T cell lines were analyzed.

Results

CMV protein-spanning 15-mer peptide pools induced simultaneous activation of both CD8+ and CD4+ CMV-specific T cells, while full-length CMV protein only efficiently activated CD4+ CMV-specific T cells. Isolation of IFNγ-secreting cells at the peak of the IFNγ response after 4 hours stimulation with CMV pp65 and IE1 peptide pools resulted in efficient enrichment of CMV-specific T cells. The T cell lines contained high frequencies of CD8+ and CD4+ T cells recognizing multiple CMV pp65 and IE1 epitopes, and produced IFNγ and TNFα upon specific restimulation.

Conclusion

This study provides a feasible strategy for the rapid generation of clinical grade CD8+ and CD4+ T cell lines with high specificity for multiple CMV pp65 and IE1 epitopes, which may be used for effective adoptive immunotherapy.

Introduction

In healthy individuals, recurrent reactivations of cytomegalovirus (CMV) are controlled by CMV-specific CD8+ and CD4+ T cells [1]. However, CMV reactivation can cause serious disease in the absence of an adequate CMV-specific T cell response in immunocompromised recipients of allogeneic stem cell transplantation (alloSCT) [2,3]. Pharmacological treatment of CMV reactivation is limited by toxicity and not sufficient for long-term anti-viral protection [4-6]. It has been shown that reconstitution of CD8+ and CD4+ T cells recognizing epitopes derived from immunodominant CMV proteins pp65 and IE1 in the first year after transplantation was associated with sustained protection from CMV disease [7-11]. Several clinical studies have demonstrated that reconstitution of donor-derived CMV-specific T cells by adoptive transfer can be a safe and effective treatment for CMV reactivation after alloSCT [12-21].

Clinical trials have indicated that adoptively transferred CMV-specific CD4+ T cells may promote development of a CMV-specific CD8+ T cell response in vivo [18-21]. Furthermore, adoptively transferred CMV-specific CD8+ T cells showed better persistence in the presence of CMV-specific CD4+ T cells [12]. These observations indicate the requirement of a CMV-specific CD4+ T cell response in the development of a sustained CMV-specific CD8+ T cell response and anti-viral protection, which has also been described after primary CMV infections in renal transplant recipients [22,23]. CMV-specific CD4+ T cells do not only support the development and function of CD8+ T cells, but can also exert direct cytolytic activity [24]. These data provide a rationale for the generation of CMV-specific T cell lines including both CD8+ and CD4+ T cells for adoptive immunotherapy.

Polyclonal CMV-specific T cell responses in healthy individuals are directed against a broad repertoire of epitopes from multiple CMV proteins [1,25]. Furthermore, restoration of immune protection against CMV in immunodeficient individuals has been shown to be associated with an enlarged repertoire of CMV-specific T cells [26]. Adoptive transfer of a coordinated CD8+ and CD4+ T cell response against multiple CMV epitopes may therefore be most effective for treatment of CMV disease in alloSCT recipients.

Several strategies have been developed to isolate virus-specific memory T cells from donor peripheral blood for the generation of virus-specific T cell lines without the need for long-term in vitro culture. Clinical grade peptide-MHC class I multimers have become available, which can be used for isolation of epitope-specific CD8+ T cell populations due to the high affinity for a specific T cell receptor (TCR) [13,27,28]. However, to provide T cell therapy for every patient at risk of developing CMV disease and to generate T cell lines against multiple epitopes, this method requires knowledge of defined epitopes restricted by prevalent MHC

class I molecules, and production of numerous peptide-MHC multimers. Furthermore, antigen-specific CD4+ T cells can not be isolated using this method due to the lack of functional peptide-MHC class II multimers. Alternatively, virus-specific memory T cells can be isolated based on activation-induced IFNγ production. Stimulation with minimal peptides corresponding to defined MHC class I restricted epitopes followed by IFNγ-based isolation has been demonstrated to result in efficient isolation of CMV-specific CD8+ T cells, but requires epitope knowledge as well [16,29]. Although this method may be extended to the isolation of CMV-specific CD4+ T cells, only a limited number of minimal peptides corresponding to defined MHC class II restricted epitopes has been characterized.

The use of full-length CMV proteins for stimulation circumvents the need of knowing the exact epitopes, and can provide CMV-specific T cell lines irrespective of the HLA type of the patient. However, the generation of a coordinated CD8+ and CD4+ CMV-specific T cell response from donor peripheral blood has been shown to be difficult. CMV antigen endogenously synthesized by transduction of specific cDNA into antigen-presenting cells was predominantly presented in MHC class I, while exogenously added CMV proteins or lysates resulted in selected presentation of CMV peptides in MHC class II [14,17-21,30]. Interestingly, 15-mer peptides have recently been described to simultaneously induce activation of both CD8+ and CD4+ T cells specific for CMV epitopes, and may be used for the generation of combined CD8+ and CD4+ T cell lines [31-33]. Synthetic protein-spanning pools of overlapping 15-mer peptides can be produced under good manufacturing practice (GMP) conditions more easily than recombinant proteins or vectors for genetic modification, and may therefore allow feasible and cost-effective production of CMV-specific T cells for adoptive immunotherapy.

The aim of this study was to develop a method for the generation of clinical grade coordinated CD8+ and CD4+ T cell lines with high specificity for immunodominant CMV proteins pp65 and IE1, irrespective of the HLA type of the patient. Full-length protein-spanning pools of overlapping 15-mer peptides induced simultaneous activation of both CMV-specific CD8+ and CD4+ T cells. To achieve high frequencies of CMV-specific T cells, activated T cells were isolated at the peak of the IFNγ response by the IFNγ capture assay. This strategy resulted in coordinated CD8+ and CD4+ T cell lines recognizing multiple CMV pp65 and IE1 epitopes, which can be generated irrespective of the patient HLA type, and may be used for treatment of CMV reactivation after alloSCT.

Material and Methods

Donor cells

After informed consent, peripheral blood was obtained from CMV seropositive healthy individuals, and mononuclear cells (PBMC) were cryopreserved after Ficoll-Isopaque separation. Stable Epstein-Barr virus (EBV)-transformed B cell lines (EBV-LCL) were generated using standard procedures [34]. Monocytes were labeled with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and isolated from PBMC by immunomagnetic separation using the midi-MACS system (Miltenyi Biotec).

CMV antigens

Peptides used were derived from CMV pp65 protein (P06725, 561 amino acids) or CMV IE1 protein (P13202, 491 amino acids). Minimal peptides corresponding to previously described MHC I or MHC II restricted pp65 epitopes, and MHC I restricted IE1 epitopes were synthesized using standard solid-phase strategies (Department of Immunohematology, Leiden University Medical Center, Leiden, The Netherlands). The minimal MHC I restricted pp65 peptides used were YSEHPTFTSQY (HLA-A*0101), NLVPMVATV (HLA-A*0201), QYDPVAALF (HLA-A*2402), TPRVTGGGAM (HLA-B*0702), RPHERNGFTVL (HLA-B*0702), IPSINVHHY (HLA-B*3501), minimal MHC I restricted IE1 peptides were VLEETSVML (HLA-A*0201), ELRRKMMYM (HLA-B*0801), QIKVRVDMV (HLA-B*0801), and minimal MHC II restricted pp65 peptides were LLQTGIHVRVSQPSL, ALPLKMLNIPSINVH, IIKPGKISHIMLDVA, PQYSEHPTFTSQYRIQ, PPWQAGILARNLVPM, KYQEFFWDANDIYRI [24,35,36]. Full pp65 or IE1 protein-spanning pools of 15-mer peptides overlapping with 11 amino acids (PeptivatorTM, Miltenyi Biotec, and PepMixTM, JPT Peptide Technologies, Berlin, Germany), and full-length recombinant pp65 protein (Miltenyi Biotec) were used.

Flow cytometric analyses

Cells were stained with FITC-labeled CD3, CD4, CD14, IFNγ (BD Biosciences, San Jose, CA, USA), PE-labeled CD56, TNFα (BD), CD40L (Beckman Coulter, Fullerton, CA, USA), PerCP-labeled CD3, CD8 (BD), APC-labeled CD4 (Beckman Coulter), CD19, IFNγ, and IL-2 (BD) monoclonal antibodies (mAbs). PE- and APC-labeled CMV peptide-MHC tetramers were produced as described previously [27]. Between $5x10^4$ and $5x10^5$ fluorescent events were analyzed for each sample using a FACSCalibur and Cellquest software (BD). The limit of detection was defined as a cluster of at least 10 specific events, after correction for background events in negative control samples.

Intracellular cytokine staining

After thawing, PBMC were resuspended in culture medium, consisting of Iscove's modified Dulbecco's medium (IMDM, Lonza, Basel, Switzerland) supplemented with 10% pooled human serum, 100 U/ml penicillin/streptomycin (Lonza), and 3 mM L-glutamine (Lonza). For measurement of activated CMV-specific T cells in PBMC by cumulative intracellular cytokine staining, cells were stimulated in culture medium with 10^{-5} or 10^{-6} M CMV peptides or protein for 6 hours at 37°C and 5% CO_2. During the last 5 hours of peptide stimulation, 10 μg/ml brefeldin A (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. For real-time kinetic measurement of intracellular IFNγ, cells were stimulated for 2 to 48 hours, and 10 μg/ml BFA was added only during the last 2 hours of stimulation. To allow detection of CMV-specific T cells in the T cell lines, we used as stimulator cells autologous EBV-LCL or monocytes labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, The Netherlands) for distinction in FACS analysis as described before [37]. Stimulator cells were incubated overnight in culture medium with 10^{-6} M CMV peptides, washed, and incubated in culture medium with the T cell line in an effector/stimulator ratio of 1/5 for 4 hours in the presence of 10 μg/ml BFA. After stimulation, cells were harvested and washed in phosphate-buffered saline (PBS) and stained with PE-labeled tetramers for 15 minutes at 37°C. Subsequently, mAbs for cell-surface staining were added and incubated for 30 minutes at 4°C. Cells were washed in PBS and fixed with 1% paraformaldehyde for 8 minutes at 4°C. For permeabilization, samples were washed in PBS with 0.1% saponin (Sigma-Aldrich) and incubated for 30 minutes at 4°C. Cells were stained intracellularly with mAbs for 30 minutes at 4°C, washed, and fluorescent events were analyzed by flow cytometry.

Isolation and culture of IFNγ-secreting cells

For isolation of IFNγ-secreting cells, PBMC were stimulated in culture medium with 10^{-6} M CMV pp65 and IE1 protein-spanning peptide pools for 4 hours at 37°C and 5% CO_2. After stimulation, cells were harvested and thoroughly washed in PBS, and IFNγ-secreting cells were stained by the IFNγ capture assay (Miltenyi Biotec) performed according to the manufacturer's instructions. Briefly, cells were labeled with IFNγ-catch reagent and cultured for 45 minutes at 37°C. Cells were counterstained with PE-labeled IFNγ mAb. For subsequent isolation, the IFNγ-PE-labeled cells were bound to anti-PE microbeads, and isolated by immunomagnetic separation using the midi-MACS system (Miltenyi Biotec). The isolated IFNγ-enriched and IFNγ-depleted cell fractions were both cultured at $1x10^6$ cells/ml in culture medium, containing 50 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) and

10 ng/ml IL-15 (Peprotech, Rocky Hill, NJ, USA) and $10x10^6$ cells/ml 30 Gy-irradiated feeder cells derived from the IFNγ-depleted fraction. Cultures were supplemented with fresh medium, 50 IU/ml IL-2, and 10 ng/ml IL-15 every 3-4 days.

Results

Activation efficiency of CMV pp65-specific T cells in PBMC

To investigate whether a broad repertoire of CMV-specific T cells could be activated in PBMC from healthy CMV seropositive donors, irrespective of their HLA type, specific IFNγ production was determined following stimulation with different CMV pp65 antigens. Intracellular staining after stimulation with MHC class II binding immunodominant CMV pp65 peptides showed that CMV-specific CD4+ T cells produced IFNγ and concomitantly expressed CD40L, as shown for a representative example (Figure 1A). Stimulation with either full-length recombinant CMV pp65 protein or a CMV pp65 protein-spanning pool of overlapping 15-mer peptides induced IFNγ production by CMV-specific CD4+ T cells in PBMC from all 11 donors tested (Figure 1B). The HLA types of the donors were heterogeneous, containing 13 different HLA-DR and HLA-DQ alleles, indicating that CMV antigen was presented by various HLA alleles. Frequencies of activated CMV-specific CD4+ T cells were comparable using the protein or peptide pool, and ranged from 0.02% to 4.5% of CD4+ T cells. Addition of minimal peptides to the peptide pool and protein for stimulation did not further increase the frequency and magnitude of the CMV pp65-specific CD4+ T cell response. Since these paired measurements showed a similar pattern of frequencies of IFNγ-producing CD4+ T cells, it is likely that the CD4+ T cells activated with the 15-mer peptide pool were similar to the CD4+ T cells activated with the protein, and included the CD4+ T cells activated with the minimal peptides.

To determine whether stimulation with CMV pp65 protein or 15-mer peptide pool could simultaneously induce activation of a broad repertoire of CMV-specific CD8+ T cells irrespective of the HLA type, specific IFNγ production by CD8+ T cells was determined in PBMC from the same CMV seropositive donors. Their heterogeneous HLA types comprised 19 different HLA-A and HLA-B alleles. As expected, intracellular staining after stimulation with minimal MHC class I binding CMV pp65 peptides showed specific production of IFNγ by CMV-specific CD8+ T cells (Figure 1C). Stimulation with either the full-length recombinant CMV pp65 protein or the CMV pp65 protein-spanning pool of overlapping 15-mer peptides also induced IFNγ production by CMV-specific CD8+ T cells. However, paired measurements showed that frequencies of activated CMV-specific CD8+ T cells were higher after stimulation with the pp65 15-mer peptide pool compared to the recombinant pp65

protein, indicating that part of the CMV pp65-specific CD8+ T cells were not activated using full-length protein. Stimulation with the combination of minimal peptides, peptide pool, and protein induced similar frequencies of IFNγ-producing CD8+ T cells compared to stimulation with peptide pool only. These data suggest that stimulation with the pp65 15-mer peptide pool resulted in activation of CMV pp65-specific CD8+ T cells, including the CD8+ T cells activated with the minimal peptides or the protein.

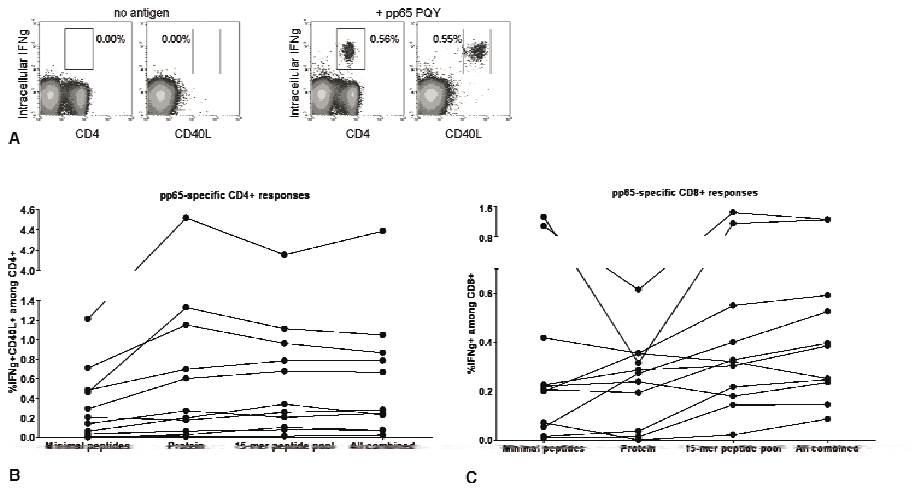


Figure 1. Activation of CMV pp65-specific T cells in PBMC. **(A)** Detection of CMV pp65-specific CD4+ T cells by intracellular IFNγ and CD40L staining upon stimulation of PBMC from donor 9 with MHC class II restricted immunodominant pp65 peptide PQY. **(B)** Frequencies of IFNγ-producing CD40L+ cells among CD4+ T cells or **(C)** frequencies of IFNγ-producing cells among CD8+ T cells in PBMC of 11 healthy CMV seropositive donors after stimulation with minimal MHC class II or I restricted pp65 peptides, pp65 protein, 15-mer pp65 peptide pool, or these antigens combined. Paired measurements from each donor are connected with lines.

To analyze whether stimulation with the CMV pp65 peptide pool indeed induced activation of multiple individual CMV-specific CD8+ T cell populations, peripheral blood samples were selected from 10 donors containing high frequencies of CD8+ T cells specific for 5 known CMV pp65 epitopes. The CMV-specific CD8+ T cells were efficiently activated after stimulation with the corresponding minimal MHC class I binding CMV peptides, as illustrated by representative examples of peptide-MHC tetramer staining combined with intracellular IFNγ staining (Figure 2). Production of IFNγ co-incided with a decrease in tetramer staining, indicating TCR downregulation. After stimulation with the CMV pp65 peptide pool (10^{-6} M), CD8+ T cells specific for 4/5 CMV pp65 epitopes were efficiently activated as shown by IFNγ production, which was also found following stimulation of the

CMV pp65-specific CD8+ T cells in the other donors. However, part of the CD8+ T cells specific for pp65-YSE/A1 did not produce IFNγ (range 20-83% IFNγ-producing tetramer+ T cells). Increasing the concentration of peptide pool to 10^{-5} M did not result in a higher frequency of pp65-YSE/A1-specific CD8+ T cells producing IFNγ. After stimulation with recombinant pp65 protein, only CD8+ T cells specific for pp65-NLV/A2 and pp65-TPR/B7 produced IFNγ and showed modest TCR downregulation, while CD8+ T cells specific for pp65-YSE/A1, pp65-RPH/B7, and pp65-IPS/B35 did not show any IFNγ production or TCR downregulation (Figure 2).

These results demonstrate that stimulation of donor PBMC with the CMV pp65 protein-spanning 15-mer peptide pool efficiently induced activation of CD8+ and CD4+ T cells specific for multiple CMV pp65 epitopes, irrespective of the donor HLA type. In contrast, stimulation with the full-length CMV pp65 protein resulted in efficient activation of pp65-specific CD4+ T cells, but only a fraction of the pp65-specific CD8+ T cells.

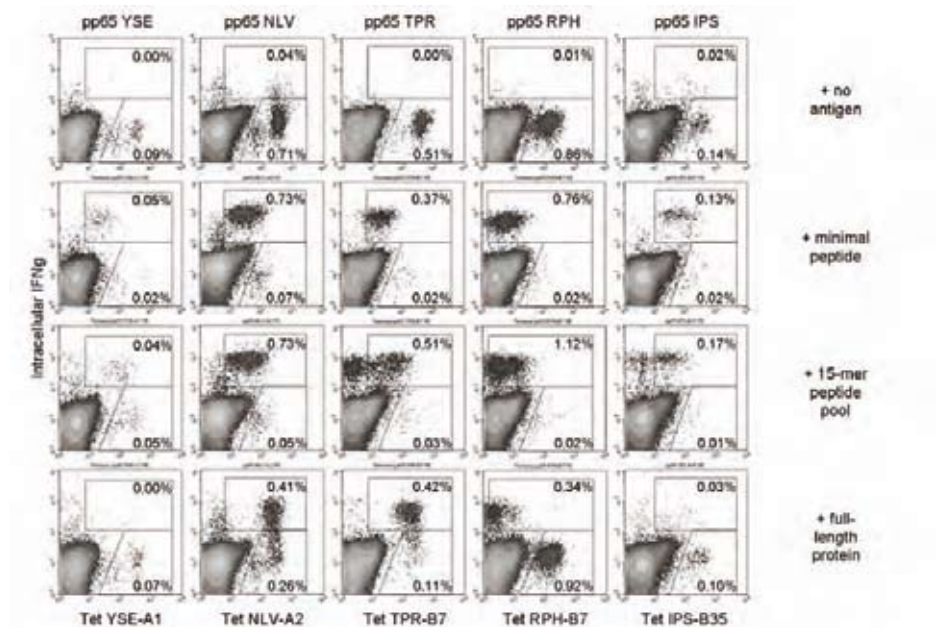


Figure 2. Activation of individual CMV pp65-specific CD8+ T cell populations in PBMC. Dot plots show representative examples of intracellular IFNγ staining of PBMC from different donors, containing CD8+ T cell populations specific for 5 immunodominant CMV pp65 epitopes, after stimulation with 10^{-6} M of the relevant minimal peptide, with 10^{-6} M 15-mer CMV pp65 peptide pool, or with 10^{-6} M full-length recombinant CMV pp65 protein. Samples were stained with peptide-MHC tetramers after stimulation, and dot plots were gated on total lymphocytes.

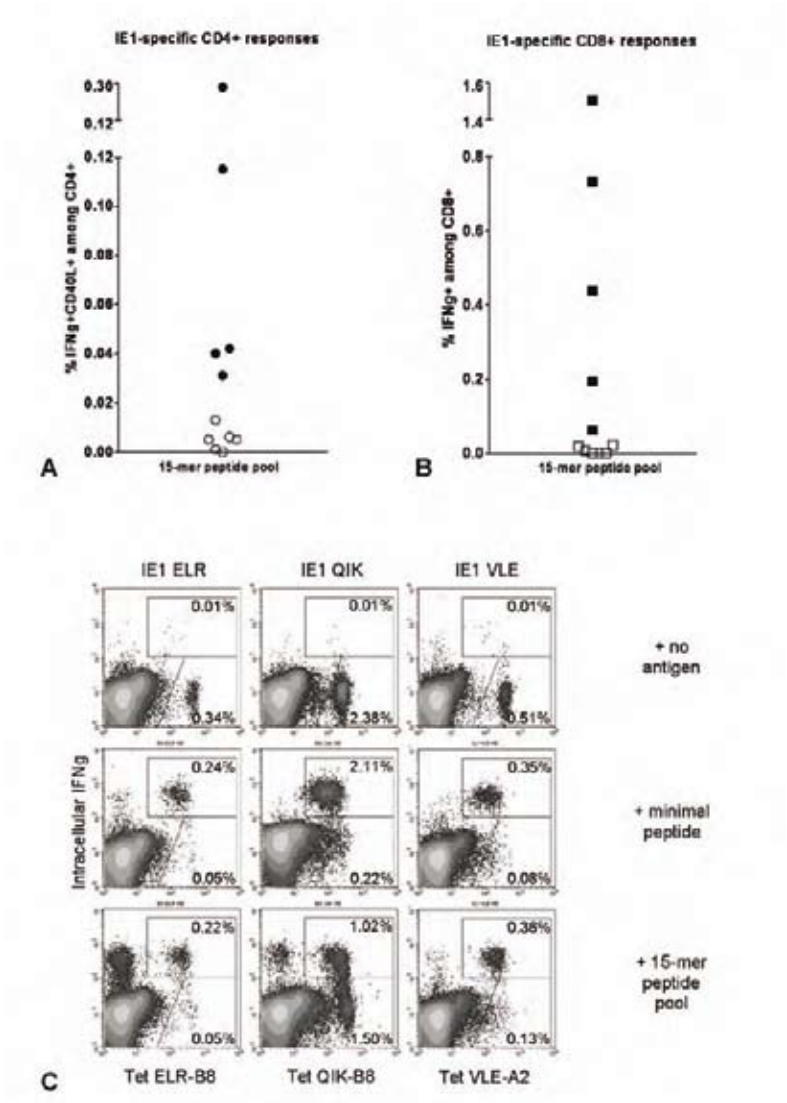


Figure 3. Activation of CMV IE1-specific T cells in PBMC. **(A)** Frequencies of IFNγ-producing CD40L+ cells among CD4+ T cells or **(B)** frequencies of IFNγ-producing cells among CD8+ T cells in PBMC of 11 healthy CMV seropositive donors after stimulation with 15-mer CMV IE1 peptide pool. Filled symbols represent specific IFNγ-producing populations, and open symbols represent frequencies below the limit of detection. **(C)** Dot plots show representative examples of intracellular IFNγ staining of PBMC from different donors, containing CD8+ T cell populations specific for 3 immunodominant CMV IE1 epitopes, after stimulation with 10^{-6} M of the relevant minimal peptide, or with 10^{-6} M 15-mer CMV IE1 peptide pool. Samples were stained with peptide-MHC tetramers after stimulation, and dot plots were gated on total lymphocytes.

Activation of CMV IE1-specific T cells in PBMC

To further extend the repertoire of CMV-specific T cells that could be activated in peripheral blood from CMV seropositive donors for IFNγ-based isolation, we next investigated whether stimulation of PBMC with a CMV IE1 protein-spanning pool of overlapping 15-mer peptides induced IFNγ production by CD8+ and CD4+ T cells specific for CMV IE1 epitopes. After CMV IE1 peptide pool stimulation, specific IFNγ-production by CD4+ T cells was detected in PBMC from 5/11 donors tested, with frequencies between 0.03% and 0.28% of CD4+ T cells (Figure 3A). CMV IE1-specific CD8+ T cells producing IFNγ upon stimulation with the CMV IE1 peptide pool were also detected in PBMC from 5/11 donors tested, with frequencies of CD8+ T cells ranging from 0.06% to 1.5% (Figure 3B). The efficiency of CMV IE1-specific T cell activation using the CMV IE1 peptide pool could not be properly evaluated due to the limited number of known MHC class I and II restricted CMV IE1 epitopes.

To analyze whether stimulation with the CMV IE1 peptide pool induced activation of multiple individual CMV-specific CD8+ T cell populations as well, peripheral blood samples were selected from 6 donors containing high frequencies of CD8+ T cells specific for 3 known CMV IE1 epitopes. As illustrated for representative examples, the CMV IE1-specific CD8+ T cells were efficiently activated after stimulation with the corresponding minimal MHC class I binding CMV IE1 peptides (Figure 3C). After stimulation with the CMV IE1 peptide pool, CD8+ T cells specific for 2/3 CMV IE1 epitopes were efficiently activated as shown by IFNγ production, while part of the CD8+ T cells specific for IE1-QIK/B8 did not produce IFNγ. Similar results were obtained for the CMV IE1-specific CD8+ T cell populations in PBMC from the other donors.

These results show that CMV IE1-specific T cells producing IFNγ after CMV IE1 peptide pool stimulation were observed in peripheral blood from 45% of healthy CMV seropositive donors. The efficiency of activation of CD8+ T cells specific for 3 CMV IE1 epitopes was consistent with the results obtained with the CMV pp65 peptide pool.

Kinetics of IFNγ response by CMV-specific T cells upon activation

To determine the optimal time-point for simultaneous isolation of a broad repertoire of CMV-specific CD8+ and CD4+ T cells by the IFNγ capture assay, the kinetics of IFNγ production following stimulation with CMV peptides was investigated. Real-time kinetic analysis after stimulation with the CMV pp65 or IE1 15-mer peptide pool showed a rapid and synchronized induction of IFNγ production by both CD8+ and CD4+ CMV-specific T cells, which was maximal after 4 hours and decreased thereafter, as depicted for

representative examples (Figure 4A-B). These data indicate that peptides derived from the 15-mer peptide pools were simultaneously presented in MHC class I and II following incubation. Similar kinetics of IFNγ production by CMV pp65-specific CD8+ T cells have previously been described after stimulation with minimal MHC I binding CMV pp65 peptides [29], and were shown to be similar after stimulation with minimal MHC I binding CMV IE1 peptides or minimal MHC II binding CMV pp65 peptides as well. These measurements show that kinetics of IFNγ production was similar for CMV-specific CD8+ and CD4+ T cells, with maximal IFNγ production 4 hours after stimulation with CMV peptides. Therefore, simultaneous isolation of CMV-specific CD8+ and CD4+ T cells by the IFNγ capture assay is likely to be most efficient after 4 hours of stimulation with CMV 15-mer peptide pools.

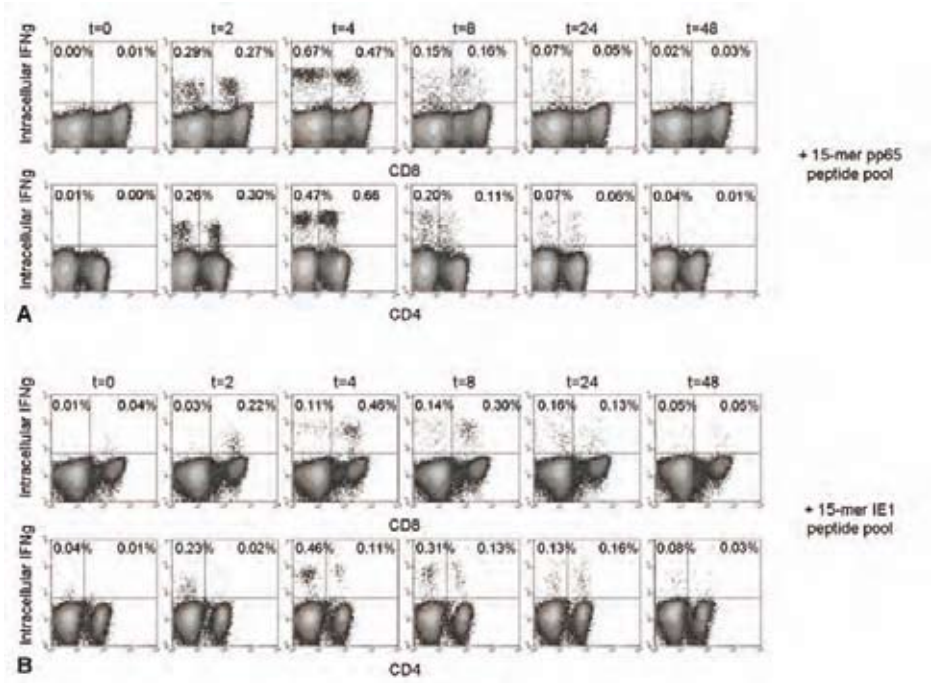


Figure 4. Kinetics of IFNγ response by CMV-specific T cells upon activation. Real-time intracellular IFNγ staining, with addition of BFA only during the last 2 hours of incubation, after stimulation of PBMC for 0, 2, 4, 8, 24, and 48 hours **(A)** with 15-mer pp65 peptide pool, or **(B)** with 15-mer IE1 peptide pool.

IFNγ-based enrichment and expansion of CMV pp65- and IE1-specific T cells

A clinical grade protocol was developed for the generation of T cell lines comprising a broad repertoire of CD8+ and CD4+ T cells specific for both CMV pp65 and IE1 epitopes. Isolation of IFNγ-secreting cells from PBMC was performed by IFNγ capture assay during the peak of IFNγ production after 4 hours of stimulation with CMV 15-mer peptide pools. Since low frequencies of IE1-specific T cells in PBMC were observed and IE1-specific T cell lines have not been described in detail before, T cell lines specific for IE1 were first generated from 3 CMV seropositive donors, resulting in high frequencies of CD8+ and CD4+ IE1-specific T cells after 14 days of culture (Table 1). Subsequently, T cell lines specific for both pp65 and IE1 were generated from 4 CMV seropositive donors, as shown for a representative example (Figure 5). Before enrichment, low frequencies of CD8+ T cells specific for CMV pp65 and IE1 epitopes and CD4+ T cells specific for CMV pp65 were detected in PBMC (Figure 5A). In the T cell line, high frequencies of CD8+ T cells specific for the same pp65 and IE1 epitopes were detected by peptide-MHC tetramer staining (Figure 5B). To determine the total frequency of CMV-specific T cells, the T cell lines was restimulated with autologous EBV-LCL loaded with CMV pp65 or IE1 peptide pools, which induced IFNγ production by high frequencies of CD8+ T cells specific for CMV pp65 and IE1 and CD4+ T cells specific for CMV pp65 (Figure 5B). The IFNγ-depleted fractions contained only low frequencies of CMV tetramer-positive CD8+ T cells, and CD8+ and CD4+ T cells producing IFNγ upon CMV-specific restimulation (Figure 5C). These data show efficient enrichment of CMV-specific T cells from PBMC, resulting in a T cell line containing CD8+ and CD4+ T cells specific for multiple pp65 and IE1 epitopes, with an overall specificity of 91% of T cells producing IFNγ upon CMV-specific restimulation.

Table 1. Generation of 7 coordinated CD8+ and CD4+ T cell lines with high specificity for CMV pp65 and IE1 epitopes derived from healthy CMV seropositive donors.

Day 0			Day 14								
			Composition [1]		Specificity for pp65 within CD8+ or CD4+ T cells [2]			Specificity for IE1 within CD8+ or CD4+ T cells [2]			Overall specificity within the total T cell population [3]	
Donor	Nr. PBMC	Antigen	% CD8+	% CD4+	% tetramer+ / CD8+	% IFNg+ / CD8+	% IFNg+ / CD4+	% tetramer+ / CD8+	% IFNg+ / CD8+	% IFNg+ / CD4+	% CMV-specific T cells	Nr. CMV-specific T cells
1	6.2x10^7	IE1	95	2				97	93	29	90	9.2x10^7
2	1.0x10^7	IE1	94	4				87	71	7	93	5.3x10^7
3	6.0x10^7	IE1	31	68				0	15	58	43	7.5x10^7
4	8.7x10^7	pp65+IE1	91	19	46	43	70	0	57	1	90	3.1x10^7
5	4.4x10^7	pp65+IE1	89	8	52	50	60	57	43	2	91	8.5x10^7
6	5.0x10^7	pp65+IE1	33	70	76	74	84	0	4	1	83	3.3x10^7
3	5.0x10^7	pp65+IE1	58	38	1	28	62	0	4	38	50	3.7x10^7

IFNg-secreting cells were isolated from donor PBMC after 4 hours of stimulation with CMV pp65 and IE1 15-mer peptide pools. After 14 days of culture, the T cell lines were analyzed:
1) The composition of the T cell lines was determined by staining of CD8+ and CD4+ T cells.
2) The specificity of the T cell lines for CMV pp65 and IE1 was determined by peptide-MHC tetramer staining without stimulation and by intracellular IFNg staining upon restimulation with autologous EBV-LCL or monocytes, which were unloaded, or loaded with 15-mer CMV pp65 or IE1 peptide pool.
3) The overall specificity of CD8+ and CD4+ T cells for CMV pp65 and IE1 was calculated from the previous columns, and the total number of CMV-specific T cells was calculated.



Figure 5. IFNγ-based enrichment and cytokine production of CMV-specific T cells. **(A)** Peptide-MHC tetramer staining without stimulation and intracellular IFNγ staining upon stimulation of PBMC from donor 5 with 15-mer pp65 or IE1 peptide pool. After stimulation with 15-mer pp65 and IE1 peptide pools, the IFNγ-enriched **(B)** and IFNγ-depleted **(C)** fraction were cultured for 15 days, and specificity was determined by peptide-MHC tetramer staining without stimulation and intracellular IFNγ staining upon restimulation with autologous EBV-LCL, which were unloaded, or loaded with 15-mer pp65 or IE1 peptide pool. **(D)** Intracellular staining of IFNγ, TNFα, and IL-2 upon stimulation of PBMC from donor 3 with MHC class II restricted pp65-IIK peptide (left), upon restimulation of the IL-2 and IL-15 cultured CMV-specific T cell line generated from donor 3 (middle), and upon restimulation of the same CMV-specific T cell line after 14 days of culture with IL-15, in the absence of exogenous IL-2 (right), with autologous EBV-LCL loaded with pp65-IIK peptide.

Using this method, T cell lines with high specificity for a broad repertoire of CMV CD8+ and CD4+ epitopes were reproducibly generated from all donors, irrespective of the HLA type (Table 1). The alloreactive recognition of third party donor PBMC by the CMV-specific T cell lines was reduced to <10% compared with the donor PBMC which had been used as starting material in a ^{3}H-incorporation assay. Furthermore, the T cell lines retained the capacity to proliferate upon antigen-specific restimulation, and showed efficient and specific lysis of CMV peptide-loaded target cells (data not shown). The composition of the T cell lines correlated with the frequencies of pp65- and IE1-specific CD8+ and CD4+ T cells in donor PBMC, illustrating that the repertoire of CMV-specific T cells in donor PBMC was represented in the T cell lines, irrespective of the HLA type of the donor.

The phenotype and capacity to produce cytokines was compared between CMV-specific T cells in donor PBMC and in cultured T cell lines. CMV-specific T cells in donor PBMC predominantly showed a CD45RO+, CD27+ or CD27-, CD28+ or CD28-, CD62L-, and CCR7- effector memory T cell phenotype. After 6 days of culture following activation and isolation, the expression of CD27, CD28, CD62L, CCR7 transiently increased, as we have recently described [29]. However, after 14 days of culture, the phenotype of CMV-specific T cells was similar to their original phenotype in donor PBMC again. Intracellular staining showed that CMV-specific CD8+ and CD4+ T cells in donor PBMC produced both IFNγ and TNFα upon stimulation, and some CMV-specific T cell populations also produced IL-2, as shown for a representative CMV pp65-specific CD4+ T cell population (Figure 5D). Following restimulation of the cultured CMV-specific T cell lines with autologous EBV-LCL loaded with CMV peptide, CMV-specific T cells produced both IFNγ and TNFα, while no production of IL-2 was detected. To determine whether the capacity to produce IL-2 was downregulated by addition of exogenous IL-2 during culture, T cell lines were cultured for 14 days with IL-15 in the absence of IL-2. Thereafter, restimulation of the cultured CMV-specific T cell lines with autologous EBV-LCL loaded with CMV peptide suggested that IL-2 production by CMV-specific T cells was partly restored (Figure 5D).

These results demonstrate that coordinated CD8+ and CD4+ T cell lines with high specificity for multiple CMV pp65 and IE1 epitopes can be rapidly generated from all CMV seropositive donors using this method. The phenotype and function of CMV-specific T cells in generated T cell lines was comparable to CMV-specific T cells in donor PBMC.

Discussion

In this study, we developed a method for the simultaneous isolation of coordinated CD8+ and CD4+ T cell responses against multiple epitopes derived from both CMV pp65 and IE1

protein from donor peripheral blood, which may be used for treatment of CMV disease in alloSCT recipients. While peptide-MHC multimers can only be used for isolation of CD8+ T cell populations specific for single and defined epitopes, IFNγ-based isolation can be used for the simultaneous isolation of a broad repertoire of CMV-specific CD8+ and CD4+ T cells. We here demonstrate that CMV protein-spanning pools of overlapping 15-mer peptides can be used to efficiently activate a broad repertoire of CMV-specific CD8+ and CD4+ T cells, irrespective of the donor HLA type, while the use of CMV lysate or transduced antigen-presenting cells have not shown efficient simultaneous activation of both T cell subsets in previous studies. Subsequently, activated CMV-specific T cells were isolated at the peak of the IFNγ response by IFNγ capture assay. Using this method, highly specific T cell lines were generated from all CMV seropositive donors, and the composition of the T cell lines correlated with the frequencies of pp65- and IE1-specific CD8+ and CD4+ T cells in donor PBMC. The phenotype and function of CMV-specific T cells after culture was comparable to CMV-specific T cells in donor PBMC, although the capacity to produce IL-2 upon activation appeared to be downregulated during culture with exogenous IL-2. However, the IL-2 production capacity may be restored after adoptive transfer in vivo, since the negative feedback caused by addition of exogenous IL-2 during culture appeared to be reversible, which was consistent with previously described data [38].

The efficient activation of specific CD4+ T cells using 15-mer peptides or protein was anticipated, since processing of exogenous protein followed by presentation in MHC class II has been extensively described, and 15-mer peptides may also bind directly in MHC class II molecules at the cell surface [39]. The efficient activation of specific CD8+ T cells using 15-mer peptides, but not protein, can not easily be explained. Activation of CMV-specific CD8+ T cells may not only result from the presence of contaminating shorter peptide fragments, since purified 15-mer peptides have also been shown to induce CD8+ T cell activation [40]. Although the mechanism of processing and MHC class I presentation of exogenously added long synthetic peptides is not known, several models have been proposed, including entrance of MHC class I in the recycling endocytic MHC class II pathway where peptidase-trimmed exogenous antigens can exchange with peptides in the MHC class I molecules [41,42]. Alternatively, long peptides may reach the cytosol followed by the classical route of MHC class I loading [43].

Adoptive transfer of donor-derived CMV-specific T cell lines may be safe, since no adverse events associated with administration have been reported. However, total cell numbers infused were largely variable, and the frequency of CMV-specific T cells was often not determined or was limited. Identification of CMV CD8+ T cell epitopes and the availability of peptide-MHC tetramers have supported the development of efficient protocols for

reproducible generation of T cell lines containing more than 70% CMV-specific CD8+ T cells [13,15,16,29]. In contrast, high specificity for CMV antigens was not demonstrated for the CD4+ T cells administered in clinical trials [14,18-20]. The risk of graft-versus-host disease may be determined by the repertoire of T cells with unknown specificity, and therefore by the absolute number of T cells with unknown specificity isolated from donor PBMC. The very low numbers of T cells with unknown specificity isolated from donor PBMC using the strategy described in this study are assumed to result in a minimal risk of graft-versus-host disease. The high frequencies of CD8+ and CD4+ T cells specific for the defined CMV antigens in the generated T cell lines are not only desirable to increase the safety of clinical application, but may also allow a more detailed evaluation of in vivo persistence and the relation between efficacy and the number of specific T cells infused.

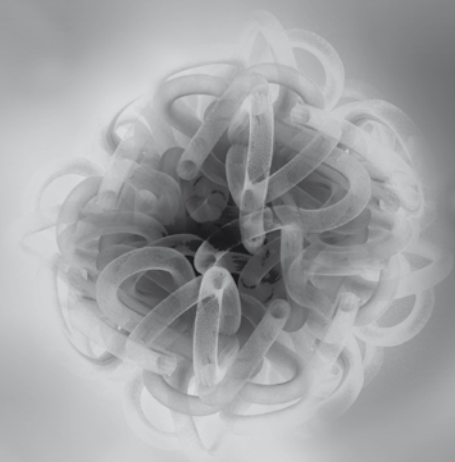
This study provides a feasible strategy for the generation of coordinated CD8+ and CD4+ T cell lines specific for multiple CMV pp65 and IE1 epitopes, irrespective of the HLA type of the patient, which may be used for treatment of CMV infection after alloSCT. The applicability of this strategy may be extended by the isolation of T cells specific for other pathogens, provided that they produce IFNγ upon activation.

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4

Combined CD8+ and CD4+ adenovirus hexon-specific T cells associated with viral clearance after stem cell transplantation as treatment for adenovirus infection

Maarten L. Zandvliet, J.H. Frederik Falkenburg,
Ellis van Liempt, Louise A. Veltrop-Duits,
Arjan C. Lankester, Jayant S. Kalpoe,
Michel G.D. Kester, Dirk M. van der Steen,
Maarten J. van Tol, Roel Willemze,
Henk-Jan Guchelaar, Marco W. Schilham,
Pauline Meij

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Abstract

Background. Human adenovirus (HAdV) can cause morbidity and mortality in immunocompromised patients after allogeneic stem cell transplantation (alloSCT). Reconstitution of HAdV-specific CD4+ T cells has been reported to be associated with sustained protection from HAdV disease, but epitope specificity of these responses has not been characterized. Since mainly CD4+ T cells and no CD8+ T cells specific for HAdV have been detected after alloSCT, the relative contribution of HAdV-specific CD4+ and CD8+ T cells in protection from HAdV disease remains to be elucidated.

Design and Methods. The presence of HAdV hexon-specific T cells was investigated in peripheral blood of pediatric and adult alloSCT recipients, who showed spontaneous resolution of disseminated HAdV infection. Subsequently, a clinical grade method was developed for rapid generation of HAdV-specific T cell lines for adoptive immunotherapy.

Results. Clearance of HAdV viremia coincided with emergence of a coordinated CD8+ and CD4+ T cell response against HAdV hexon epitopes in patients after alloSCT. Activation of HAdV hexon-specific CD8+ and CD4+ T cells with a hexon protein-spanning peptide pool followed by IFNγ-based isolation allowed rapid expansion of highly specific T cell lines from healthy adults, including donors with very low frequencies of HAdV hexon-specific T cells. HAdV-specific T cell lines recognized multiple MHC class I and II restricted epitopes, including known and novel epitopes, and efficiently lysed HAdV-infected target cells.

Conclusion. This study provides a rationale and strategy for the adoptive transfer of donor-derived HAdV hexon-specific CD8+ and CD4+ T cells for treatment of disseminated HAdV infection after alloSCT.

Introduction

Human adenovirus (HAdV) can cause serious morbidity in immunocompromised patients, in particular in pediatric patients after allogeneic stem cell transplantation (alloSCT), and progression to disseminated HAdV disease is associated with high mortality [1-5]. Efficacy of antiviral agents such as ribavirin and cidofovir has not been proven in controlled trials yet, and administration is limited by toxicity. It has been demonstrated that reconstitution of HAdV-specific T cells is essential to control HAdV infection after alloSCT [5-9]. Manipulation of immune reconstitution by adoptive transfer of donor-derived HAdV-specific T cells may therefore be an effective strategy to provide short- and long-term antiviral protection.

In healthy individuals, low frequencies of T cells recognizing target cells infected with HAdV or loaded with HAdV lysate have been reported [10-12]. The majority of HAdV-specific T cells was CD4+, although HAdV-specific CD8+ T cells could also be detected. Further characterization demonstrated that most HAdV-specific T cells recognized major capsid proteins, predominantly the abundant HAdV hexon protein [13-15]. Only recently, a number of immunodominant CD8+ and CD4+ epitopes of HAdV hexon have been defined [14-19]. Since these epitopes are largely conserved, specific T cells were shown to be cross-reactive towards HAdV serotypes from different HAdV subgroups, and may therefore provide protection against a wide range of HAdV serotypes. HAdV hexon-specific T cells have been reported to be detectable in 72% of healthy donors, but may be present at very low frequencies in the remaining donors [20].

In alloSCT recipients, higher frequencies of HAdV-specific T cells have been detected after clearance of HAdV infection, but epitope specificity of these responses has not been further characterized [7-9]. Furthermore, mainly CD4+ T cells and no CD8+ T cells specific for HAdV were detected. Since HAdV-specific CD4+ T cells are capable to directly lyse HAdV infected target cells, the relative contribution of HAdV-specific CD4+ and CD8+ T cells in protection from HAdV disease after alloSCT remains to be elucidated [21,22].

For the generation of donor-derived HAdV-specific T cell lines for clinical application, peripheral blood has been stimulated with HAdV antigens or HAdV transduced antigen-presenting cells, resulting in enrichment of either CD4+ or CD8+ T cells specific for HAdV [20,23-29]. Although alloreactivity was reduced using these strategies, the frequency of HAdV-specific T cells in the cell lines was not determined or was limited, even when IFNγ-based isolation steps were included.

In this study, we demonstrate that CD8+ and CD4+ T cell responses against HAdV hexon epitopes were associated with clearance of HAdV infection in pediatric and adult patients

after alloSCT, providing a rationale for HAdV hexon-specific adoptive immunotherapy. For the generation of clinical grade combined CD8+ and CD4+ HAdV-specific T cell lines, efficient activation of both CD8+ and CD4+ HAdV-specific T cells by stimulation with an HAdV hexon protein-spanning peptide pool was followed by IFNγ-based isolation. Cultured T cell lines derived from all healthy donors tested contained high frequencies of CD8+ and CD4+ T cells specific for multiple HAdV hexon epitopes, and efficiently lysed HAdV-infected target cells. The adoptive transfer of these donor-derived HAdV hexon-specific T cell lines may be used for treatment of disseminated HAdV disease after alloSCT.

Design and Methods

Donor and patient cells

Donor and patient cells were obtained after informed consent, with approval from the local institutional review board. Peripheral blood mononuclear cells (PBMC) were obtained after Ficoll-Isopaque separation. Measurement of HAdV DNA in patient plasma was performed by RQ-PCR as previously described [30]. Fibroblasts were cultured from skin biopsies in DMEM with 10% fetal calf serum (Lonza, Basel, Switzerland).

HAdV antigens

Peptides were derived from the HAdV serotype 5 hexon protein (AP_000211). MHC I restricted peptides were TDLGQNLLY (HLA-A*0101), YVLFEVFDVV (HLA-A*0201), LLYANSAHAL (HLA-A*0201), TYFSLNNKF (HLA-A*2401), MPNRPNYIAF (HLA-B*0702/HLA-B*3501), KPYSGTAYNAL (HLA-B*0702), and IPYLDGTFY (HLA-B*3501) [15,18,19]. The 30-mer peptides used corresponded to amino acids 571-600, 691-720, 796-825, 856-885, and 901-930 [16,17]. Protein-spanning pools of 30-mer peptides overlapping with 15 amino acids or 15-mer peptides overlapping with 11 amino acids (Miltenyi Biotec, Bergisch Gladbach, Germany) were used [17]. A titrated stock of HAdV serotype 5 was produced as previously described [8].

Flow cytometry

Cells were stained with FITC-labeled CD3, CD4, CD27, CD28 (BD Biosciences, San Jose, CA, USA), CD45RO (Caltag, Burlingame, CA, USA), CD62L (BenderMedSystems, Vienna, Austria), CCR7 (R&DSystems, Minneapolis, MN, USA), PE-labeled CD28 (BD), CD45RA, CD40L (Beckman Coulter, Fullerton, CA, USA), CCR7 (R&DSystems), PerCP-labeled CD8 (BD), APC-labeled CD4 (Beckman Coulter), CD45RA, CD45RO, and IFNγ (BD) monoclonal antibodies (mAbs). PE- and APC-labeled HAdV 5 hexon peptide-MHC tetramers were

produced as described previously [31]. Fluorescent events were analyzed for each sample using a FACSCalibur and Cellquest software (BD). The limit of detection was defined as a cluster of at least 10 specific events, after correction for background events in negative control samples.

Intracellular IFNγ staining

PBMC were stimulated with 10^{-6} M HAdV 5 hexon peptides for 6 hours. During the last 5 hours, 10 μg/ml brefeldin A (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. For detection of low frequencies of HAdV-specific T cells, PBMC were cultured 1 week with 10^{-7} M HAdV 5 hexon peptides and 100 IU IL-2/ml (Chiron, Amsterdam, The Netherlands) prior to analysis. For the analysis of T cell lines, we used as stimulator cells autologous Epstein-Barr virus-transformed B cell lines (EBV-LCL) labeled with CFSE (Molecular Probes, Leiden, The Netherlands) for distinction in FACS analysis as previously described [32]. Stimulator cells were loaded overnight with 10^{-6} M HAdV 5 hexon peptides and the T cells were incubated with stimulator cells for 4 hours with 10 μg/ml BFA. After stimulation, cell-surface staining with mAbs was performed, followed by intracellular staining as previously described [33].

Isolation and culture of IFNγ-secreting cells

$25x10^6$ PBMC were stimulated with 10^{-6} M HAdV 5 hexon protein-spanning 15-mer peptide pool for 4 hours. Cells were thoroughly washed in PBS and IFNγ-secreting cells were labeled using the IFNγ capture assay (Miltenyi Biotec) according to the manufacturer's instructions, and isolated using the midi-MACS system (Miltenyi Biotec). The IFNγ-enriched and IFNγ-depleted cell fractions were both cultured with 50 IU/ml IL-2 and 30 Gy-irradiated feeder cells derived from the IFNγ-depleted fraction. Fresh medium and IL-2 were supplemented every 3-4 days.

Cytotoxicity assay

Standard ^{51}Cr release assay was performed as described previously [34]. Briefly, fibroblasts incubated with 200 U/ml IFNγ (Boehringer Ingelheim, Alkmaar, The Netherlands) for 5 days, were incubated overnight with 10^{-6} M HAdV 5 hexon peptides or HAdV 5 virus particles (MOI 500). After washing, fibroblasts were labeled with $Na_2{}^{51}CrO_4$ (GE Healthcare, London, UK) for 1 hour at 37°C, and incubated with effector T cell lines. After 10 hours of incubation, supernatant was harvested for ^{51}Cr analysis.

Proliferation assay

Uncultured donor PBMC or HAdV-specific T cell lines were incubated with allogeneic 30Gy-irradiated PBMC to analyze residual alloreactivity. At day 6 of incubation, ^{3}H-thymidine (Amersham International, Amersham, UK) was added for a further 16 hours of incubation and ^{3}H-thymidine uptake was measured subsequently. The residual alloreactivity was calculated by comparing the ^{3}H-thymidine uptake of the HAdV-specific T cell lines with the uncultured PBMC.

IFNγ ELISA

As stimulator cells, EBV-LCL were loaded with the relevant HAdV hexon peptide at 10^{-6} M for 2 hours. After washing, stimulator cells were incubated with the HAdV-specific T cell line. After overnight incubation, supernatant was harvested and the IFNγ concentration was analyzed by enzyme-linked immunosorbent assay (ELISA, CLB, Amsterdam, The Netherlands).

Results

HAdV hexon-specific T cells in healthy donors

To analyze the HAdV hexon-specific T cell response in PBMC from healthy adult donors, we determined frequencies of CD8+ and CD4+ T cells specific for HAdV serotype 5 hexon epitopes. Analysis of the kinetics of IFNγ production after stimulation showed maximal IFNγ production 4 hours after stimulation with the HAdV 5 hexon protein-spanning 15-mer peptide pool, which was similar for CMV-specific T cells as described recently [33]. Therefore, cumulative intracellular IFNγ staining was measured 6 hours after stimulation with the hexon 15-mer peptide pool. Intracellular IFNγ staining showed hexon-specific CD8+IFNγ+ T cell populations in 6/16 healthy donors (Figure 1A and 1B). Stimulation with minimal peptides corresponding to known hexon CD8+ epitopes induced responses in 3/16 donors (Figure 1B). Addition of minimal peptides to the hexon 15-mer pool did not increase the number of donors responding, indicating that the hexon 15-mer pool efficiently induced activation of hexon-specific CD8+ T cells. Protein-spanning 15-mer peptide pools derived from cytomegalovirus or Epstein-Barr virus were used as irrelevant control peptides in cytomegalovirus and/or Epstein-Barr virus seronegative individuals. No T cell responses were observed using these control peptides (data not shown), confirming the specificity of HAdV hexon-specific IFNγ production. The presence of hexon-specific CD8+ T cells was confirmed in 5 of the 6 positive donors by staining with peptide-MHC tetramers

corresponding to known hexon CD8+ epitopes, with frequencies of 0.06-0.46% of CD8+ T cells (data not shown).

CD4+ T cells specific for HAdV hexon were detected by intracellular IFNγ and CD40L staining upon stimulation of PBMC with HAdV hexon peptide pool (Figure 1A). Hexon-specific CD4+IFNγ+CD40L+ T cell populations could be detected in 13/16 donors (Figure 1C, range 0.03-0.39% of CD4+ T cells). Using 5 selected hexon 30-mer peptides, which have been described to contain immunodominant hexon CD4+ epitopes [17], lower frequencies of hexon-specific CD4+ T cells were activated. These data indicate that significantly more CD4+ T cells, presumably specific for other hexon CD4+ epitopes, were activated using the hexon protein-spanning 15-mer peptide pool than with the 5 selected 30-mer peptides. Addition of the 5 selected 30-mer peptides to the hexon 15-mer pool did not result in increased frequencies of IFNγ-producing CD4+ T cells, indicating that the hexon 15-mer pool also efficiently induced activation of CD4+ T cells specific for previously described hexon CD4+ epitopes.

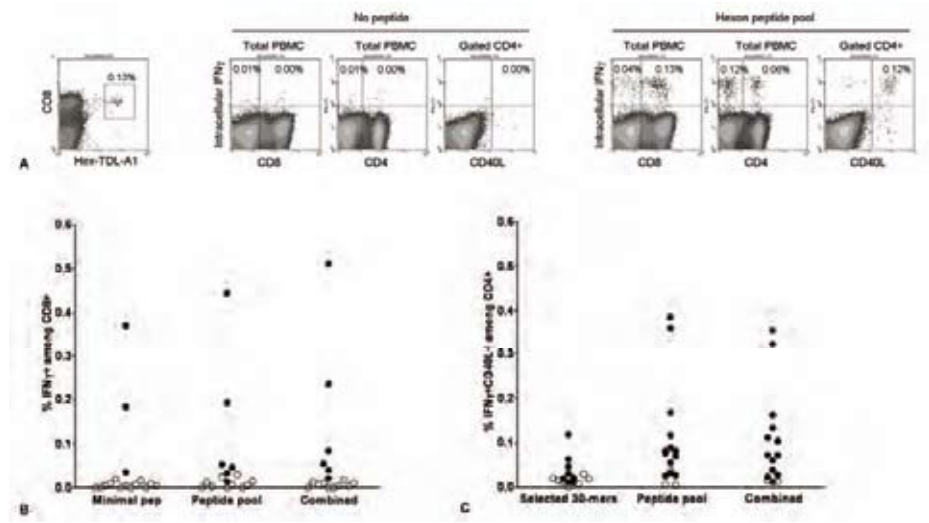


Figure 1. HAdV hexon-specific T cells in healthy donors. **A)** Example (donor 4) of detection of HAdV hexon-specific CD8+ T cells by peptide-MHC tetramer staining and HAdV hexon-specific CD8+ and CD4+ T cells by intracellular IFNγ and CD40L staining upon stimulation with the HAdV hexon 15-mer peptide pool. **B)** Frequencies of IFNγ-producing cells among CD8+ T cells in PBMC of 16 healthy donors after stimulation with HAdV hexon minimal peptides, 15-mer peptide pool, or these 2 sets of peptides combined. **C)** Frequencies of IFNγ-producing CD40L+ cells among CD4+ T cells in PBMC of 16 healthy donors after stimulation with HAdV hexon selected 30-mers, 15-mer peptide pool, or these 2 sets of peptides combined. Filled symbols represent specific IFNγ-producing populations, and open symbols represent frequencies below the limit of detection.

These data show that low frequencies of HAdV hexon-specific CD4+ T cells were detected directly ex vivo in peripheral blood from 81% of healthy adults, while hexon-specific CD8+ T cells could be detected directly ex vivo in 38% of donors. To determine whether the HAdV hexon-specific T cell response was below the threshold of detection in the donors where no HAdV hexon-specific responses could be detected directly ex vivo, PBMC were stimulated for 1 week with HAdV hexon peptide pool and IL-2 for specific expansion. Subsequently, restimulation with HAdV hexon peptide pool followed by intracellular IFNγ staining showed hexon-specific CD8+ and CD4+ T cells in PBMC from all healthy adults tested (data not shown), indicating that low frequencies of HAdV hexon-specific T cells were present in PBMC from all healthy adults.

HAdV-specific T cell response in alloSCT recipients

We next analyzed whether HAdV hexon-specific T cell responses were associated with clearance of HAdV infection in alloSCT recipients, using the same method as for the healthy individuals. From previously described cohorts of patients, we selected 7 pediatric and 6 adult patients, who developed HAdV viremia as determined by positive HAdV DNA plasma load, and subsequently showed spontaneous resolution of disseminated infection [4,8]. HAdV-specific T cell responses could not be analyzed in patients who deceased from HAdV viremia, since only for a few patients peripheral blood samples were available and the lymphocyte numbers in these samples were too low for analysis. Peripheral blood samples obtained during positive HAdV plasma load were available for 10 patients, and peripheral blood samples obtained after resolution of HAdV viremia were available for all 13 patients. During HAdV viremia hexon-specific CD8+ T cells could not be detected directly ex vivo and hexon-specific CD4+ T cells were detected in 2 patients (Figure 2A). After 1 week of culture with the hexon peptide pool and IL-2 for specific expansion, the presence of low frequencies of hexon-specific CD4+ T cells was observed in 5 patients, while no hexon-specific CD8+ T cells could be detected. Following resolution of HAdV viremia, direct intracellular staining showed hexon-specific CD8+ T cells in 7 patients, and hexon-specific CD4+ T cells in all patients (Figure 2B). After 1 week culture of these cells with hexon peptides and IL-2, both hexon-specific CD8+ and CD4+ T cells were shown to be present in PBMC from 12 of the 13 patients. In 4 patients, part of hexon-specific CD8+ T cells were directed against previously described epitopes, as detected by peptide-MHC tetramer staining after expansion (data not shown).

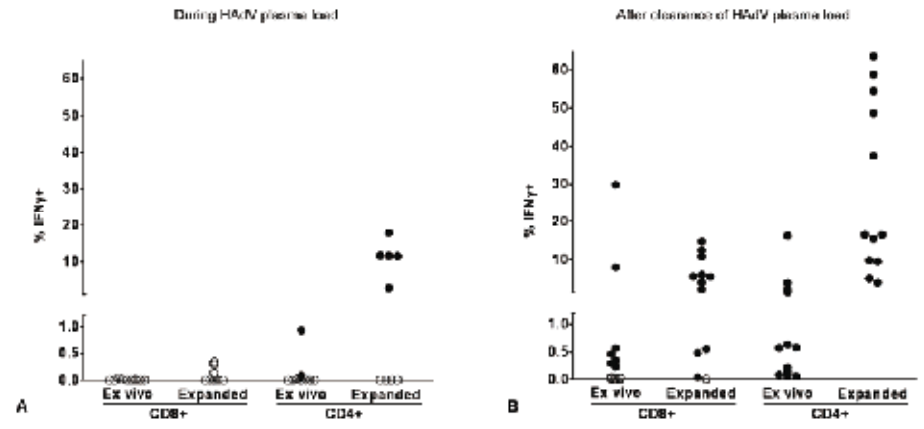


Figure 2. HAdV-specific T cell responses in alloSCT recipients. Frequencies of CD8+ and CD4+ T cells producing IFNγ upon HAdV hexon peptide pool stimulation of PBMC derived from 7 pediatric and 6 adult patients **A)** during or **B)** after HAdV viremia as detected by HAdV DNA plasma load. HAdV-specific IFNγ production was analyzed directly in PBMC (*ex vivo*) or after 1 week culture of PBMC with HAdV hexon peptide pool and IL-2 (expanded). Filled symbols represent specific IFNγ-producing populations, and open symbols represent frequencies below the limit of detection.

A more detailed analysis of development of HAdV-specific T cell responses is shown for 5 patients, of whom multiple peripheral blood samples were available (Figure 3). During HAdV viremia, direct intracellular IFNγ staining upon hexon peptide pool stimulation showed no or low frequencies of hexon-specific CD8+ or CD4+ T cells in these patients. After clearance of the HAdV plasma load, high frequencies up to 30% of CD8+ T cells and 16% of CD4+ T cells producing IFNγ upon hexon peptide pool stimulation appeared in peripheral blood. The hexon-specific T cells emerged directly after resolution of HAdV viremia in most patients, although proliferation of high frequencies of hexon-specific T cells was shown to continue for several months after clearance of the HAdV plasma load in pediatric patient 1 (Figure 3A). The relation between the development of hexon-specific T cells and control of HAdV infection was illustrated by the minor HAdV reactivation at day 152 after transplantation in pediatric patient 2, which was followed by an increase in frequencies of HAdV hexon-specific CD8+ and CD4+ T cells and concomitant viral clearance (Figure 3C). These results demonstrate that a coordinated CD8+ and CD4+ T cell response specific for HAdV hexon epitopes was associated with clearance of HAdV infection in both pediatric and adult patients following alloSCT.

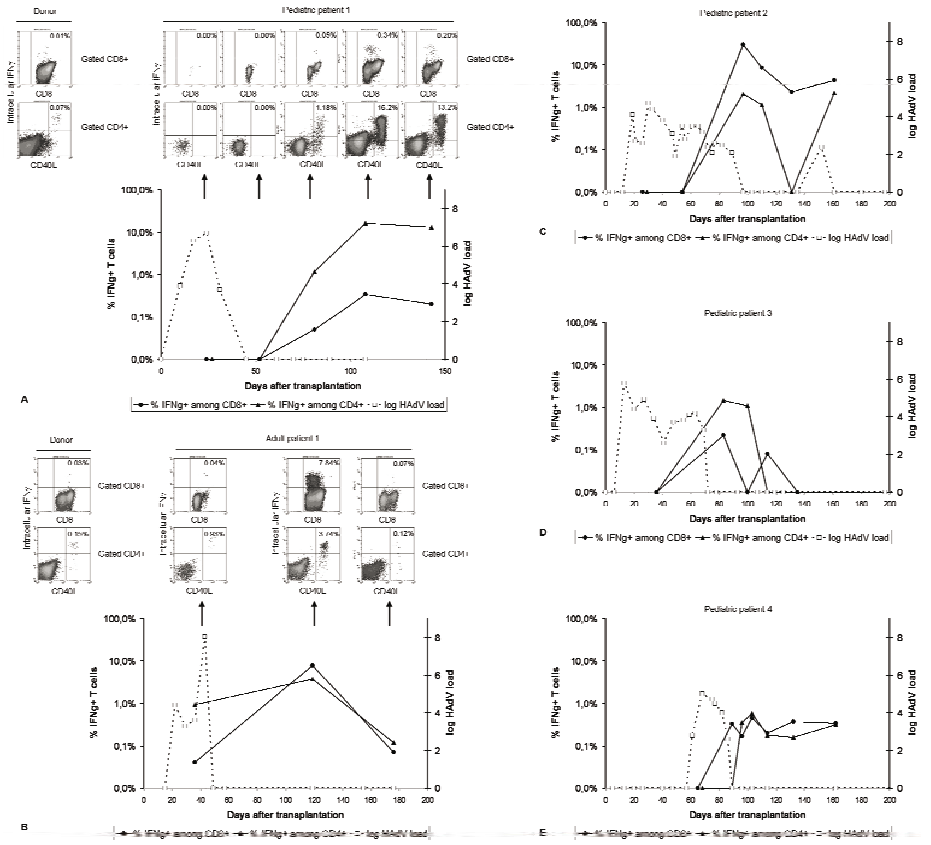


Figure 3. Detailed analysis of HAdV-specific T cell responses in alloSCT recipients. The dot plots show direct intracellular IFNγ and CD40L staining following HAdV hexon peptide pool stimulation of **A)** PBMC from pediatric patient 1 (patient 16 reference [8]), and **B)** PBMC from adult patient 1 (patient 3 reference [4]), obtained during and after resolution of HAdV viremia. The graphs show frequencies of IFNγ-producing cells among CD8+ T cells (black circles) and among CD4+ T cells (black triangles), and the HAdV DNA plasma load (open squares). Similar graphs are presented in **C)**, **D)**, and **E)** for pediatric patient 2, 3, and 4.

Generation of HAdV hexon-specific CD8+ and CD4+ T cell lines

As the presence of CD4+ as well as CD8+ HAdV hexon-specific T cells was associated with viral clearance after alloSCT, a clinical grade protocol was developed for the generation of HAdV hexon-specific T cell lines containing both T cell subsets. PBMC from healthy adult donors were stimulated with the hexon peptide pool, and IFNγ-secreting cells were isolated and cultured with autologous feeders and IL-2. The data of a representative T cell line generated are shown (Figure 4A-C). Before enrichment, low frequencies of HAdV-specific T

cells were detected in PBMC (Figure 4A). In the T cell line generated from the enriched cell population (Figure 4B), 74% of CD8+ T cells (58% of total cells) were directed against the MPN/HLA-B35 epitope [19]. Restimulation of the T cell line with autologous EBV-LCL loaded with the hexon peptide pool induced specific IFNγ production by 94% of CD8+ T cells, indicating that additional hexon CD8+ epitopes were recognized. Furthermore, hexon-specific IFNγ production upon restimulation was observed by 91% of CD4+ T cells. Only low frequencies of hexon-specific T cells could be detected in the IFNγ-depleted fraction (Figure 4C). Results from all 6 donors are summarized (Table 1). HAdV-specific T cell lines could be generated from donors with relatively high frequencies as well as from donors with low or undetectable frequencies of hexon-specific CD8+ and CD4+ T cells. To analyze residual alloreactivity, the HAdV-specific T cell lines derived from donor 1, 2, 3, and 5 were incubated with PBMC from 3 different allogeneic donors. The proliferation of the HAdV-specific T cell lines compared with the uncultured PBMC was only 0.0-6.8%, indicating absent to very low levels of alloreactive capacity (data not shown).

Table 1. Generation of T cell lines containing CD8+ and CD4+ T cells with high specificity for HAdV hexon derived from 6 healthy adult donors.

			Day 1	Day 14/15						
	Donor PBMC		Cell nr.	Cell nr.	Composition			Specificity		
Do-nor	% IFNγ+ / CD8+*	% IFNγ+ / CD4+*	Isolated	Cultured	% CD8+	% CD4+	% NK	% tetramer+ / CD8+*	% IFNγ+ / CD8+*	% IFNγ+ / CD4+*
1	0.04	0.06	$8.4x10^4$	$2.9x10^6$	77	20	3	74	94	91
2	0.00	0.01	$1.2x10^5$	$4.3x10^6$	36	10	48	49	89	61
3	0.02	0.02	$2.3x10^5$	$3.0x10^6$	53	24	23	10	49	13
4	0.51	0.12	$5.2x10^5$	$2.0x10^7$	74	15	8	47	88	45
5	0.00	0.04	$1.6x10^5$	$1.2x10^6$	3	92	5	24	25	92
6	0.24	0.11	$2.4x10^5$	$1.9x10^6$	38	35	20	86	87	79

* To analyze the frequency of HAdV hexon-specific T cells in donor PBMC and in generated T cell lines, the percentage of tetramer-positive T cells was determined after gating on CD8+ T cells, and the percentage of IFNγ-positive T cells was determined after gating on either CD8+ T cells or CD4+ T cells.

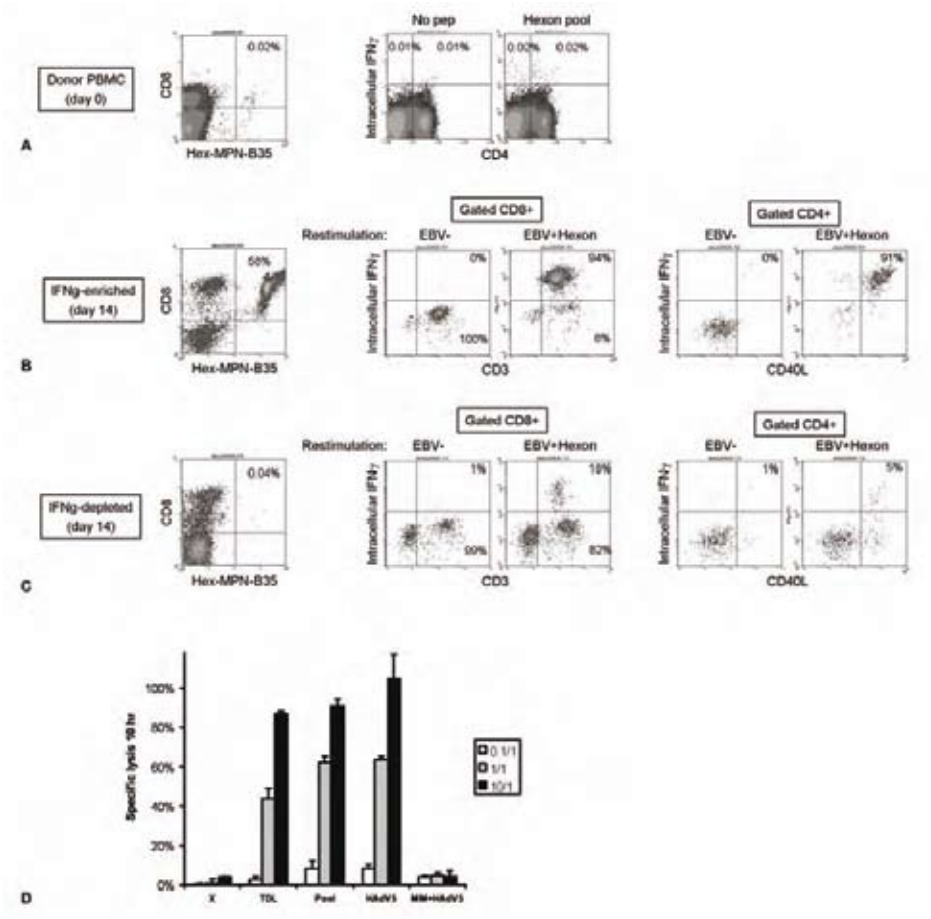


Figure 4. Example of a HAdV-specific T cell line containing CD8+ and CD4+ T cells with high specificity for HAdV hexon. **(A)** Peptide-MHC tetramer staining and intracellular IFNγ staining upon stimulation of PBMC from healthy donor 1 with HAdV hexon peptide pool. Specificity of IFNγ-enriched **(B)** and IFNγ-depleted **(C)** fraction after 14 days of culture as determined by peptide-MHC tetramer staining and intracellular IFNγ staining upon restimulation with autologous EBV-LCL, which were unloaded or loaded with HAdV hexon peptide pool. **(D)** Specific lysis of allogeneic HLA-A1 matched or mismatched (MM) fibroblasts, unloaded (X), loaded with HLA-A1 restricted minimal peptide (TDL), loaded with HAdV 5 hexon peptide pool (Pool), or infected with HAdV 5 (HAdV5) after 10 hours incubation with the HAdV-specific T cell line derived from donor 4 at 3 effector/target ratios.

To investigate whether HAdV-specific T cell lines recognized HAdV-infected target cells which endogenously processed the synthesized HAdV antigen, specific lysis was determined of partially matched allogeneic fibroblasts that were loaded with the minimal

hexon peptide, with the hexon 15-mer peptide pool, or infected with HAdV serotype 5. HAdV-specific T cell lines very efficiently lysed both HAdV peptide-loaded and HAdV-infected fibroblasts, while fully mismatched HAdV-infected fibroblasts were not recognized (Figure 4D). Specific lysis of MHC class I restricted peptide-loaded fibroblasts demonstrated cytotoxic capacity of HAdV hexon-specific CD8+ T cells. Specific lysis by HAdV hexon-specific CD4+ T cells was shown in the T cell line derived from donor 5, containing predominantly CD4+ T cells, which specifically lysed autologous EBV-LCL loaded with the hexon 15-mer peptide pool (67% specific lysis). Lysis by this T cell line could be blocked using anti-MHC class II antibodies (95% block).

The phenotype of HAdV hexon-specific CD8+ and CD4+ T cells in donor PBMC and the T cell lines was investigated. In donor PBMC, HAdV hexon-specific CD8+ and CD4+ T cells were CD56-, CD45RO+, CD27+, CD28+, and CD62L+/-. After isolation and culture, the phenotype remained identical except for the expression of CD62L, which first increased and subsequently decreased on HAdV-specific CD8+ T cells and increased on HAdV-specific CD4+ T cells. This phenotypic analysis demonstrated that HAdV-specific T cells did not differentiate into end-stage effector T cells during in vitro activation and culture.

These results show that IFNγ-based isolation after stimulation with hexon 15-mer peptide pool allowed rapid generation of T cell lines with high frequencies of both HAdV hexon-specific CD8+ and CD4+ T cells from all healthy adult donors tested, including donors with very low frequencies of circulating HAdV hexon-specific T cells. Furthermore, the HAdV hexon-specific T cell lines induced specific and efficient lysis of HAdV-infected target cells.

Characterization of HAdV hexon epitopes recognized

To determine the repertoire of HAdV hexon epitopes recognized by HAdV-specific T cell lines, staining was performed with peptide-MHC tetramers covering all known HAdV hexon CD8+ epitopes restricted by prevalent MHC class I molecules. CD8+ T cell populations specific for 7 previously described hexon epitopes were detected in the HAdV-specific T cell lines derived from 6 healthy donors (Table 2). Analysis of specific IFNγ production by CD8+ T cells upon restimulation with separate hexon overlapping 30-mer peptides showed recognition of 4 additional hexon peptides by the HAdV-specific T cell lines, of which 2 were recognized by CD8+ T cells from 2 different donors (Table 2). To investigate HLA-restriction of these epitopes, CD8+ T cell recognition of a panel of EBV-LCL sharing MHC class I molecules with the donor, loaded with specific hexon 30-mer peptide, was determined. Subsequently, minimal peptides were predicted to bind by algorithms, and were shown to be recognized by specific CD8+ T cells (Figure 5). Specific CD8+ T cells were shown to be stained with IPFSSNFMSM/HLA-B*3501 tetramers, which confirmed HLA-

restriction of this peptide. The HLA-restriction of the other peptides (FRKDVNMVL/HLA-B7, ETYFSLNNKF/HLA-B52, and YSYKARFTL/HLA-B63) was confirmed by specific recognition of peptide-loaded EBV-LCL that expressed these HLA molecules (Figure 5). Despite the recognition of FRKDVNMVL peptide in HLA-B7 by specific CD8+ T cells, production of fluorescently labeled FRKDVNMVL/HLA-B*0702 tetramers was not successful. The HAdV hexon sequences recognized by CD8+ T cells in HAdV-specific T cell lines derived from all 6 donors are presented (Table 2). Both previously described and newly identified hexon CD8+ epitopes were largely conserved between different HAdV species (Table 3). Furthermore, CD4+ T cells in all T cell lines recognized multiple hexon 30-mer peptides, indicating a broad specificity which included conserved HAdV hexon regions. These data demonstrate that HAdV-specific T cell lines generated by IFNγ-based isolation after hexon peptide pool stimulation recognized multiple CD8+ and CD4+ HAdV hexon epitopes, including both known and novel epitopes, thereby exploiting the full donor HLA repertoire.

Table 2. Characterization of HAdV hexon CD8+ epitopes recognized by T cell lines as determined by peptide-MHC I tetramer staining or recognition of specific hexon peptides.

	HAdV hexon CD8+ epitopes recognized					
	Previously described			Newly identified		
Donor	Peptide	AA	HLA restriction	Peptide	AA	HLA restriction
1	MPN IPY	320-329 705-713	B35 B35	IPF	873-882	B35
2	YVL MPN IPY	917-926 320-329 705-713	A2 B35 B35	IPF	873-882	B35
3	YVL KPY MPN TYF	917-926 114-124 320-329 37-45	A2 B7 B7 A24	FRK	585-593	B7
4	TDL	886-894	A1	YSY	78-86	B63
5	TYF	37-45	A24			
6	TDL	886-894	A1	ETY YSY	36-45 78-86	B52 B63

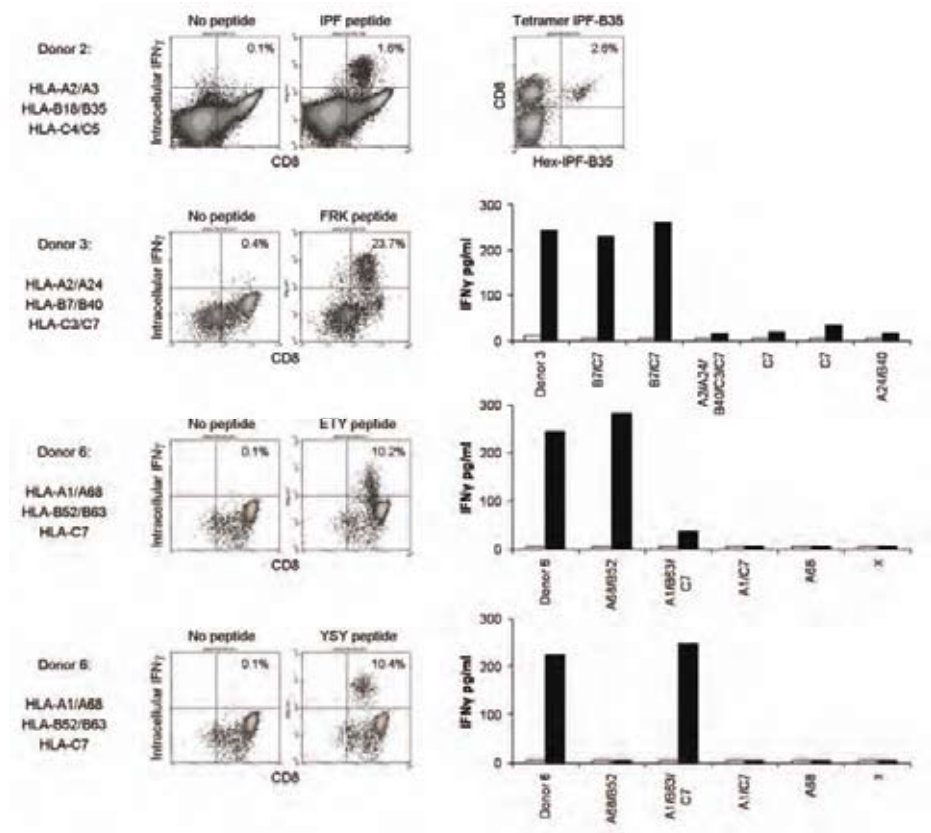


Figure 5. Confirmation of newly identified HAdV hexon CD8+ T cell epitopes. Intracellular IFNγ staining of HAdV-specific T cell lines after restimulation with minimal MHC class I restricted HAdV hexon peptides. In addition, the HAdV-specific T cell line derived from donor 2 was stained with IPF/HLA-B35 tetramers. To confirm HLA-restriction of the other epitopes, IFNγ release was determined by ELISA upon incubation with autologous or partially HLA-matched allogeneic EBV-LCL without peptide (white bars), or loaded with the relevant minimal HAdV hexon peptide (black bars).

Table 3. Conservation of HAdV hexon CD8+ epitopes between different HAdV species.

		Previously described HAdV hexon CD8+ epitopes					
HAdV species	HAdV serotype	AA 37-45	AA 114-124	AA 320-329	AA 705-713	AA 886-894	AA 917-926
C	5	TYFSLNNKF	KPYSGTAYNAL	MPNRPNYIAF	IPYLDGTFY	TDLGQNLLY	YVLFEVFDVV
A	12	---T-G---	---------S-	A---A-----	---------	------M--	----------
B	35	---N-G---	---------S-	--------G-	---------	------M--	-L--------
C	2	---------	----------	---------	---------	---------	----------
D	30	-----G---	---------S-	--------G-	---------	------M--	-L--------
E	4	-----G---	---------S-	--------G-	---------	------M--	--V-------
F	40	-----G---	---------S-	A-------G-	V--------	------M--	----------

		Newly identified HAdV hexon CD8+ epitopes			
HAdV species	HAdV serotype	AA 36-45	AA 78-86	AA 585-593	AA 873-882
C	5	ETYFSLNNKF	YSYKARFTL	FRKDVNMVL	IPFSSNFMSM
A	12	D---T-G---	---------	-------I-	----------
B	35	D---N-G---	----V-Y--	---------	----------
C	2	----------	---------	---------	----------
D	30	D-----G---	---------	-------I-	----------
E	4	D-----G---	----V-Y--	-------I-	----------
F	40	D-----G---	----V----	-------I-	----------

HAdV hexon sequences were compared between serotype 5 (AP_000211), serotype 12 (AP_000121), serotype 35 (AP_000585), serotype 2 (AP_000175), serotype 30 (ABA00012), serotype 4 (AAO24097), and serotype 40 (NP_040862). The sequence derived from serotype 5 is depicted for each HAdV hexon CD8+ epitope, and amino acids that differ are shown for the other serotypes.

Discussion

Based on our observation that resolution of HAdV viremia coincided with appearance of a coordinated CD8+ and CD4+ HAdV hexon-specific T cell response in patients following alloSCT, we developed a method for generation of CD8+ and CD4+ T cell lines with high and defined specificity for HAdV hexon for adoptive immunotherapy. HAdV-specific CD8+ T cells have been detected before in healthy adults and in patients following alloSCT [7,15,18-20,27]. Our study further shows a kinetic association between HAdV-specific CD8+ T cell responses and clearance of HAdV infection in alloSCT recipients.

Since in previous studies predominantly HAdV-specific CD4+ T cells were detected in peripheral blood from healthy individuals, and since these CD4+ T cells showed direct cytotoxic capacity, the role of CD8+ T cells in protection from HAdV disease has been questioned [1,10,12,17,21,22]. In this study, HAdV hexon-specific T cells were detected with

high sensitivity using peptide-MHC tetramer staining and intracellular cytokine staining following stimulation with a HAdV hexon peptide pool. Using these methods, low frequencies of HAdV hexon-specific CD8+ and CD4+ T cells were detected directly ex vivo in peripheral blood from 38% and 81% of healthy adults, respectively. Subsequently, activation of HAdV hexon-specific T cells with the hexon peptide pool followed by IFNγ-based isolation allowed rapid expansion of both CD8+ and CD4+ T cells specific for HAdV hexon epitopes from all healthy donors tested. These data suggest that coordinated responses of CD8+ and CD4+ T cells specific for HAdV hexon epitopes contribute to control of HAdV infection in healthy individuals, and persist as memory T cells afterwards, which is similar to T cell responses described against most other viral infections.

While the development of HAdV-specific T cell responses in patients after alloSCT has previously been shown to be associated with protection from HAdV disease, only HAdV-specific CD4+ T cells, and no HAdV-specific CD8+ T cells have been detected [7-9]. To further investigate this, we analyzed T cell responses specific for the immunodominant HAdV hexon protein in patients that showed spontaneous resolution of disseminated HAdV infection. Using the same sensitive methods of detection as for the healthy donors, combined CD8+ and CD4+ T cell responses specific for HAdV hexon epitopes were demonstrated to be associated with clearance of HAdV infection in patients after alloSCT. The HAdV hexon-specific CD8+ and CD4+ T cells were shown to develop synchronously upon clearance of the HAdV plasma DNA load, supporting the coordinated action of HAdV-specific CD8+ and CD4+ T cells in viral control. It is unlikely that immunosuppressive drugs hampered the detection of HAdV hexon-specific T cells directly ex vivo, since only part of the patients used immunosuppressive drugs during the time points of analysis, and expansion of HAdV hexon-specific T cells was observed in PBMC from all patients. These data indicated that both CD8+ and CD4+ T cells specific for HAdV hexon epitopes contribute to resolution of HAdV infection in alloSCT recipients, and provide a rationale for the adoptive transfer of donor-derived HAdV hexon-specific CD8+ and CD4+ T cells for treatment of HAdV infection after alloSCT.

Despite the low to undetectable frequencies of HAdV hexon-specific T cells in healthy individuals, using HAdV hexon peptide pool stimulation followed by IFNγ-based isolation, highly specific T cell lines could be generated from all donors tested. In contrast to previous studies, we demonstrated high and defined specificity of the T cell lines for HAdV hexon epitopes (median 87% of CD8+ T cells and 70% of CD4+ T cells). The risk of graft-versus-host disease may be determined by the repertoire of non-HAdV-specific T cells, and therefore by the absolute number of non-HAdV-specific T cells isolated from donor PBMC. The very low numbers of non-HAdV-specific T cells isolated using this method are assumed

to result in a minimal risk of graft-versus-host disease. This assumption was supported by the absence or very low levels of residual alloreactive capacity of the HAdV-specific T cell lines generated. Furthermore, no adverse events associated with administration of HAdV- or cytomegalovirus-specific T cell lines generated by IFNγ-based isolation have been reported [23,35,36].

The vigorous expansion and central memory phenotype (CD45RO+, CD27+, CD28+, CD62L+/-) of HAdV hexon-specific T cells indicate a HAdV-specific memory T cell response present in most healthy individuals, that rapidly reacts upon specific activation. While expression of CD27 and CD28 was reported to be transiently upregulated upon activation and subsequently downregulated on CMV-specific T cells during culture [33,37], we here demonstrated that HAdV-specific T cells retained expression of CD27 and CD28. This phenotypic analysis indicated that cells did not differentiate to the end-stage effector cell type that might show impaired proliferative capacity in vivo.

Stimulation of donor PBMC with the HAdV hexon protein-spanning pool of 15-mer peptides (11-mer overlapping) efficiently induced activation of HAdV-specific CD8+ and CD4+ T cells. Although the frequencies of HAdV hexon tetramer-positive CD8+ T cells were not sufficiently high to perform co-staining with intracellular IFNγ directly ex vivo, the activation of cytomegalovirus-specific CD8+ T cell populations upon stimulation with 15-mer peptide pools has recently been confirmed by tetramer and IFNγ co-staining [38]. Although the mechanism of processing and presentation of these 15-mer peptides has not been investigated, the data suggested that stimulation with 15-mer peptides results in efficient presentation in both MHC class I and II, in contrast to endogenously synthesized antigen being predominantly presented in MHC class I, and exogenous antigens such as full-length protein or lysate being mainly presented in MHC class II [20,23-29]. The use of synthetic peptides, which can readily be produced under GMP regulations, may contribute to the general applicability of generating clinical grade virus-specific T cell lines. Furthermore, the flexibility of peptide synthesis allows inclusion of peptides derived from multiple viral proteins and viruses.

From all healthy adults, T cells recognizing multiple MHC class I and II restricted epitopes derived from HAdV serotype 5 hexon protein were isolated. Like the HAdV hexon epitopes described previously, the HAdV hexon epitopes identified in this study are largely conserved between different HAdV serotypes [14,17-19,26]. The newly identified epitopes increase the repertoire of known immunodominant HAdV hexon epitopes, which may improve the analysis of HAdV-specific T cell lines generated for adoptive transfer, and the monitoring of HAdV-specific T cell responses in patients after alloSCT. T cells specific for conserved HAdV epitopes have been demonstrated to be cross-reactive to a wide range of

HAdV serotypes [14,17,18,21,26]. The T cell lines generated in this study may therefore provide protection from infection with all prevalent HAdV serotypes.

The presence of both CD8+ and CD4+ HAdV hexon-specific T cells in healthy individuals and in alloSCT recipients after clearance of HAdV viremia suggests that both subsets are required for adequate antiviral protection. This study provides a strategy for the adoptive transfer of donor-derived CD8+ and CD4+ T cells specific for multiple known and unknown HAdV hexon epitopes, which allows treatment of all patients irrespective of their HLA type.

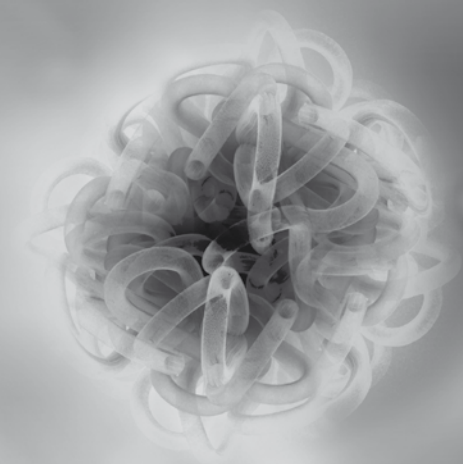
Acknowledgements

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5

Simultaneous isolation of CD8+ and CD4+ T cells specific for multiple viruses for broad antiviral immune reconstitution after allogeneic stem cell transplantation

Maarten L. Zandvliet
Ellis van Liempt
Inge Jedema
Simone Kruithof
Michel G.D. Kester
Henk-Jan Guchelaar
J.H. Frederik Falkenburg
Pauline Meij

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Abstract

Opportunistic viral infections can cause serious morbidity and mortality in immunocompromised patients after allogeneic stem cell transplantation (alloSCT). Clinical studies have demonstrated that adoptive transfer of donor-derived T cells specific for cytomegalovirus (CMV), Epstein-Barr virus (EBV), or human adenovirus (HAdV) can be a safe and effective treatment of infections with these major viral pathogens. The aim of this study was to develop a method for the simultaneous isolation of coordinated CD8+ and CD4+ memory T cell responses against a broad repertoire of viral epitopes. To ensure that the method was applicable to a wide variety of virus-specific T cells that may differ in phenotypic and functional properties, we focused on T cells specific for the persistent viruses CMV and EBV, as well as T cells specific for HAdV and influenza (FLU), which are not repetitively activated in vivo after initial viral clearance. Following in vitro activation, nearly all T cells specific for these viruses produced IFNγ and TNFα, and expressed CD137, while the populations varied in production of IL-2, degranulation, and expression of phenotypic markers. Different kinetics of IFNγ production were observed in CMV/EBV-specific T cells and HAdV/FLU-specific T cells. However, after stimulation of peripheral blood from seropositive donors with viral protein-spanning peptide pools, the activated virus-specific CD8+ and CD4+ T cells could be simultaneously isolated by either IFNγ-based or CD137-based enrichment. This study provides an efficient and widely applicable strategy for isolation of virus-specific T cells, which may be used for reconstitution of virus-specific immunity in alloSCT recipients.

Introduction

Opportunistic viral infections are a major cause of morbidity and mortality in immunocompromised patients after allogeneic stem cell transplantation (alloSCT) [1,2]. Viral reactivations or de novo infections can not be controlled in the absence of an adequate antiviral T cell response. These T cells may be eradicated or functionally impaired by the conditioning regimen and immune suppression co-inciding the alloSCT. Treatment with pharmacological agents is limited by toxicity and often not sufficient for long-term control of these viral infections [3,4]. It has been shown that development of virus-specific T cell responses after alloSCT was associated with sustained protection from viral disease [5-9]. Clinical studies have demonstrated that the adoptive transfer of donor-derived virus-specific memory T cells can be a safe and effective treatment for infections with cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human adenovirus (HAdV), which are the main viral pathogens after alloSCT [10-15]. Extensive characterization of T cell responses against these viruses in both patients and healthy individuals has shown coordinated CD8+ and CD4+ T cell responses against a broad repertoire of epitopes derived from multiple viral proteins [16-19]. Adoptive transfer of donor-derived CD8+ and CD4+ T cells against a broad repertoire of viral antigens may therefore be an effective strategy to provide immune reconstitution as prophylaxis or treatment of viral disease after alloSCT.

Isolation of virus-specific memory T cells from donor peripheral blood allows the rapid generation of highly specific T cell products. Peptide-MHC multimers with high affinity for a specific T cell receptor (TCR) can be used for isolation of epitope-specific CD8+ T cell populations [15,20]. However, to generate T cell lines against multiple viral proteins and for every eligible patient, this method requires knowledge of defined epitopes restricted by prevalent MHC class I molecules, and production of a large number of clinical grade peptide-MHC multimers. Furthermore, virus-specific CD4+ T cells can not be isolated using this method due to the lack of functional peptide-MHC class II multimers. Stimulation of donor peripheral blood with synthetic pools of overlapping 15-mer peptides spanning immunodominant CMV, EBV, or HAdV proteins has recently been shown to induce efficient and simultaneous activation of virus-specific CD8+ and CD4+ T cells, irrespective of the donor HLA type and without the need of knowing exact epitopes [21-26,41]. The activated virus-specific T cells may subsequently be isolated based on the specific expression of an activation marker to generate highly specific T cell lines for adoptive transfer.

CD8+ and CD4+ T cells specific for CMV, EBV, or HAdV can be isolated from peripheral blood based on IFNγ production induced by antigen-specific activation [11,26-28,41]. The IFNγ-based isolation method is clinical grade available, and has been used in several clinical

studies [11,29,30]. Secreted IFNγ is captured by an antibody at the cell membrane, after which a secondary IFNγ-specific antibody attached to a magnetic bead can be used to isolate the labeled cells by magnetic separation. Detailed analysis of the kinetics of IFNγ production by CMV-specific CD8+ and CD4+ T cells has shown a rapid induction of IFNγ production following activation, which was maximal after 4 hours of stimulation, and decreased thereafter [26,31]. Comparable kinetics of IFNγ production by other virus-specific T cell populations would allow simultaneous IFNγ-based isolation of coordinated CD8+ and CD4+ T cell responses against multiple viruses for broad immune reconstitution. However, it is unknown whether T cell populations specific for other viruses than CMV show similar kinetics of IFNγ production upon activation. Furthermore, the IFNγ-based isolation procedure is technically demanding, which may have hampered a wider clinical application. Therefore, other molecules that are expressed at the cell surface of virus-specific T cells upon antigen-specific stimulation, like CD137 (4-1BB) and CD154 (CD40L), have been explored for suitability as isolation marker.

Recently, CMV- and EBV-specific CD8+ T cells have been shown to specifically express the co-stimulatory molecule CD137 at the cell surface upon antigen-specific activation [32-34]. The expression of CD137 by activated CD8+ T cells was demonstrated to peak 24 hours after stimulation [32,34,35]. Prolonged expression of CD137 may provide more freedom in the timeframe for efficient isolation, which can contribute to the robustness of the isolation method. However, the kinetics of activation-induced CD137 expression was analyzed on cultured T cell clones and T cell lines, while the expression of CD137 by virus-specific T cells activated in donor peripheral blood directly ex vivo is unknown. Furthermore, conflicting results have been reported on the activation-induced expression of CD137 by virus-specific CD4+ T cells [33,34]. Since CD137 is expressed at the cell surface upon activation, a simple labeling with CD137-specific antibody and magnetic beads followed by magnetic separation was shown to result in isolation of the activated CD137-positive T cells.

The aim of this study was to define a feasible method for simultaneous isolation of CD8+ and CD4+ T cells recognizing epitopes derived from different viral proteins from peripheral blood of seropositive donors. To ensure that the method was applicable to a wide variety of virus-specific T cells that may differ in phenotypic and functional properties, we focused on T cells specific for the persistent viruses CMV and EBV, as well as T cells specific for HAdV and influenza (FLU), which are not repetitively activated in vivo after initial viral clearance. We demonstrate that both IFNγ-based and CD137-based enrichment result in efficient isolation of CD8+ and CD4+ T cells specific for multiple viral proteins after stimulation of peripheral blood with viral protein-spanning peptide pools. The simple CD137 labeling procedure has an advantage over the more laborious IFNγ labeling procedure, allowing

more feasible and cost-effective production of virus-specific T cell lines. This study provides an efficient and widely applicable strategy for the simultaneous isolation of virus-specific T cells, which may be used for reconstitution of virus-specific immunity in alloSCT recipients.

Materials and methods

Donor cells and cell lines

After informed consent, peripheral blood was obtained from healthy individuals, and mononuclear cells (PBMC) were cryopreserved after Ficoll-Isopaque separation. Stable Epstein-Barr virus (EBV)-transformed B cell lines (EBV-LCL) were generated using standard procedures [36].

Viral peptides

Minimal peptides corresponding to previously described viral epitopes were synthesized using standard solid-phase strategies (Department of Immunohematology, Leiden University Medical Center, Leiden, The Netherlands). In addition, pools of 15-mer peptides overlapping with 11 amino acids spanning viral proteins were used (PepMixTM, JPT Peptide Technologies, Berlin, Germany, and PeptivatorTM, Miltenyi Biotec, Bergisch Gladbach, Germany). The minimal MHC I and II binding peptides and protein-spanning pools used are depicted in Table 1.

Flow cytometric analyses

Cells were stained with FITC-labeled CD3, CD4, CD25, CD27, CD28, IFNγ, CD107a (BD Biosciences, San Jose, CA), CD45RO (Caltag, Burlingame, CA), CCR7 (R&D Systems, Minneapolis, MN), CD62L (Bender MedSystems, Vienna, Austria), PE-labeled CD28, TNFα (BD), CD45RA (Beckman Coulter, Fullerton, CA), CCR7 (R&D Systems), PerCP-labeled CD3, CD4, CD8 (BD), APC-labeled CD4 (Beckman Coulter), CD45RA, CD45RO, CD137, IFNγ, and IL-2 (BD) monoclonal antibodies (mAbs). PE- and APC-labeled peptide-MHC I tetramers were produced as described previously [20]. PE-labeled CMV-pp65-ALP-DR1 tetramer was obtained through the NIH Tetramer Facility (Emory University, Atlanta, GA) and PE-labeled FLU-HA-PKY-DR4 tetramer was kindly provided by T.N.M. Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Between $5x10^4$ and $5x10^5$ fluorescent events were analyzed for each sample using a FACSCalibur and Cellquest software (BD).

Analysis of cytokine production and degranulation

After thawing, PBMC were resuspended in culture medium, consisting of Iscove's modified Dulbecco's medium (IMDM, Lonza, Basel, Switzerland) supplemented with 10% pooled human serum, 100 U/ml penicillin/streptomycin (Lonza), and 3 mM L-glutamine (Lonza). For cumulative intracellular cytokine staining, PBMC were stimulated in culture medium with 10^{-6} M viral peptides for 6 hours at 37°C and 5% CO2. After 1 hour of peptide stimulation, 10 μg/ml brefeldin A (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added for the remaining 5 hours of incubation. For detection of degranulation, CD107a mAb was added during peptide stimulation. For real-time kinetic measurement of cytokine production, cells were stimulated for 2 to 48 hours, and 10 μg/ml BFA was added only during the last 2 hours of stimulation. After stimulation, cell-surface staining with mAbs was performed, followed by intracellular staining as described previously [31]. To analyze production of additional cytokines by virus-specific T cells, supernatant was harvested after peptide stimulation and analyzed by the human Th1/Th2/Th17 multi-analyte profiler ELISArray™ kit (SA Biosciences, Frederick, MD), performed according to the manufacturer's instructions. To allow analysis of frequencies of virus-specific T cells in T cell lines by intracellular cytokine staining, we used as stimulator cells autologous EBV-LCL labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, The Netherlands) for distinction in FACS analysis as described before [37]. The stimulator cells were loaded overnight in culture medium with 10^{-6} M viral peptides, washed, and the T cell line was incubated with stimulator cells in an effector/stimulator ratio of 1:5 for 4 hours in the presence of 10 μg/ml BFA. After stimulation, cell-surface staining with mAbs was performed, followed by intracellular staining as described previously [31].

Isolation and culture of virus-specific T cells

PBMC were stimulated with 10^{-6} M viral peptides in culture medium supplemented with 10 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) for 4 to 96 hours at 37°C and 5% CO2. For isolation based on IFNγ production, cells were harvested after stimulation, thoroughly washed in PBS, and IFNγ-secreting cells were stained and isolated by the IFNγ secretion assay (Miltenyi Biotec) performed according to the manufacturer's instructions. Briefly, cells were labeled with IFNγ-catch reagent and cultured for 45 minutes at 37°C. Subsequently, cells were counterstained with PE-labeled IFNγ mAb, bound to anti-PE microbeads, and isolated using the midi-MACS system (Miltenyi Biotec). For isolation based on CD137 expression, cells were harvested after stimulation, thoroughly washed in PBS, and stained with APC-labeled CD137 mAb (BD) for 30 minutes at 4°C. Subsequently, cells were bound

to anti-APC microbeads (Miltenyi Biotec), and isolated using the midi-MACS system. The enriched and depleted cell fractions were both cultured at $1x10^6$ cells/ml in culture medium, containing 50 IU/ml IL-2 and 10 ng/ml IL-15 (Peprotech, Rocky Hill, NJ) and $5x10^6$ cells/ml 25 Gy-irradiated feeder cells derived from the depleted fractions. Cultures were supplemented with fresh medium containing 50 IU/ml IL-2 and 10 ng/ml IL-15 every 3-4 days.

Results

Variability in phenotype and function of virus-specific T cells

To investigate whether T cells specific for the persistent viruses CMV and EBV, which are frequently activated in vivo, differed from T cells specific for HAdV and FLU, which are not repetitively activated in vivo after viral clearance, we analyzed phenotypic and functional properties of these T cells. Peripheral blood samples were selected from 15 healthy CMV- and EBV-seropositive individuals, which were positive for at least one of the prevalent MHC molecules HLA-A1, -A2, -B7, -B8, or -B35. Although their serostatus was unknown for HAdV and FLU, the majority of the adult population has been exposed to these viruses. Virus-specific CD8+ T cell populations were analyzed using peptide-MHC I tetramers (n=18), which are presented in Table 1. The frequencies of CD8+ T cells specific for CMV (median 0.40%) and EBV (median 0.20%) epitopes were higher compared to frequencies of CD8+ T cells specific for HAdV and FLU (median 0.02%) epitopes. Phenotypic analysis showed that virus-specific CD8+ T cell populations were heterogeneous with regard to the expression of phenotypic markers CD27, CD28, CCR7, CD62L, CD45RA, and CD45RO, indicating the presence of different T cell subsets. Most CD8+ T cells specific for HAdV or FLU epitopes expressed CD27 (median 82%), and CD28 (median 85%) (Figure 1A-B). Furthermore, these T cells expressed CCR7, CD62L, and CD45RO (median 60%, 58%, 75% respectively). These results were mainly consistent with a central memory phenotype for HAdV- and FLU-specific CD8+ T cells. EBV-specific CD8+ T cells also showed expression of CD27, CD28, CCR7, CD62L and CD45RO, although T cells specific for EBV lytic epitopes showed lower percentages of CD62L expression (median 35%), indicative of a central memory or effector memory phenotype. In contrast, CD8+ T cells specific for CMV epitopes showed less expression of CD27 (median 51%), CD28 (median 32%), CCR7 (median 30%) and CD62L (median 30%), and showed higher percentages of CD45RA positive CD8+ T cells (median 68%). These results were consistent with an effector phenotype for CMV-specific CD8+ T cells.

Table 1. Viral peptides used for stimulation of specific CD8+ and CD4+ T cells.

Antigen	Virus	Protein	Sequence	Restriction
MHC I binding peptides	CMV	pp50	VTEHDTLLY	HLA-A1
	CMV	pp65	NLVPMVATV	HLA-A2
			TPRVTGGGAM	HLA-B7
			IPSINVHHY	HLA-B35
	CMV	IE1	VLEETSVML	HLA-A2
			ELRRKMMYM	HLA-B8
			QIKVRVDMV	HLA-B8
	EBV	BMLF1	GLCTLVAML	HLA-A2
	EBV	BRLF1	YVLDHLIVV	HLA-A2
	EBV	BZLF1	RAKFKQLL	HLA-B8
			EPLPQGQLTAY	HLA-B35
	EBV	EBNA1	HPVGEADYFEY	HLA-B35
	EBV	EBNA3A	RPPIFIRRL	HLAB7
			FLRGRAYGL	HLA-B8
			YPLHEQHGM	HLA-B35
	HAdV 5	Hexon	TDLGQNLLY	HLA-A1
			MPNRPNYIAF	HLA-B35
	FLU	Matrix	GILGFVFTL	HLA-A2
MHC II binding peptides	CMV	pp65	ALPLKMLNIPSINVH	HLA-DR1
			IIKPGKISHIMLDVA	HLA-DR4
			PQYSEHPTFTSQYRIQ	HLA-DR11
			KYQEFFWDANDIYRI	HLA-DR52
	FLU	HA	PKYVKQNTLKLAT	HLA-DR4
Protein-spanning 15-mer peptide pools	CMV	pp65 [1) 2)]	P06725	N.A.
		IE1 [1) 2)]	P13202	N.A.
	EBV	BZLF1 [1)]	P03206	N.A.
		EBNA1 [1)]	P03211	N.A.
		EBNA3A [2)]	P12977	N.A.
		EBNA3C [2)]	P03204	N.A.
	HAdV 5	Hexon [1)]	P04133	N.A.
	FLU	Matrix [2)]	Q67157	N.A.
		Nucleocapsid [2)]	Q2LPC2	N.A.

1) Obtained from Miltenyi Biotec, Bergisch Gladbach, Germany.
2) Obtained from JPT Peptide Technologies, Berlin, Germany.
N.A.: Not applicable.

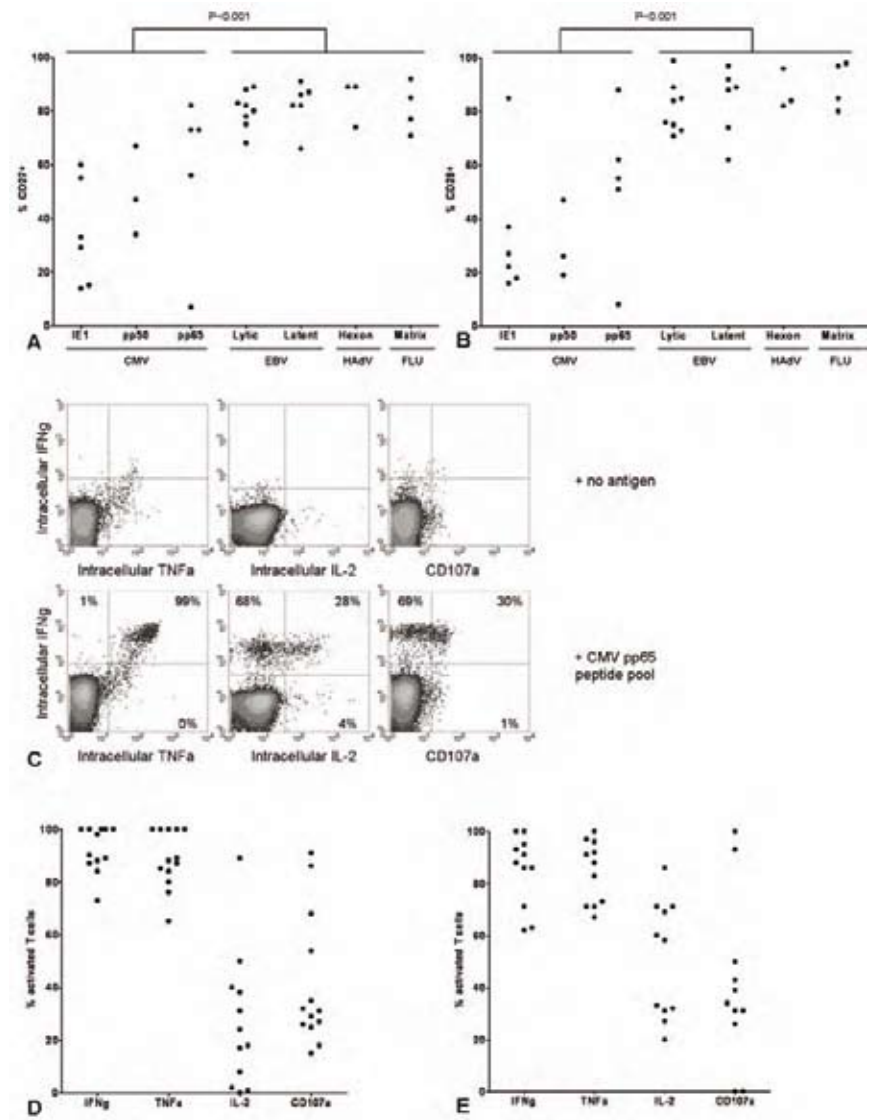


Figure 1. Variability in phenotype and function of virus-specific T cells. **(A)** The percentage of CD27+ cells and **(B)** the percentage of CD28+ cells is shown of peptide-MHC I tetramer-positive CD8+ T cell populations specific for CMV (n=14), EBV (n=14), HAdV (n=3), and FLU (n=4) epitopes. **(C)** Representative example of intracellular staining of IFNγ, TNFα, and IL-2, and surface CD107a staining of PBMC without stimulation and after stimulation with the CMV pp65 protein-spanning peptide pool. **(D)** The percentage of IFNγ-, TNFα-, and IL-2-producing T cells as measured by intracellular staining, and the percentage of degranulating T cells as measured by surface CD107a staining, of virus-specific CD8+ T cells activated after stimulation with minimal MHC I binding peptides (n=13), and **(E)** of virus-specific CD8+ and CD4+ T cells activated after stimulation with viral protein-spanning peptide pools (n=11).

To compare the functional profile of the T cells specific for the different viruses, cumulative intracellular staining and cell surface staining were performed following stimulation of PBMC from the healthy donors with viral peptides. Kinetic analyses showed maximal cumulative measurement of IFNγ, TNFα, IL-2, and CD107a after 6 hours of stimulation. As depicted for a representative example of PBMC after stimulation with CMV pp65 protein-spanning peptide pool in Figure 1C, activated CMV pp65-specific T cells produced both IFNγ and TNFα, while only part of these T cells additionally produced IL-2 or showed degranulation by surface expression of CD107a. This functional profile was observed for the different specificities (CMV, EBV, HAdV, and FLU), and both after stimulation with minimal MHC class I binding peptides (Figure 1D), as well as after stimulation with protein-spanning pools of overlapping 15-mer peptides (Figure 1E). Analysis of supernatant harvested after stimulation with minimal MHC class I binding peptides did not show production of other cytokines by virus-specific T cells. No correlation was observed between the phenotype or viral protein recognized by the CD8+ T cells, and their cytokine production or degranulation upon activation (data not shown).

These results demonstrate that the T cells specific for the different viral proteins produced both IFNγ and TNFα upon activation, while the populations varied in production of IL-2, degranulation, and expression of phenotypic markers. It appeared that CMV-specific CD8+ T cells had a more differentiated phenotype. However, no differences were observed in cytokine production or degranulation between the T cells specific for the different viruses.

Kinetics of IFNγ production by virus-specific T cells

Since nearly all virus-specific T cells produced IFNγ upon stimulation and the IFNγ-based isolation method is clinical grade available, the kinetics of IFNγ production were investigated to determine whether the different CD8+ and CD4+ T cell populations can be isolated simultaneously. IFNγ production kinetics were analyzed following stimulation of PBMC directly ex vivo with minimal MHC I or II binding peptides corresponding to known CMV, EBV, HAdV and FLU epitopes. When no minimal MHC binding peptides were available for stimulation of virus-specific T cells, 15-mer peptide pools were used, which were previously shown to induce IFNγ production with similar kinetics [26]. Real-time kinetic analysis after stimulation of PBMC with minimal MHC I binding CMV or EBV peptides showed a rapid decrease in peptide-MHC tetramer staining, which coincided with the induction of IFNγ production by CMV- or EBV-specific CD8+ T cells. Production of IFNγ was maximal 4 hours after stimulation and rapidly decreased thereafter (Figure 2A-C). In contrast, FLU-specific CD8+ T cells showed a slightly slower induction of IFNγ production

after stimulation with minimal MHC I binding FLU peptide, which transiently decreased after 24 hours of stimulation, but continued up to 48 hours after stimulation (Figure 2D-E). The frequencies of HAdV-specific CD8+ T cells in PBMC were not sufficiently high to analyze kinetics of IFNγ production.

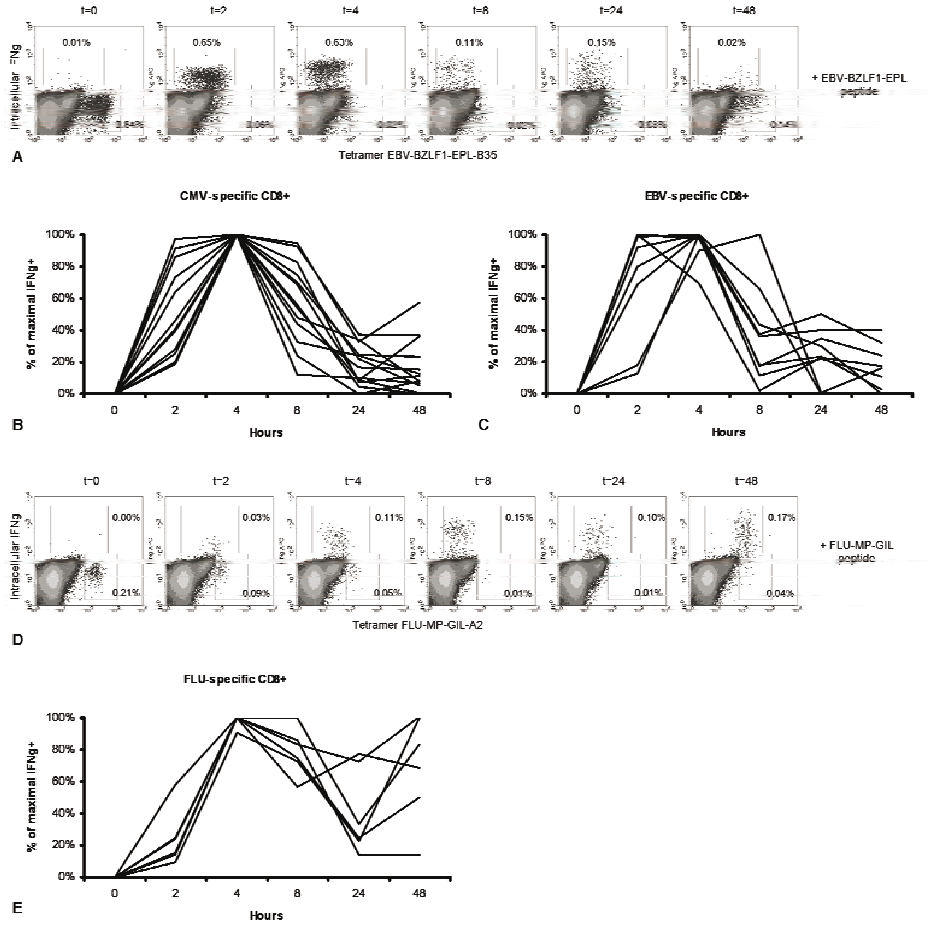


Figure 2. Kinetics of IFNγ production by virus-specific CD8+ T cells. Real-time intracellular IFNγ staining, with addition of BFA only during the last 2 hours of incubation, after stimulation of PBMC for 0, 2, 4, 8, 24, and 48 hours **(A)** with HLA-B35 restricted EBV-BZLF1-EPL peptide, **(B)** with minimal MHC class I binding CMV peptides (n=13), **(C)** with minimal MHC class I binding EBV peptides (n=9), **(D)** with HLA-A2 restricted FLU-MP-GIL peptide, or **(E)** with minimal MHC class I binding FLU peptides or FLU peptide pools (n=6). In (B/C/E), the frequency of IFNγ-positive T cells is expressed compared to the maximal frequency of IFNγ-positive T cells at one of the time points.

In line with the results from CD8+ T cells, CMV-specific CD4+ T cells showed a rapid induction of IFNγ production after stimulation with minimal MHC II binding peptides or CMV peptide pools, which peaked 4 hours after stimulation, and decreased thereafter (Figure 3A-B). After stimulation with the HAdV hexon peptide pool, HAdV-specific CD4+ T cells showed a slower induction of IFNγ production, which continued up to 48 hours after stimulation (Figure 3C-D). The frequencies of EBV- and FLU-specific CD4+ T cells in PBMC were not sufficiently high for kinetic analysis of IFNγ production.

These kinetic measurements illustrate a difference in kinetics of IFNγ production between T cells specific for the persistent viruses CMV and EBV, showing rapid and brief IFNγ production following activation, and T cells specific for HAdV and FLU which are not repetitively activated in vivo after initial infection, showing a more prolonged IFNγ production. Despite these kinetic differences, our data suggest that the majority of virus-specific T cells can be simultaneously isolated based on IFNγ production after 4 hours of peptide stimulation.

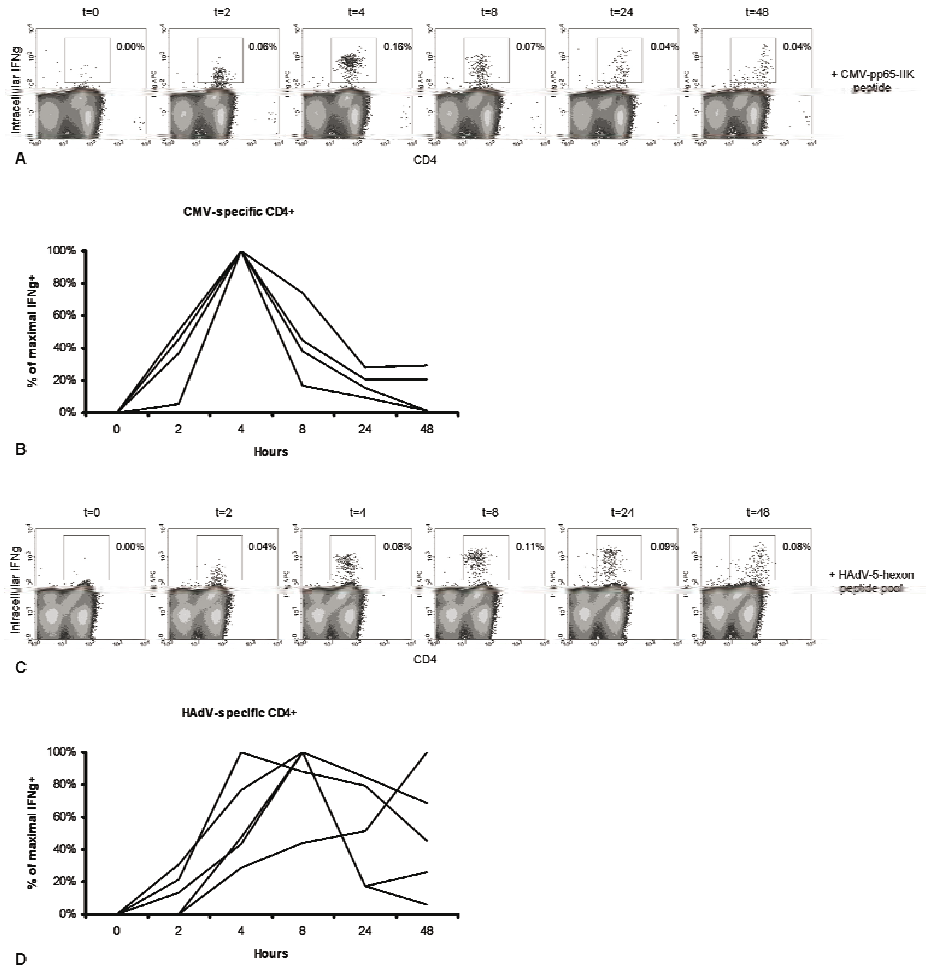


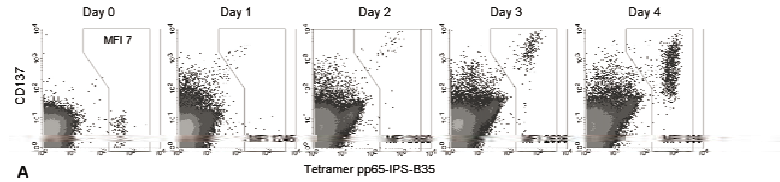
Figure 3. Kinetics of IFNγ production by virus-specific CD4+ T cells. Real-time intracellular IFNγ staining, with addition of BFA only during the last 2 hours of incubation, after stimulation of PBMC for 0, 2, 4, 8, 24, and 48 hours **(A)** with HLA-DR4 restricted CMV-pp65-IIK peptide, **(B)** with minimal MHC class II binding CMV peptides or CMV peptide pool (n=4), or **(C)** and **(D)** with HAdV 5 hexon peptide pool (n=5). In (B/D), the frequency of IFNγ-positive T cells is expressed compared to the maximal frequency of IFNγ-positive T cells at one of the time points.

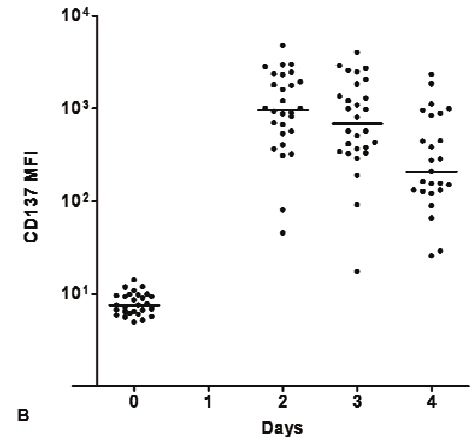
Kinetics of CD137 expression by virus-specific T cells

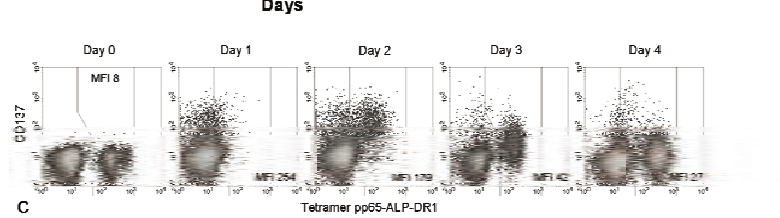
To investigate whether CD8+ and CD4+ T cells specific for different viruses can also be isolated from PBMC based on activation-induced CD137 expression, the expression and kinetics of CD137 after stimulation of T cells specific for CMV, EBV, HAdV, and FLU were determined. As shown for a representative example in Figure 4A, virus-specific CD8+ T cells expressed high levels of CD137 following peptide stimulation of PBMC directly ex vivo. Tetramer-positive CD8+ T cells expressed CD137 at day 1 after stimulation, but only part of the specific T cells could be discerned due to a decrease in tetramer staining. The expression of CD137 was shown to be maximal at day 2, and slowly decreased thereafter. As shown in Figure 4B, similar results were obtained for the CD8+ T cell populations specific for epitopes derived from CMV, EBV, HAdV, and FLU proteins (Table 1), demonstrating very low background CD137 expression before stimulation (day 0), and maximal mean fluorescence intensity (MFI) of CD137 staining at day 2 after stimulation. No CD137 MFI values are reported for day 1, since discrimination of specific T cells was not sufficient in most samples due to the decrease in tetramer staining.

Due to the lack of functional peptide-MHC class II tetramers, the specific expression of CD137 can not be analyzed for most virus-specific CD4+ T cell populations. The CMV pp65-ALP-DR1 and FLU HA-PKY-DR4 tetramers were available to stain virus-specific CD4+ T cells. However, the frequency of CD4+ T cells specific for these epitopes was not high enough for the analysis of CD137 expression in PBMC directly ex vivo. Therefore, a CMV pp65-ALP-specific CD4+ T cell clone was diluted in a PBMC background to analyze CD137 expression. After peptide stimulation, the CMV-specific CD4+ T cells showed CD137 expression, which also peaked at day 2, and decreased thereafter (Figure 4C). To investigate CD137 expression by virus-specific CD4+ T cells following stimulation of PBMC directly ex vivo, staining of CD4, CD25 and CD137 was performed to discern the specifically activated CD4+ T cells. As shown in Figure 4D, following stimulation of donor PBMC directly ex vivo with MHC II binding peptide KYQ, activated CD4+ T cells (CD25+CD137+) could be clearly observed from day 1 after stimulation. All CD137+CD4+T cells expressed high levels of CD25, indicating that these were the activated KYQ-specific CD4+ T cells, while most CD4+CD137- T cells did not express CD25. Similar results were obtained after stimulation of other CMV- and HAdV-specific CD4+ T cell populations (n=7, data not shown).

These data demonstrate that both CD8+ and CD4+ T cell populations recognizing the different viruses expressed CD137 upon antigen-specific stimulation, with uniform kinetics that resulted in maximal CD137 expression at day 1 to 2 after activation. Therefore, it is likely that the majority of virus-specific CD8+ and CD4+ T cells can also be simultaneously isolated based on activation-induced CD137 expression.







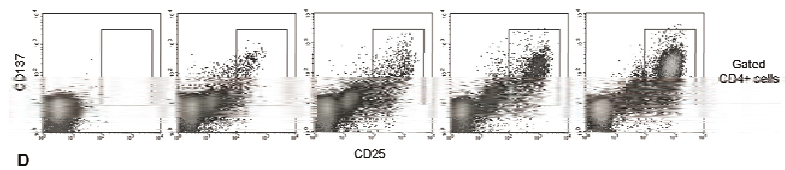


Figure 4. Kinetics of CD137 expression by virus-specific T cells. **(A)** Representative example of CD137 and tetramer staining of CD8+ T cells specific for CMV-pp65-IPS-B35 in donor PBMC and **(B)** the mean fluorescence intensity (MFI) of CD137 staining of peptide-MHC tetramer-positive CD8+ T cell populations (n=28) specific for CMV, EBV, HAdV, or FLU epitopes in donor PBMC before stimulation, and 2, 3, and 4 days after stimulation with minimal MHC I binding peptides (Table 1). Horizontal lines mark the median. **(C)** Representative example of CD137 and tetramer staining of a CD4+ T cell clone specific for CMV-pp65-ALP-DR1 mixed in PBMC as background before stimulation and 1, 2, 3, and 4 days after stimulation with the specific CMV-pp65 peptide. **(D)** Representative example of CD137 and CD25 staining of donor PBMC before stimulation, and 1, 2, 3, and 4 days after stimulation with HLA-DR52 binding CMV-pp65-KYQ peptide, gated on CD4+ cells.

Comparison between IFNγ-based and CD137-based isolation of virus-specific T cells

Since production of IFNγ and expression of CD137 was observed following stimulation of all virus-specific CD8+ and CD4+ T cells, both IFNγ-based and CD137-based enrichment may be used for isolation of CD8+ and CD4+ T cells specific for multiple viral antigens. To define the optimal procedure for isolation of different virus-specific CD8+ and CD4+ T cell populations, the efficiency of IFNγ-based and CD137-based isolation was compared at different time-points after activation. Donor PBMC were stimulated with a minimal MHC class I or II binding peptide derived from CMV, EBV, or FLU, followed by IFNγ-based or CD137-based isolation after 4, 24, 48, or 96 hours. After 8 days of subsequent culturing, peptide-MHC tetramer staining was performed to detect the frequencies of peptide-specific CD8+ or CD4+ T cells in the enriched and depleted fractions. An example of IFNγ-based isolation of virus-specific CD8+ T cells is depicted in Figure 5A, showing high frequencies of FLU-specific CD8+ T cells in the fractions isolated after 4, 24, or 48 hours of stimulation, while a lower frequency of specific T cells was observed in the fraction isolated after 96 hours of stimulation. Figure 5B shows the corresponding fractions after CD137-based isolation, with a low frequency of FLU-specific CD8+ T cells in the fraction isolated after 4 hours of stimulation, and high frequencies of specific T cells in the fractions isolated after 24, 48, or 96 hours of stimulation. Both the IFNγ-depleted and CD137-depleted fractions contained low frequencies of residual peptide-specific CD8+ T cells. As shown in Figure 5C-D, both IFNγ-based and CD137-based isolation resulted in enrichment of virus-specific CD4+ T cells as well. The highest frequencies of CMV-specific CD4+ T cells in both IFNγ-enriched fractions and CD137-enriched fractions were observed after 48 or 96 hours of stimulation (Figure 5C-D). The IFNγ-depleted and CD137-depleted fractions contained low frequencies of residual peptide-specific CD4+ T cells. The results of IFNγ-based and CD137-based isolation of 6 different virus-specific CD8+ and CD4+ T cell populations are summarized in Figure 5E-F. The highest frequencies of virus-specific CD8+ and CD4+ T cells were observed after 24 or 48 hours of peptide stimulation followed by either IFNγ-based or CD137-based isolation (Figure 5E). Consistently, the highest absolute numbers of virus-specific CD8+ and CD4+ T cells were observed in these isolated fractions (Figure 5F). All samples were analyzed following the same time interval after initial stimulation, and also following the same time interval after isolation, to compensate for differences in the length of initial stimulation (4 to 96 hours). Similar results were obtained, indicating that the differences observed resulted from differences in isolation efficiency and did not result from differences in culture period. The modest frequencies of specific CD4+ T cells after isolation may be explained by the very low frequencies of CD4+ T cells specific for these selected epitopes in donor PBMC, which could not be detected by tetramer staining before isolation.

These data demonstrate that stimulation of donor PBMC with viral peptides for 24 or 48 hours followed by either IFNγ-based or CD137-based enrichment can be used for the efficient isolation of CD8+ and CD4+ T cells specific for multiple viral epitopes.

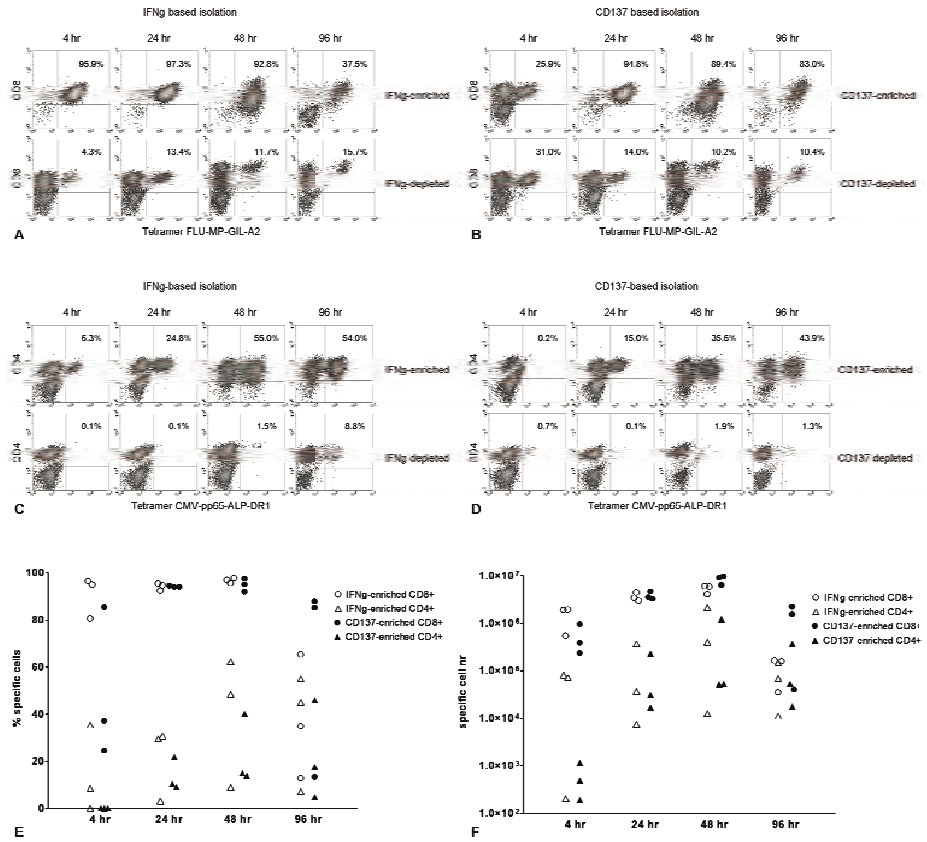


Figure 5. Comparison between IFNγ-based and CD137-based isolation of virus-specific T cells. Donor PBMC were stimulated for 4, 24, 48, or 96 hours with minimal MHC I binding CMV-pp65-NLV, EBV-BZLF1-RAK, or FLU-MP-GIL peptide, or MHC II binding CMV-pp65-ALP or FLU-HA-PKY peptide, followed by IFNγ-based isolation or CD137-based isolation. After 8 days of culture, the frequency of peptide-specific CD8+ or CD4+ T cells was determined by peptide-MHC tetramer staining in the enriched and depleted fractions. **(A)** Example of the tetramer-staining of FLU-MP-GIL-A2-specific CD8+ T cells after 8 days of culture of the IFNγ-enriched and IFNγ-depleted fractions, and **(B)** of the CD137-enriched and CD137-depleted fractions. **(C)** Example of the tetramer-staining of CMV-pp65-ALP-DR1-specific CD4+ T cells after 8 days of culture of the IFNγ-enriched and IFNγ-depleted fractions, and **(D)** of the CD137-enriched and CD137-enriched fractions. **(E)** The frequency and **(F)** absolute number of peptide-specific CD8+ T cells (circles) or CD4+ T cells (triangles) after 8 days of culture in the IFNγ-enriched fractions (open symbols) or CD137-enriched fractions (black symbols).

Generation of multivirus-specific CD8+ and CD4+ T cell lines

Since separate CD8+ and CD4+ T cell populations specific for viral epitopes were efficiently isolated, we next investigated whether CD8+ and CD4+ T cells specific for epitopes from multiple viral proteins could be isolated simultaneously. PBMC from 4 healthy CMV- and EBV-seropositive donors were stimulated with a combination of 3 peptide pools spanning viral proteins derived from CMV, EBV, HAdV, or FLU. After 24 or 48 hours of incubation, IFNγ-based or CD137-based isolation was performed, and the enriched fractions were cultured with IL-2 and IL-15. After 8 days of culture, the T cell lines contained 44-94% CD8+ T cells and 1-33% CD4+ T cells. The specificity of the T cell lines was analyzed by staining with peptide-MHC tetramers for immunodominant viral epitopes (Table 1) and by intracellular cytokine staining upon restimulation with the viral protein-spanning peptide pools. The overall specificity of the T cell lines was high, since a median of 84% of CD8+ T cells (range 56-100%) and 46% of CD4+ T cells (range 29-84%) were specific for the 3 viral proteins. As shown in Figure 6A-D, no significant differences in frequencies of virus-specific CD8+ and CD4+ T cells were observed between the T cell lines generated by IFNγ-based or CD137-based isolation after 24 or 48 hours of incubation. Although dominant virus-specific T cell populations were present in the T cell lines, CD8+ and CD4+ T cells specific for each of the 3 viral proteins could be detected. For example, the T cell lines described in Figure 6C contained predominantly CMV-specific CD8+ T cells directed against CMV pp65-NLV-A2 and CMV pp65-IPS-B35, but smaller populations of CD8+ T cells specific for EBV EBNA1-HPV-B35 and HAdV hexon-MPN-B35 could also be detected (Figure 6E). Likewise, the T cell lines described in Figure 6D contained mainly EBV-specific CD8+ T cells directed against EBV BZLF1-EPL-B35, but other CD8+ T cell populations were specific for EBV EBNA1-HPV-B35 and EBV EBNA3A-YPL-B35 (Figure 6F). Furthermore, no functional differences were observed between T cells isolated based on IFNγ or CD137 after 24 or 48 hours of incubation (Figure 6G). Specific T cells produced IFNγ and part of the cells additionally produced TNFα, IL-2 or showed degranulation by surface expression of CD107a, which was similar to the T cells in the starting material. Low frequencies of residual virus-specific CD8+ and CD4+ T cells were detected in the IFNγ-depleted and CD137-depleted fractions.

These data demonstrate that stimulation of donor PBMC with viral protein-spanning peptide pools followed by either IFNγ-based or CD137-based enrichment can be used to generate T cell lines for adoptive immunotherapy containing high frequencies of CD8+ and CD4+ T cells specific for multiple viral proteins.

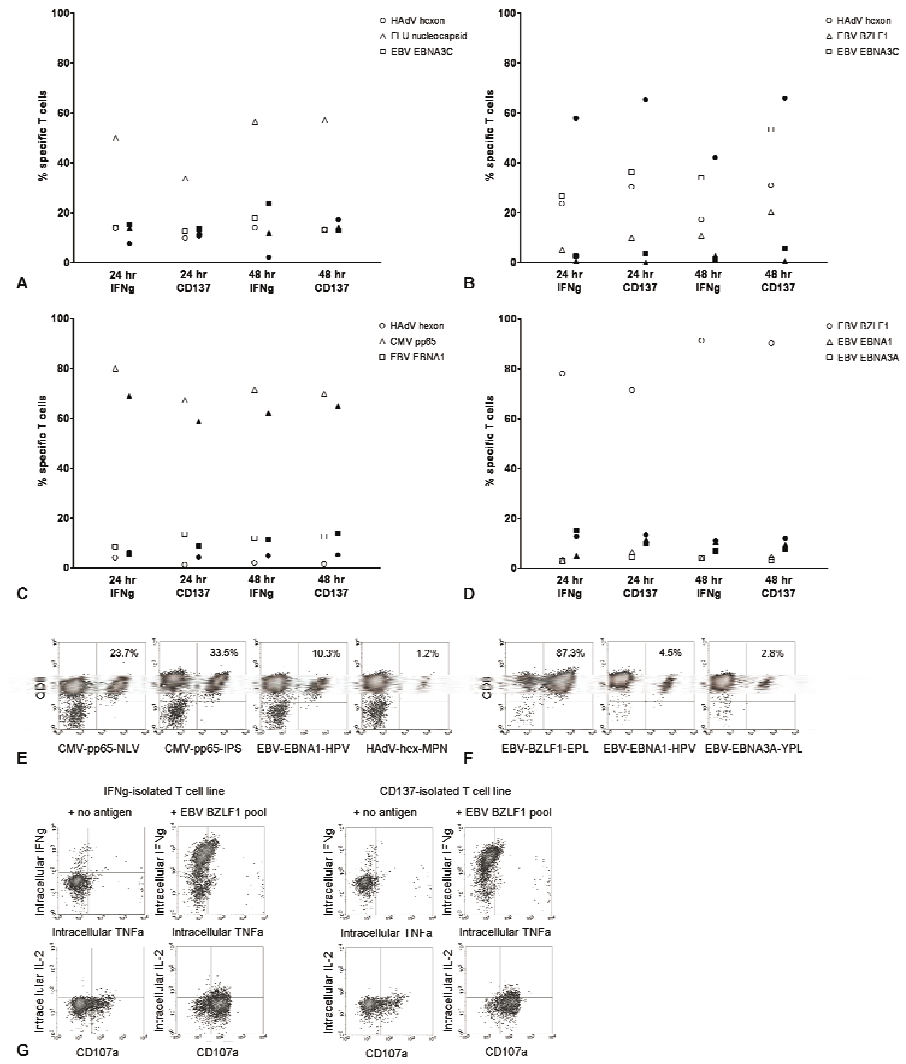


Figure 6. Generation of multivirus-specific CD8+ and CD4+ T cell lines. **(A-D)** PBMC from 4 healthy donors were stimulated for 24 or 48 hours with 15-mer peptide pools spanning 3 proteins derived from CMV, EBV, HAdV, or FLU, followed by IFNγ-based isolation or CD137-based isolation. After 8 days of culture, the frequency of T cells specific for each of the 3 viral proteins was determined by peptide-MHC tetramer staining and by intracellular IFNγ-staining upon restimulation with the viral peptide pools. The frequency of specific cells among CD8+ T cells (open symbols) and among CD4+ T cells (black symbols) are shown for each of the 3 viral proteins. **(E-F)** Peptide-MHC tetramer staining of multiple virus-specific CD8+ T cell populations in the T cell lines shown in (C-D), generated by CD137-based isolation after 48 hours of stimulation. **(G)** Intracellular staining of IFNγ, TNFα, IL-2, and surface CD107a staining without restimulation and after EBV BZLF1 peptide pool restimulation of the T cell lines shown in (D), generated by IFNγ-based or CD137-based isolation after 48 hours of stimulation.

Discussion

In this study, we developed an efficient method for the simultaneous isolation of CD8+ and CD4+ T cells specific for a broad repertoire of viral antigens from peripheral blood, which may be used for adoptive transfer to alloSCT recipients to provide antiviral immune reconstitution. To ensure that the method was applicable to a wide variety of virus-specific T cells that may differ in phenotypic and functional properties, we focused on T cells specific for the persistent viruses CMV and EBV, as well as T cells specific for HAdV and FLU, which are not repetitively activated in vivo after initial viral clearance. Phenotypic analysis of the different virus-specific CD8+ T cells showed heterogeneous CD8+ T cell populations, containing diverse T cell subsets. The CMV-specific CD8+ T cell populations contained more CD27- and CD28- T cells compared to EBV-, HAdV- and FLU-specific CD8+ T cells. Functional analysis did not show a correlation between the phenotype or specificity of the CD8+ T cells and their functional profile. Nearly all virus-specific CD8+ and CD4+ T cells produced both IFNγ and TNFα upon activation. However, differences were observed in the kinetics of IFNγ production upon activation, which appeared to correlate with specificity. CMV- or EBV-specific T cells showed a rapid and brief IFNγ production, while T cells specific for HAdV or FLU showed a more prolonged IFNγ production. The more differentiated phenotype of CMV-specific T cells is consistent with previously published data, and may result from frequent antigenic activation in vivo due to viral reactivations [38]. Likewise, the difference in kinetics of IFNγ production may result from the repetitive antigenic activation of CMV- and EBV-specific T cells in vivo due to viral persistence, while HAdV- and FLU-specific T cells are not repetitively activated in vivo.

Both CD8+ and CD4+ virus-specific T cells were shown to express high levels of CD137 after antigen-specific activation. The kinetics of CD137 expression was uniform, with CD137 expression from 24 hours to more than 96 hours after stimulation, and peak expression at 48 hours after stimulation. Previous studies have reported maximal CD137 expression on cultured T cell lines and clones recognizing various antigens after 24 hours of stimulation, which decreased thereafter [32,34,35]. In contrast to these studies, we analyzed CD137 expression on virus-specific T cells activated in PBMC directly ex vivo, which may explain the difference in CD137 kinetics observed. Although it has been previously shown that CD4+ T cells specific for CMV pp65 could be isolated based on CD137 expression [33], another study has reported very low CD137 expression on activated CD4+ T cells specific for EBV EBNA1, which was not sufficient for isolation [34]. We here demonstrated high levels of CD137 expressed by CMV- and HAdV-specific CD4+ T cells following activation. The frequencies of EBV- and FLU-specific CD4+ T cells in PBMC were not sufficiently high for

analysis of CD137 expression directly ex vivo. However, CD137-based isolation of virus-specific CD4+ T cells confirmed CD137 expression by CD4+ T cells specific for CMV, EBV, HAdV, and FLU epitopes, including epitopes derived from the EBV EBNA1 protein.

Based on the analysis of IFNγ production and CD137 expression, we anticipated efficient isolation of the virus-specific CD8+ and CD4+ T cell populations using either IFNγ-based or CD137-based isolation, although CD137-based isolation might be superior due to more uniform kinetics. Indeed, both methods of enrichment resulted in high frequencies and absolute numbers of virus-specific T cells in the isolated fractions. CD137-based isolation was most efficient after 24 or 48 hours of stimulation, which was consistent with the kinetics of CD137 expression. However, IFNγ-based isolation also resulted in highest frequencies and numbers of virus-specific T cells in the isolated fractions after 24 or 48 hours of stimulation, while the kinetic analysis showed maximal IFNγ production after 4 to 8 hours of stimulation for most T cell populations. The detection of IFNγ production by intracellular staining has previously been shown to be synchronous with the detection of IFNγ secretion by the IFNγ capture assay, which is used for IFNγ-based isolation [31]. Apparently, the virus-specific T cells that produce IFNγ after 24 to 48 hours are the cells that can be expanded most efficiently, providing high numbers of virus-specific T cells in the final product. Although, both IFNγ-based and CD137-based isolation were demonstrated to result in efficient enrichment of CD8+ and CD4+ T cells specific for multiple viral epitopes from peripheral blood, the simple CD137 labeling procedure has a logistic advantage over the more laborious IFNγ labeling procedure. This may save costs of training personnel and of reagents and may make the treatment strategy more widely applicable. However, only IFNγ-based isolation is currently available for the generation of clinical grade virus-specific T cell lines.

Previous studies have used EBV-LCL transduced with cDNA encoding additional viral proteins as stimulator cells to generate multivirus-specific T cell lines [13,28,39,40]. Although the different approaches can not be directly compared, the efficiency of isolation of CD8+ and CD4+ T cell populations against multiple viral proteins using the method described in this study appears to be at least similar. The availability of clinical grade synthetic protein-spanning pools of overlapping peptides and IFNγ-based or CD137-based isolation kits will allow the generation of T cell lines with high specificity against multiple viral proteins in a large number of treatment centers.

This study provides an efficient and widely applicable method for the simultaneous isolation of both CD8+ and CD4+ T cells specific for a broad repertoire of antigens from multiple viral pathogens from donor peripheral blood. These multivirus-specific T cell lines may be adoptively transferred to alloSCT recipients for prophylaxis of viral disease.

Alternatively, selected viral protein-spanning peptide pools can be used for isolation and adoptive transfer of CD8+ and CD4+ T cells specific for a single virus for treatment of refractory viral disease after alloSCT.

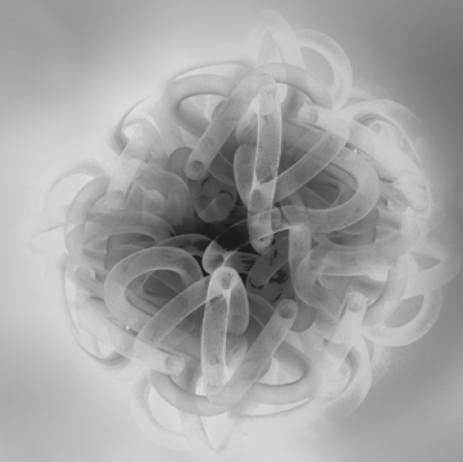
Acknowledgements

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6

Efficiency and mechanism of antigen-specific CD8+ T cell activation using synthetic long peptides

Maarten L. Zandvliet
Michel G.D. Kester
Ellis van Liempt
Arnoud H. de Ru
Peter A. van Veelen
Marieke Griffioen
Henk-Jan Guchelaar
J.H. Frederik Falkenburg
Pauline Meij

Submitted

Abstract

Synthetic long peptides that contain immunogenic T cell epitopes have been used to induce activation of antigen-specific CD8+ T cells in vitro for immune monitoring or adoptive transfer, or in vivo following peptide vaccination. However, the efficiency and mechanisms of presentation of exogenous long peptides in HLA class I remain to be elucidated. In this study, we demonstrated that the efficiency of antigen-specific CD8+ T cell activation using extended peptide variants of common viral epitopes is variable. We demonstrated that processing and HLA class I presentation of the long peptides was not dependent on the proteasome and TAP, illustrating that the classical route of HLA class I presentation was not required for activation of specific CD8+ T cells by exogenous synthetic long peptides. Although long peptides were shown to bind to the relevant HLA class I molecules, peptide trimming was likely to be essential for optimal HLA class I presentation and T cell activation. Since the proteasome was not required for processing of exogenous peptides, it is very likely that peptide trimming was mediated by peptidases, which may be located either extracellularly at the cell surface, or in endosomal compartments. Furthermore, the results suggested that processing of the correct minimal peptides was facilitated by binding in HLA class I molecules. This mechanism of HLA-guided processing may be important in HLA class I presentation of exogenous long peptides to induce activation of specific CD8+ T cells.

Introduction

The availability of labeled peptide-HLA class I multimers has facilitated the detection of antigen-specific CD8+ T cells in peripheral blood mononuclear cells (PBMC) for immune monitoring, as well as the isolation of antigen-specific CD8+ T cells from PBMC for adoptive immunotherapy [1,2]. However, this technique requires knowledge of the minimal HLA class I binding peptides recognized, and production of a large number of peptide-HLA multimers to cover CD8+ T cell responses directed against multiple epitopes. Furthermore, antigen-specific CD4+ T cells can not be identified using this method due to the lack of functional peptide-HLA class II multimers. To detect or isolate antigen-specific T cells without the need of knowing the exact epitopes, stimulation of PBMC with whole proteins can be performed, after which the responding T cells can be identified based on their activation-induced effector functions. Following antigen-specific stimulation, activated T cells are routinely detected by analysis of cytokine production using intracellular staining, surface mobilization of the degranulation marker CD107a, expression of the co-stimulatory activation markers CD40L or CD137, or proliferation by dilution of dyes such as carboxyfluorescein diacetate succinimidyl ester (CFSE) [3-6]. Furthermore, isolation of antigen-specific T cells based on activation-induced IFNg production or CD40L or CD137 expression is widely applied [7-9].

The use of whole proteins for stimulation of PBMC circumvents the need of knowing the exact epitopes, and may induce activation of antigen-specific CD8+ and CD4+ T cells irrespective of the HLA type. In contrast to the use of full-length recombinant proteins, pools of overlapping synthetic long peptides consisting of 15-20 amino acids have been demonstrated to efficiently induce activation of both CD8+ and CD4+ antigen-specific T cells in PBMC [10-14]. Currently, a wide variety of overlapping peptide pools spanning whole infectious and tumor-associated proteins is being used for the detection and isolation of CD8+ and CD4+ antigen-specific T cells [11-20]. The processing of exogenous antigen followed by presentation in HLA class II has been extensively described, and the exogenously added long peptides may also directly bind in HLA class II molecules at the cell surface [21]. However, the pathways that lead to processing and presentation of exogenously added long peptides in HLA class I are not clear.

Synthetic long peptides have also been used to induce or boost antigen-specific T cell responses in vivo by peptide vaccination. Superior in vivo CD8+ T cell responses have been reported following vaccination with long peptides compared with the corresponding minimal HLA class I binding peptides, which was attributed to selective uptake, processing, and presentation by professional antigen-presenting cells (APCs) [22-25]. The selective

presentation of long peptides in the stimulatory context of activated professional APCs in the draining lymph node was suggested to result in optimal induction of a specific CD8+ T cell response. In contrast, vaccination with minimal HLA class I binding peptides resulted in systemic presentation by non-professional APCs, and suboptimal development of specific CD8+ T cells. A tolerizing effect of vaccination with minimal HLA class I binding peptides has been described before [26]. Although these data suggest that long peptides are preferentially processed and presented by professional APCs, it has been demonstrated that exogenous long peptides can also be presented in HLA class I by non-professional APCs [27]. The capacity to internalize, process, and present synthetic long peptides has not been directly compared between different types of APC. The mechanisms that result in HLA class I presentation in vivo after vaccination with long peptides remain to be elucidated [28].

Various mechanisms explaining the presentation of exogenous long peptides in HLA class I molecules have been proposed. Elution of endogenously synthesized and processed peptides from surface HLA class I molecules has demonstrated the presentation of different length variants of minimal HLA class I binding motifs extended at the N- or C-terminus, indicating that some long peptide variants may directly bind in HLA class I [29-33]. Long peptides that bind in HLA class I molecules have been shown to adapt a centrally bulged conformation, or to protrude from one side of the peptide binding groove [34-38]. Furthermore, degradation of exogenous long peptides by serum peptidases or membrane-bound peptidases may allow extracellular binding of trimmed peptides to cell surface HLA class I molecules [39-42]. Binding of long peptides in HLA class I molecules and peptidase editing can also occur simultaneously. The binding of long peptide variants in HLA class I may even facilitate the processing of the correct minimal peptide bound in the HLA class I groove, which has been described before as the model of HLA-guided processing [43]. Alternatively, following uptake of exogenous long peptides, transport over endosomal membranes to the cytosol may result in the classical route of presentation which is dependent on proteasomal degradation and the transporter associated with antigen processing (TAP) [44]. Internalized peptides may also enter endosomal compartments which have fused with the endoplasmic reticulum (ER), and therefore contain the machinery for peptide loading of HLA class I molecules [45]. Finally, entrance of HLA class I molecules in the recycling endocytic HLA class II pathway has been demonstrated to allow exchange with exogenous peptides, which can be edited by endosomal peptidases [46-49].

In this study, we demonstrated that the efficiency of CD8+ T cell activation by several extended variants of minimal HLA class I binding peptides was highly variable. Since processing and HLA class I presentation was demonstrated not to be dependent on the proteasome and TAP, our data illustrated that the classical route of HLA class I presentation

was not required for activation of specific CD8+ T cells by exogenous synthetic long peptides. Although long peptides were shown to bind to the relevant HLA class I molecules, peptide trimming was likely to be required for optimal HLA class I presentation and T cell activation. The results indicated that HLA-guided processing may be important in HLA class I presentation of exogenous long peptides to allow activation of specific CD8+ T cells.

Materials and methods

Synthetic peptides

Minimal HLA class I binding peptides corresponding to previously described cytomegalovirus (CMV) epitopes were synthesized. In addition, extended peptide variants of these minimal HLA class I binding peptides were constructed by addition of 6 naturally flanking amino acids at the N-terminal end, at the C-terminal end, or at both ends. Peptide sequences are shown in Table 1. The peptides were synthesized using standard solid-phase strategies (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands).

T cells and antigen-presenting cells

After informed consent, peripheral blood was obtained from healthy CMV-seropositive individuals, and mononuclear cells (PBMC) were isolated by Ficoll-Isopaque separation. To generate CMV peptide-specific CD8+ T cell lines, peptide-HLA class I tetramers containing minimal CMV peptide were used to stain CMV-specific CD8+ T cells in donor PBMC. Subsequently, the CMV peptide-HLA tetramer-positive CD8+ T cells were selected by flow cytometric sorting using a FACSDiva and Cellquest software (BD Biosciences, San Jose, CA, USA). The peptide-specific CD8+ T cells were cultured in culture medium, consisting of Iscove's modified Dulbecco's medium (IMDM, Lonza, Basel, Switzerland) supplemented with 5% pooled human serum, 5% fetal calf serum (Lonza), 100 U/ml penicillin/streptomycin (Lonza), 3 mM L-glutamine (Lonza), and 100 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands). Stable Epstein-Barr virus (EBV)-transformed B cell lines (EBV-LCL) were generated using standard procedures [50]. The transporter associated with antigen processing (TAP)-competent T1 and TAP-deficient T2 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) [51,52].

Table 1. Minimal peptides and extended peptide variants with 6 naturally flanking amino acids at the N-terminal end (6-PEP), at the C-terminal end (PEP-6), or at both ends (6-PEP-6).

Source protein	HLA-restriction	Peptide	Sequence
CMV pp65	HLA-A1	YSE	YSEHPTFTSQY
		6-YSE	LQRGPQYSEHPTFTSQY
		YSE-6	YSEHPTFTSQYRIQGKL
		6-YSE-6	LQRGPQYSEHPTFTSQYRIQGKL
CMV pp65	HLA-A2	NLV	NLVPMVATV
		6-NLV	AGILARNLVPMVATV
		NLV-6	NLVPMVATVQGQNLK
		6-NLV-6	AGILARNLVPMVATVQGQNLK
CMV IE1	HLA-A2	VLE	VLEETSVML
		6-VLE	RVLCCYVLEETSVML
		VLE-6	VLEETSVMLAKRPLI
		6-VLE-6	RVLCCYVLEETSVMLAKRPLI
CMV pp65	HLA-B7	TPR	TPRVTGGGAM
		6-TPR	VTTERKTPRVTGGGAM
		TPR-6	TPRVTGGGAMAGASTS
		6-TPR-6	VTTERKTPRVTGGGAMAGASTS
CMV pp65	HLA-B7	RPH	RPHERNGFTVL
		6-RPH	NPQPFMRPHERNGFTVL
		RPH-6	RPHERNGFTVLCPKNMI
		6-RPH-6	NPQPFMRPHERNGFTVLCPKNMI
CMV IE1	HLA-B8	ELR	ELRRKMMYM
		6-ELR	ARAKKDELRRKMMYM
		ELR-6	ELRRKMMYMCYRNIE
		6-ELR-6	ARAKKDELRRKMMYMCYRNIE
CMV IE1	HLA-B8	QIK	QIKVRVDMV
		6-QIK	LAELVKQIKVRVDMV
		QIK-6	QIKVRVDMVRHRIKE
		6-QIK-6	LAELVKQIKVRVDMVRHRIKE

Analysis of specific CD8+ T cell activation

As stimulator cells, $1.0x10^3$ to $3.0x10^4$ EBV-LCL were loaded with 10^{-5} to 10^{-12} M peptide for 4 hours in serumfree medium, consisting of IMDM supplemented with 2% human albumin (Octapharma, Langenfeld, Germany). Subsequently, the stimulator cells were incubated with $5.0x10^3$ peptide-specific CD8+ T cells. After overnight incubation, supernatant was harvested and IFNγ production was analyzed by enzyme-linked immunosorbent assay (ELISA, CLB, Amsterdam, The Netherlands). The peptide concentration that resulted in half of the maximal IFNγ signal was defined as the half maximal effective concentration EC_{50}. To analyze proteasome dependency, stimulator cells were incubated with 10^{-5} M lactacystin (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 2 hours before and during peptide loading.

Mass spectrometry and HPLC fractionation

Stock solutions of 10^{-2} M peptide in dimethylsulfoxide (DMSO, Lonza) were diluted to 10^{-12} M and analyzed by high resolution mass spectrometry (LTQ-FT Ultra, Thermo, Bremen, Germany). In addition, peptides were purified by high-performance liquid chromatography (HPLC) as described before [53]. Briefly, peptide stock solutions were injected on a HPLC system (Agilent, Amstelveen, The Netherlands) and subjected to reversed-phase HPLC on a home-made 10 cm x 2.1 mm Reprosil-Pur C18-AQ 3 μm nano HPLC column (Dr. Maisch GmbH, Ammerbruch, Germany). A gradient from 20% to 60% acetonitrile containing 0.1% trifluoric acetic acid was run at 0.1 ml/min, while fractions of 1.0 minute were collected in 96 wells plates. The collected HPLC fractions were loaded on EBV-LCL to analyze activation of peptide-specific CD8+ T cells.

HLA class I binding assay

The efficiency of peptide binding in HLA class I molecules was analyzed by competition between the test peptide and a fluorescently labeled reference peptide for binding HLA class I monomers as described before [54]. Briefly, 10^{-4} to 10^{-12} M test peptide and 10^{-10} M fluorescently labeled strongly binding reference peptide were added to HLA class I heavy chain and beta-2-microglobulin to allow folding of stable peptide-HLA monomers. Peptide-HLA monomers were purified by size-exclusion chromatography and fluorescent signal was measured. The concentration of test peptide that resulted in a decrease of half of the fluorescent signal was defined as the half maximal inhibitory concentration IC_{50}.

Elution of peptides from HLA

To verify the content of the HLA molecules in the stable peptide-HLA monomers, 200 pmol of peptide-HLA monomers were acidified with 10% acetic acid and peptides were separated from proteins by size-exclusion centrifugation (Amicon Ultrafree-MC 5 kDa, Millipore, Billerica, MA, USA). Peptide concentrates were injected on the reversed phase HPLC system run, while fractions of 0.5 minute (flow 0.2 ml/min) were collected in siliconized vials and stored at -80°C. The collected HPLC fractions were loaded on EBV-LCL to analyze activation of peptide-specific CD8+ T cells. To confirm peptide sequences by mass spectrometry, reactive fractions were injected on a home-made 20 cm x 50 μm Reprosil-Pur C18-AQ 3 μm nano HPLC column. Subsequently, the eluent was sprayed into the LTQ-FT Ultra mass spectrometer to acquire full scan mass spectra. The selected ions were then fragmented in the linear ion trap using collision-induced dissociation.

Transduction of minigenes for analysis of endogenous peptide expression

To allow endogenous expression of peptide sequences, retroviral vectors were constructed encoding the minimal or extended peptide variants listed in Table 1 fused to the C-terminus of ubiquitin, which results in efficient liberation of the exact peptide sequence as described previously [55,56]. Briefly, plasmid encoding for ubiquitin with SIINFEKL peptide fused to the C-terminus was kindly provided by Dr. J. Neefjes (Divison of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). From this template, oligonucleotides were produced using a ubiquitin forward primer and ubiquitin-overlapping peptide-encoding reverse primers. These ubiquitin-peptide-encoding oligonucleotides were cloned into the retroviral MP71 vector, containing the truncated human nerve growth factor receptor (NGFR) selection marker gene linked by an IRES sequence, as described previously [57]. Retroviral vector supernatants were produced by transfecting packaging F-NX-A cells with retroviral vectors. Subsequently, EBV-LCL, T1, or T2 cells were transduced with retroviral vector supernatants. The cells were stained with PE-labeled NGFR monoclonal antibodies (BD) for 30 minutes at 4°C and the NGFR-positive cells were selected by flow cytometric sorting using a FACSDiva and Cellquest software (BD).

Results

Activation of specific CD8+ T cells using synthetic long peptides

To investigate the capacity of synthetic long peptides to induce activation of peptide-specific CD8+ T cells, extended peptide variants were produced of immunodominant minimal HLA class I binding peptides by addition of 6 naturally flanking amino acids to the N-terminal end, to the C-terminal end, or to both ends (Table 1). EBV-LCL were loaded with 10^{-5} to 10^{-12} M of the minimal HLA class I binding peptides or the long peptide variants. The experiments were performed in serumfree medium to exclude peptide modification by serum peptidase activity. After 4 hours of incubation, peptide-specific CD8+ T cells were added at an effector to stimulator ratio of 1:6 for 16 hours, and the concentration of IFNγ was analyzed in supernatant to determine specific CD8+ T cell activation. As shown for the HLA-A2 restricted CMV pp65-derived NLV epitope in Figure 1A, the minimal HLA class I binding peptide NLV was most efficient in CD8+ T cell activation, resulting in half of maximal IFNg signal at a peptide concentration of $2.4x10^{-10}$ M, which was defined as the half maximal effective concentration EC_{50}. The N-terminally extended peptide 6-NLV was slightly less efficient in CD8+ T cell activation, with a 10-fold increase of the EC_{50} to $2.4x10^{-9}$ M. Extending the minimal peptide with 6 amino acids at the C-terminus or at both ends (NLV-6 or 6-NLV-6) resulted in a low efficiency of CD8+ T cell activation, as indicated by a 4,000- and 25,000-fold (EC_{50} $1.0x10^{-6}$ M and $5.9x10^{-6}$ M) increase of the EC_{50} compared to the minimal peptide, respectively.

The results for 7 viral epitopes are depicted in Figure 1B, showing a large variability in the efficiency of CD8+ T cell activation after incubation with the extended peptide variants, ranging from efficiencies that are similar to the minimal peptide (for example 6-YSE: no increase in EC_{50}) to very low activation efficiencies (for example 6-ELR-6: 75,000-fold increase in EC_{50}). For each epitope tested, the N-terminally extended peptide induced CD8+ T cell activation more efficiently compared to the C-terminally extended peptide, and the peptides extended at both ends showed lowest efficiency of T cell activation. These data demonstrate that all extended peptide variants of HLA class I binding epitopes could result in activation of specific CD8+ T cells. However, the efficiency of CD8+ T cell activation was highly variable, and appeared to be better for N-terminally extended peptides than for C-terminally extended peptides.

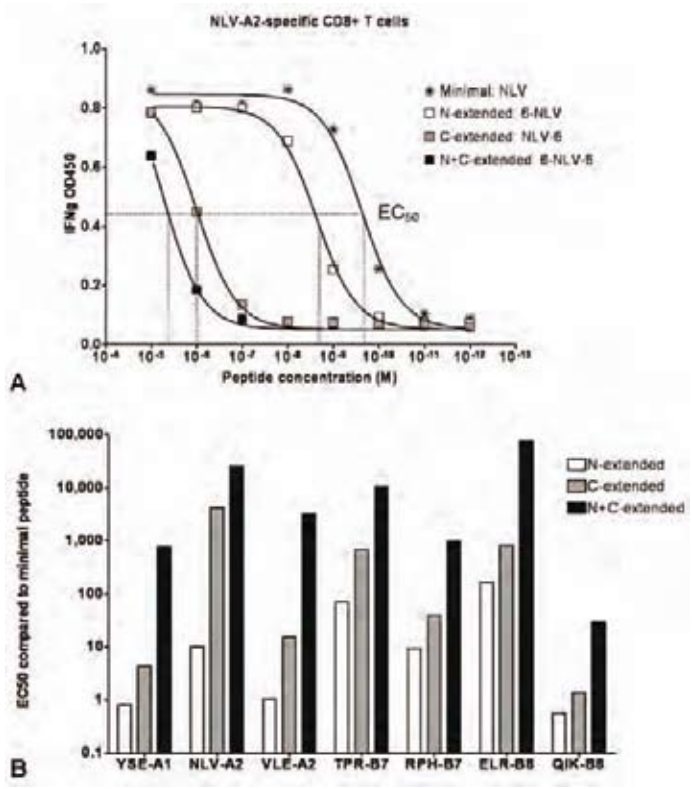


Figure 1. Activation of peptide-specific CD8+ T cells with minimal and N- and C-terminally extended peptides. **(A)** The concentration of IFNγ in supernatant is shown after incubation of NLV-specific CD8+ T cells with EBV-LCL loaded with 10^{-5} to 10^{-12} M of the HLA-A2 binding peptide NLV and extended NLV peptide variants. The curves were used to determine the half maximal effective concentration EC_{50} for each peptide variant. **(B)** The EC_{50} values for peptide variants corresponding to 7 viral epitopes are presented as compared to the EC_{50} value of the minimal peptide.

Absence of contaminating shorter peptide fragments

To exclude the possibility that contaminating shorter peptide fragments, which may have been produced during peptide synthesis, contributed to the activation of the antigen-specific CD8+ T cells, the presence of shorter peptides in the stock solutions of the extended peptide variants was investigated. Analysis by high resolution mass spectrometry demonstrated that all stock solutions contained the correct peptide sequences. No traces of minimal peptide were detected in any of the extended peptide stock solutions (data not shown).

To confirm that the extended peptides could induce CD8+ T cell activation, the minimal and extended peptides containing the NLV epitope were injected on a HPLC system, and eluted

fractions were collected to analyze CD8+ T cell reactivity. As shown in Figure 2A, the minimal peptide NLV eluted after 45 minutes, and was collected in fraction 14 and 15. After loading of the eluted fractions on HLA-A2-positive EBV-LCL, fractions 14 and 15 were recognized by NLV-A2-specific CD8+ T cells (Figure 2C). As shown in Figure 2B, the N-terminally extended peptide 6-NLV eluted after 49 minutes, and was collected in fraction 20. Figure 2D shows that EBV-LCL loaded with fraction 20 were indeed recognized by NLV-A2-specific CD8+ T cells. The presence of the correct sequences and the absence of shorter contaminating peptide fragments in the eluted fractions was confirmed by mass spectrometry (data not shown). These data demonstrate that CD8+ T cell activation resulted from stimulation with the synthetic long peptides, and was not due to the presence of contaminating shorter peptides.

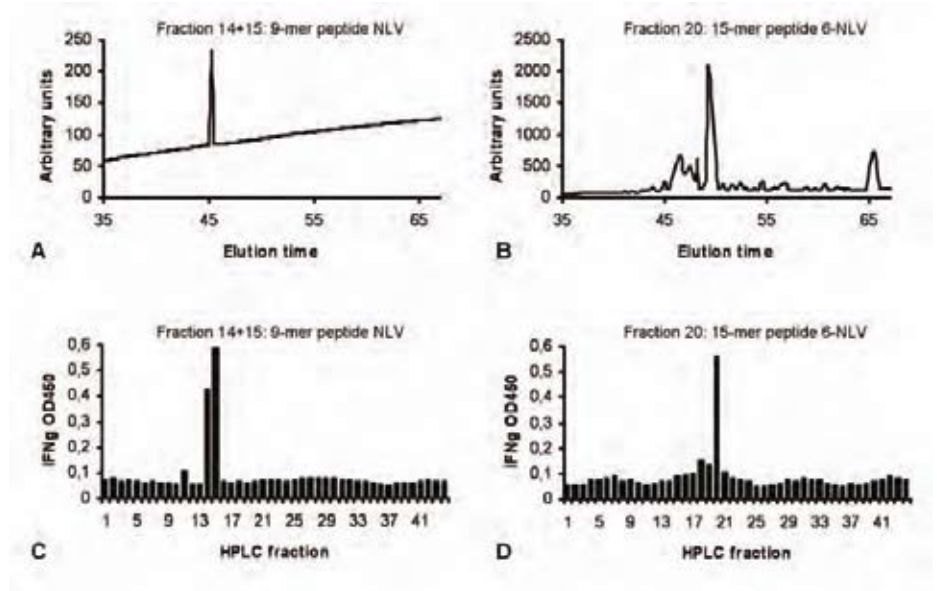


Figure 2. Absence of contaminating shorter peptide fragments. **(A)** Detection of the minimal peptide NLV and **(B)** the N-terminally extended peptide 6-NLV during fractionation on a HPLC system. **(C)** and **(D)** Recognition of the eluted fractions loaded on HLA-A2 positive EBV-LCL by NLV-specific CD8+ T cells as determined by the concentration of IFNγ in supernatant.

Classical route of HLA class I peptide presentation

To determine whether exogenously added long peptides were processed and presented in the classical route of HLA class I presentation, the roles of the proteasome and TAP were investigated. Minigenes were constructed encoding the minimal or extended peptide variants fused to the C-terminus of ubiquitin, which results in the rapid liberation of the

exact peptide sequence, as described previously [55,56]. To investigate the role of the proteasome in the presentation of the endogenously expressed peptides, the effect of the irreversible proteasome inhibitor lactacystin was analyzed [58]. As shown in Figure 3A, lactacystin profoundly inhibited CD8+ T cell activation by EBV-LCL which endogenously expressed the C-terminally extended NLV-6 or 6-NLV-6 peptide, as expected. In contrast, proteasome inhibition only marginally decreased CD8+ T cell activation by EBV-LCL which endogenously expressed the NLV or 6-NLV peptide. The slight decrease in activation was likely to represent a non-specific effect of lactacystin on the stimulator cells. To investigate the role of TAP in the presentation of the endogenously expressed peptides, the presentation of the endogenously expressed peptides was compared between TAP-competent T1 cells and TAP-deficient T2 cells. As shown in Figure 3B, CD8+ T cell activation by T2 cells expressing endogenous peptides 6-NLV, NLV-6, and 6-NLV-6 was significantly reduced or completely inhibited compared with T1 cells. CD8+ T cell activation by TAP-deficient T2 cells expressing endogenous minimal peptide NLV was comparable to TAP-competent T1 cells, which might be due to the large amount of peptide expressed after minigene transduction. These results demonstrated that endogenously expressed peptides that are extended at the C-terminus require proteasomal processing and that transport via TAP is required for HLA class I presentation of all endogenously expressed extended peptides.

Subsequently, similar experiments were performed to investigate the role of the proteasome and TAP in the presentation of exogenously added peptides. The activation of CD8+ T cells by the exogenously added peptide variants (NLV, 6-NLV, NLV-6, 6-NLV-6) was only partially inhibited following lactacystin incubation (Figure 4A). As shown in Figure 4B, the activation of specific CD8+ T cells by TAP-deficient T2 cells loaded with all exogenously added peptides (NLV, 6-NLV, NLV-6, 6-NLV-6) was even increased compared to TAP-competent T1 cells. These results indicate that, while the processing of endogenous C-terminally extended peptides was dependent on proteasome activity, the processing of exogenous long peptides was predominantly independent of proteasome activity. Furthermore, while the presentation of endogenous peptides was dependent on transportation by TAP, the presentation of exogenous peptides was TAP-independent. Since processing and HLA class I presentation of exogenous peptides was not dependent on the proteasome and TAP, these data illustrated that the classical route of HLA class I presentation was not required for activation of specific CD8+ T cells by exogenous synthetic long peptides.

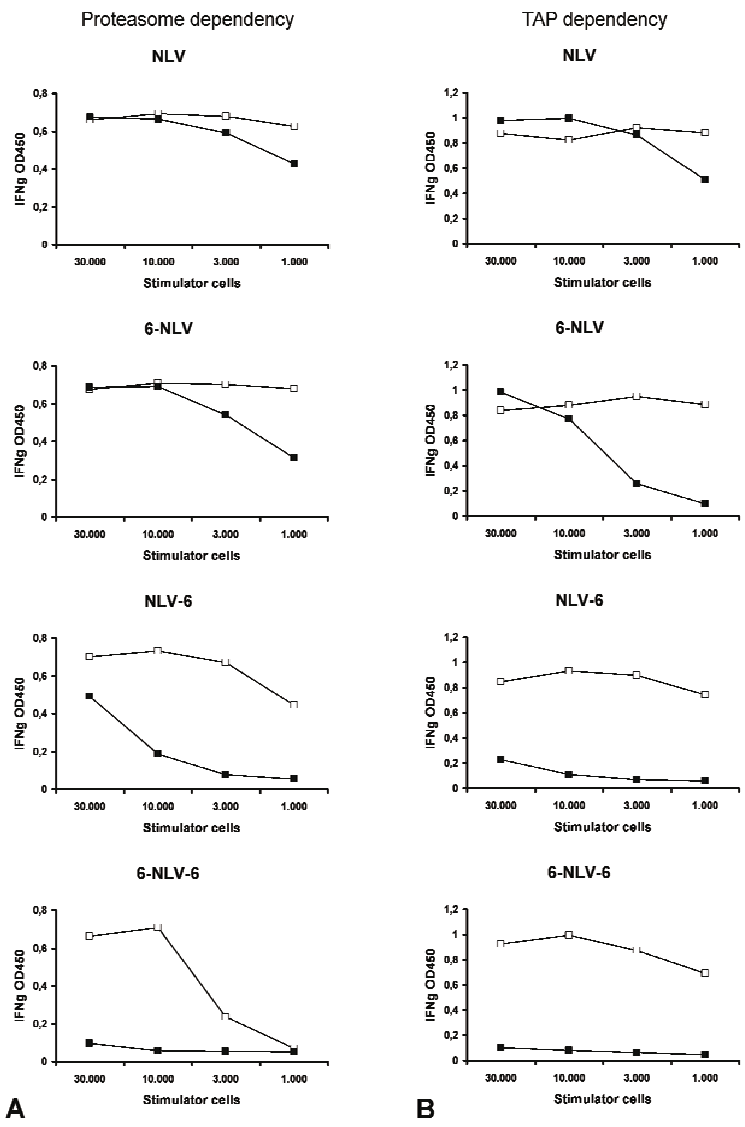


Figure 3. Role of proteasome and TAP in processing of endogenously expressed peptides. **(A)** The concentration of IFNγ in supernatant is shown after incubation of NLV-specific CD8+ T cells with $1.0x10^3$ to $3.0x10^4$ EBV-LCL transduced with minigenes encoding minimal peptide NLV or extended NLV peptide variants in the absence (white) or presence (black) of the proteasome inhibitor lactacystin. **(B)** The concentration of IFNγ in supernatant is shown after incubation of NLV-specific CD8+ T cells with $1.0x10^3$ to $3.0x10^4$ TAP-competent T1 cells (white) or TAP-deficient T2 cells (black) transduced with minigenes encoding minimal peptide NLV or extended NLV peptide variants.

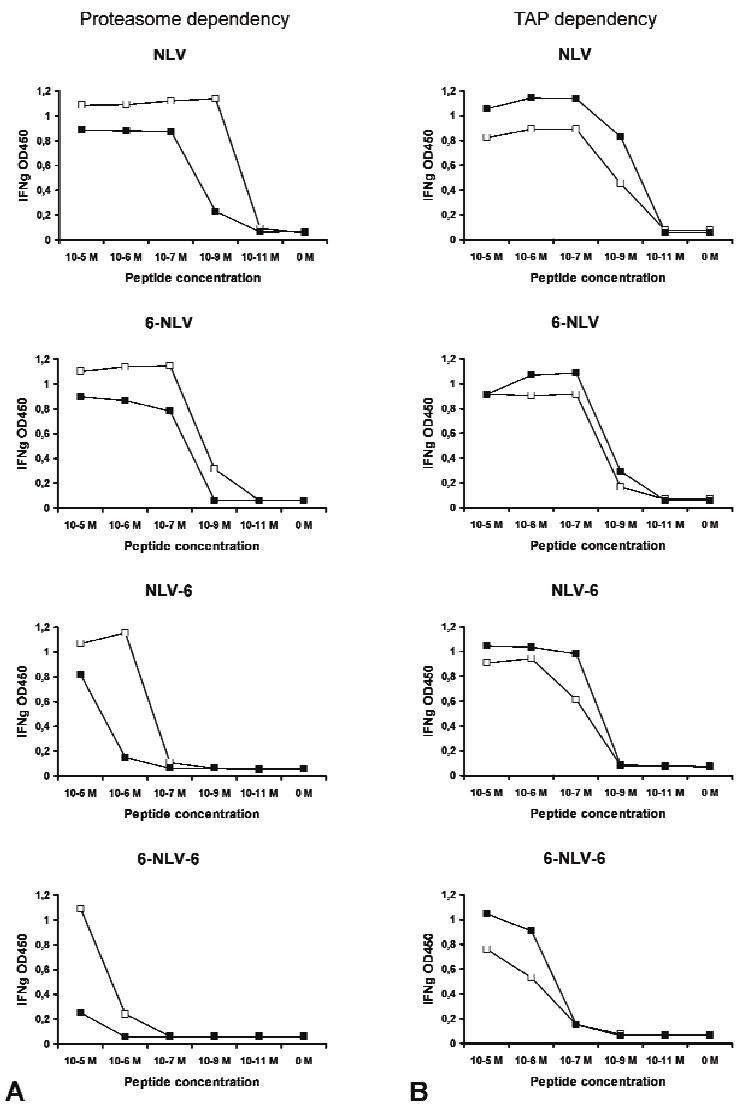


Figure 4. Role of proteasome and TAP in processing of exogenously added peptides. **(A)** The concentration of IFNγ in supernatant is shown after incubation of NLV-specific CD8+ T cells with $3.0x10^4$ EBV-LCL loaded with 10^{-5} to 10^{-11} M minimal peptide NLV or extended NLV peptide variants in the absence (white) or presence (black) of the proteasome inhibitor lactacystin. **(B)** The concentration of IFNγ in supernatant is shown after incubation of NLV-specific CD8+ T cells with $3.0x10^4$ TAP-competent T1 cells (white) or TAP-deficient T2 cells (black) loaded with 10^{-5} to 10^{-11} M minimal peptide NLV or extended NLV peptide variants.

Binding of long peptides in HLA class I

Since the classical route of HLA class I presentation was not required for activation of specific CD8+ T cells, we next determined whether the synthetic long peptides could directly bind to HLA class I molecules or needed processing by other mechanisms to allow binding of the minimal peptides in the groove of HLA class I. The HLA class I binding capacity of the peptide variants was determined using a competition assay. As shown for the HLA-A2 restricted NLV peptides in Figure 5A, the minimal peptide NLV was most efficiently bound, resulting in a decrease of half of the fluorescent signal at a peptide concentration that was 30-fold higher than the concentration of fluorescent reference peptide, which was defined as the half maximal inhibitory concentration IC_{50}. The N-terminally extended peptide 6-NLV was less efficient for binding in HLA-A2, with a 140-fold increase of the IC_{50} compared to the minimal peptide. Extending the minimal peptide with 6 amino acids at the C-terminus or at both ends (NLV-6 or 6-NLV-6) resulted in a low efficiency of HLA-A2 binding, as indicated by a 1,300- and 3,000-fold increase of the IC_{50} compared to the minimal peptide, respectively.

The efficiency of HLA class I binding of the extended peptides variants containing the 7 different epitopes is shown in Figure 5B. The binding efficiency of the long peptides was very variable, ranging from a similar binding efficiency compared to the binding of the minimal peptide (YSE-6: no increase in IC_{50}) to very low efficiencies of binding (6-TPR-6: 29,000-fold increase in IC_{50}). For epitopes NLV, VLE, and QIK the N-terminally extended peptide showed the highest binding efficiency compared to the minimal peptide, while for epitopes YSE, TPR, RPH, and ELR the C-terminally extended peptide showed the highest binding efficiency. The peptides extended at both ends showed lowest binding efficiencies. For the extended peptide variants, no relation was observed between the efficiency of HLA binding and the efficiency of CD8+ T cell activation, as expressed by the IC_{50} and the EC_{50} values (Figure 1B and 5B). Therefore, the data indicate that direct binding of long peptides in HLA class I is not sufficient for CD8+ T cell activation, and that peptide trimming is likely to be required for HLA class I presentation and CD8+ T cell activation.

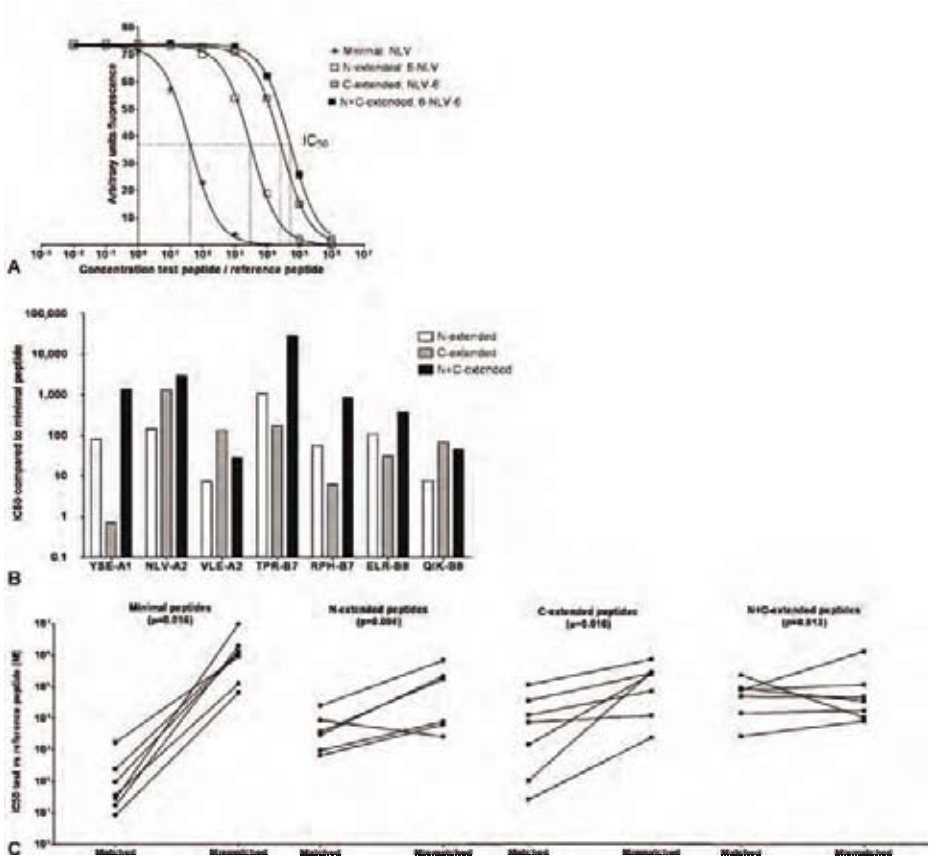


Figure 5. Binding of long peptides in HLA class I. **(A)** The fluorescent signal of a reference peptide strongly binding to HLA-A2 monomers is shown after incubation with different concentrations of the HLA-A2 binding peptide NLV and extended NLV peptide variants. The curves were used to determine the half maximal inhibitory concentration IC_{50} for each peptide variant. **(B)** The IC_{50} values for peptide variants corresponding to 7 viral epitopes are presented as compared to the IC_{50} value of the minimal peptide. **(C)** The IC_{50} values for the same minimal, N-terminally extended, C-terminally extended, and both N- and C-terminally extended peptides as in (B) are shown using the matched or mismatched HLA molecules. The p value is calculated by the Wilcoxon t-test for paired samples.

We next assessed whether the binding of extended peptides in HLA class I molecules was based on specific binding of the minimal epitope sequence. For this purpose, the IC_{50} values of the peptides in the matched HLA class I molecule were compared with IC_{50} values using the same peptides in a mismatched HLA class I molecule. As shown in Figure 5C, the IC_{50} of the minimal peptides in the matched HLA class I molecules were low, and the IC_{50} of the minimal peptides in the mismatched HLA class I molecules were significantly higher

(p=0.016). These measurements indicated specific binding of the minimal peptides in the matched HLA class I molecule, and a very low level of binding in the mismatched HLA class I molecule. Binding of the peptides extended at the N-terminus or C-terminus was also better in the matched HLA class I molecules compared to the mismatched HLA class I molecules, although the difference was only significant for C-terminally extended peptides and not for N-terminally extended peptides (p=0.016 and p=0.094, respectively), demonstrating specific interaction of the minimal epitope sequence in these extended peptides with the matched HLA class I molecule. However, binding of the peptides extended both at the N- and C-terminus was not statistically different in the matched and mismatched HLA class I molecules (p=0.813). Therefore, specific interaction between the minimal epitopes sequence in these N- and C-terminus extended long peptides and the matched HLA class I molecules could not be demonstrated.

Since no relation was observed between the efficiency of HLA binding and the efficiency of CD8+ T cell activation for the extended peptide variants, we hypothesized that peptide trimming might have occurred to result in activation of specific CD8+ T cells. The extended peptides that showed the highest efficiency of HLA class I binding compared to minimal peptides were the N-terminally extended peptides 6-VLE and 6-QIK, and the C-terminally extended peptides YSE-6 and RPH-6, with a 1- to 10-fold increased IC_{50} compared to the corresponding minimal peptides (Figure 5B). To investigate whether these extended peptides were directly presented in HLA class I, we attempted to generate stable multimeric complexes of the long peptides in HLA class I molecules. The selected long peptides were added to the correct HLA class I heavy chain and beta-2-microglobulin to produce stable peptide-HLA monomers. Analysis by size exclusion chromatography showed that stable peptide-HLA monomers were formed for YSE-6-A1, 6-VLE-A2 and RPH-6-B7, while no stable complexes were detected for 6-QIK-B8. Fluorescently labeled streptavidin was added to the stable peptide-HLA monomers to produce fluorescent peptide-HLA tetramers, which showed specific staining of VLE-A2- and RPH-B7-specific CD8+ T cells, but no specific staining of YSE-A1-specific CD8+ T cells (data not shown). Subsequently, the peptides were eluted from the peptide-HLA monomers. Mass spectrometric analysis showed that both the initial extended YSE-6, 6-VLE, and RPH-6 peptides and various (partially) degraded peptides were detected (Table 2) The minimal immunogenic YSE, VLE, and RPH peptides were dominantly present in the elution fractions. These data indicate that the long peptides had been partly degraded to the minimal HLA binding peptides during the process of peptide-HLA tetramer production. Although long peptides were shown to bind to the relevant HLA class I molecules, peptide trimming was likely to be required for HLA class I presentation and CD8+ T cell activation.

Table 2. Mass spectrometric analysis of peptides eluted from peptide-HLA monomers formed with extended peptide variants with 6 naturally flanking amino acids at the N-terminal end (6-PEP) or at the C-terminal end (PEP-6).

Peptide	Sequence	Charges detected	Relative abundance [1] (%)
Input: YSE-6	YSEHPTFTSQYRIQGKL	3+,4+	38.1
	YSEHPTFTSQYR	3+	1.5
Immunogenic [3]	YSEHPTFTSQY	2+	28.8
Immunogenic [3]	YSEHPTFTSQ	2+	31.6
Input: 6-VLE	RVLCCYVLEETSVML	Not detected [2]	Not detected [2]
	LCCYVLEETSMVL	2+	1.9
	YVLEETSMVL	1+,2+	36.1
Immunogenic [3]	VLEETSMVL	1+,2+	61.3
Input: RPH-6	RPHERNGFTVLCPKNMI	2+,3+,4+,5+	7.9
Immunogenic [3]	RPHERNGFTVL	2+,3+,4+	34.3
Immunogenic [3]	RPHERNGFTV	2+,3+,4+	5.4
	RPHERNGFT	2+,3+	3.5
	RPHERNGF	2+,3+	46.3

1) Relative abundance is expressed as percentage of total ion counts. Fragments with a relative abundance of more than 1% are presented.
2) The input peptide 6-VLE could not properly be detected due to cystein-cystein interactions.
3) These immunogenic peptides have been reported before as minimal peptides to efficiently induce activation of antigen-specific T cells.

Discussion

In this study, we demonstrated that the efficiency of antigen-specific CD8+ T cell activation using extended peptide variants of common viral epitopes can be highly variable, ranging from a similar efficiency compared to the minimal peptide to a 75,000-fold decreased efficiency. Processing and HLA class I presentation of the exogenous long peptides was demonstrated not to be dependent on the proteasome and TAP, illustrating that the classical route of HLA class I presentation was not required for activation of specific CD8+ T cells. Although long peptides were shown to bind to the relevant HLA class I molecules, peptide trimming was likely to be required for HLA class I presentation and T cell activation. Since the proteasome was not required for processing of the exogenous peptides, it is very likely that peptide trimming was mediated by peptidases, which may be located either extracellularly at the cell surface, or in endosomal compartments.

The efficiency of CD8+ T cell activation by extended peptide variants may be an important factor in the design of strategies that use synthetic long peptides for detection or isolation of T cells in vitro. An optimal strength of antigenic presentation has been shown to result in most efficient T cell activation in vitro [59-61]. While low levels of peptide presented in HLA

class I may not result in proper CD8+ T cell activation, high levels of peptide presented in HLA class I can result in overstimulation and activation-induced cell death. When the minimal epitope is known, the optimal dose of minimal peptide may be empirically determined. However, when immunogenic epitopes are not known, the large variability in efficiency of CD8+ T cell activation using the extended peptide variants in this study indicates that the inclusion of many different overlapping peptide variants in peptide pools may increase the repertoire of specific CD8+ T cells that can be properly activated.

For approaches of peptide vaccination in vivo, the relation between strength of peptide stimulation and efficiency of T cell activation has not been investigated in detail. The doses of peptide used in most murine (10-40 μg) and human (200-1500 μg) vaccination studies are likely to result in very high peptide concentrations at the site of injection, which may result in overstimulation and activation-induced cell death. A tolerizing effect of minimal peptide vaccination has been shown to be most prominent when using high doses of 50 μg peptide in murine studies, while no tolerance was induced at low peptide doses [26]. The more limited efficiency of HLA class I presentation of synthetic long peptides may therefore have contributed to the superior in vivo CD8+ T cell responses that were reported following vaccination with long peptides compared with the corresponding minimal HLA class I binding peptides [22-25].

The efficiency of HLA class I binding of the long peptides investigated in this study was shown to be very variable as well, ranging from a similar binding efficiency compared with the minimal peptide to a 29,000-fold lower binding efficiency. No relation was observed between the efficiency of HLA binding and the efficiency of CD8+ T cell activation, as expressed by the IC_{50} and the EC_{50} values, indicating that direct binding of long peptides in HLA class I may not be sufficient for CD8+ T cell activation, and that peptide trimming is likely to occur resulting in HLA class I presentation of the minimal epitope to CD8+ T cells. The elution of both the initial extended peptide variants and the minimal peptides from HLA class I monomers that were generated using the extended peptides indicated that, although direct binding of long peptides can occur, trimming of long peptides to minimal HLA class I binding epitopes is a highly efficient process. Our data do not indicate whether the long peptides were trimmed first, and subsequently bound in HLA class I molecules, or first bound in HLA class I, and were subsequently trimmed, as was described before as the model of HLA-guided processing [43]. These processes may also occur simultaneously. However, it is likely that processing of the correct minimal peptide is facilitated by binding in HLA class I molecules, since the long peptides were trimmed predominantly to the exact minimal binding and immunogenic peptides, suggesting that the HLA class I groove protected the peptide from further degradation.

The activation of specific CD8+ T cells was more efficient using N-terminally extended peptides compared with C-terminally extended peptides, while the binding of N-terminally extended peptides in HLA class I was similar or even less efficient compared with C-terminally extended peptides. These results may illustrate the efficient trimming of N-terminal amino acids by aminopeptidases, in contrast to the low efficiency of C-terminal amino acid cleavage by carboxypeptidases or endopeptidases [44]. Therefore, the difference in results between N-terminally extended peptides and C-terminally extended peptides is also consistent with the model in which peptidase trimming is required for HLA class I presentation and CD8+ T cell activation.

Although the mechanisms of trimming of exogenous peptides by peptidases remain to be elucidated in more detail, we demonstrated that EBV-LCL were capable of processing and presentation of synthetic long peptides in HLA class I, resulting in CD8+ T cell activation. Previous studies have demonstrated that PBMC can also process and present synthetic long peptides in HLA class I [10-14]. Data from the studies that explored the use of synthetic long peptides for vaccination in vivo indicated that professional APCs are more capable to acquire, process and present long peptides [22-25]. Direct comparison of the mechanisms and capacity of processing and presentation of long peptides between different APC populations may be helpful in designing improved strategies for peptide vaccination. The results from our study illustrate that trimming of exogenous long peptides by peptidases to minimal epitopes, which is facilitated by binding to HLA class I molecules, is likely to result in optimal activation of antigen-specific CD8+ T cells.

Acknowledgements

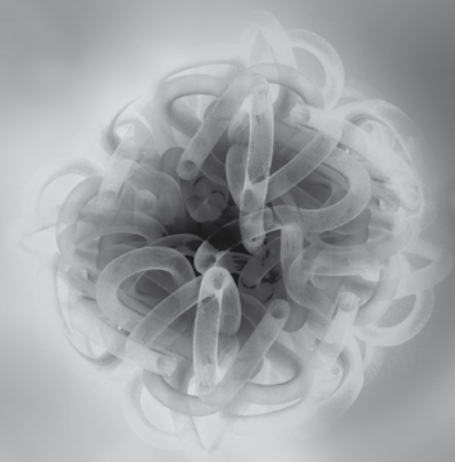
We thank L. Janssen (The Netherlands Cancer Institute, Amsterdam, The Netherlands) for kind provision of the ubiquitin-SIINFEKL template and expert technical assistance on the generation of ubiquitin-peptide-encoding oligonucleotides.

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Treatment of refractory cytomegalovirus reactivation after allogeneic stem cell transplantation with in vitro generated cytomegalovirus pp65-specific CD8+ T cell lines

Pauline Meij, Inge Jedema, Maarten L. Zandvliet,
Pim L.J. van der Heiden, H.M. Esther van Egmond,
Ellis van Liempt, Conny Hoogstraten,
Marian van de Meent, Sabrina Veld,
W.A. Erik Marijt, Peter A von dem Borne,
Arjan C. Lankester, Constantijn J.M. Halkes
J.H. Frederik Falkenburg

Submitted

Abstract

To treat patients with refractory cytomegalovirus (CMV) reactivation after allogeneic stem cell transplantation a phase I/II clinical study on adoptive transfer of in vitro generated donor or patient derived CMV pp65-specific CD8+ T cell lines was performed. Peripheral blood mononuclear cells from CMV seropositive donors or patients were stimulated with HLA-A*0201 and/or HLA-B*0702 restricted pp65 peptides (NLV/TPR) and one day after stimulation interferon-gamma (IFNγ) producing cells were enriched using the IFNγ capture assay, and cultured with autologous feeders and low dose IL-2. After 8-14 days of culture quality controls were performed and the CMV-specific T cell lines were administered or cryopreserved. The T cell lines generated contained 1-17x10^6 cells, comprising 54-96% CMV pp65-specific CD8+ T cells, and showed CMV-specific lysis of target cells. To date 14 CMV-specific T cell lines were generated of which 7 were administered to patients with refractory CMV reactivation. After administration no adverse events were observed and CMV load decreased and remained negative. In several patients a direct relation between administration of the T cell line and the in vivo appearance of CMV pp65-specific T cells could be documented. In conclusion, administration of CMV pp65-specific CD8+ T cell lines was found to be feasible and safe, and efficacy of administered CMV pp65-specific CD8+ T cell lines was demonstrated.

Introduction

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for a variety of hematological malignancies and inherited hematopoietic disorders. A significant complication of alloSCT is the development of severe graft versus host disease (GvHD), which can be treated by immune suppression. Depletion of T cells from the stem cell graft can also be performed to prevent the development of severe GvHD after alloSCT. However, T cell depletion or immune suppression significantly delays immune reconstitution in patients after alloSCT, which is associated with an increased risk of opportunistic infections including CMV infection [1-3]. Reactivation of CMV can cause serious morbidity and mortality during the prolonged period of immune deficiency following alloSCT, which is also illustrated by increased CMV-related morbidity in patients receiving a graft from a CMV seronegative donor [4,5]. The availability of antiviral agents like ganciclovir and foscarnet has contributed to a significant reduction of CMV-related morbidity and mortality following alloSCT. However, if appropriate T cell responses against CMV do not develop after alloSCT subsequent viral reactivations and refractory disease are commonly observed. Reconstitution of the CMV-specific T cell repertoire directed against immunodominant proteins, like CMV pp65, in the first year after alloSCT has been demonstrated to confer sustained protection from CMV disease. Furthermore, for the long-term protection against CMV the development of CMV-specific T cell immunity has been found to be essential [6-9]. Therefore, adoptive transfer of donor-derived virus-specific T cells is an attractive strategy for treatment of viral disease in alloSCT recipients. Clinical studies have demonstrated that the adoptive transfer of donor-derived CMV-specific memory T cells can be a safe and effective treatment for patients with refractory CMV reactivation [10-18].

In most clinical studies CMV-specific CD8+ T cell lines were generated by repetitive stimulation of the T cells in vitro for several weeks to obtain highly pure T cell lines to minimize the risk of the induction of GvHD [10-16]. However, isolation of CMV-specific T cells from peripheral blood also allows the generation of highly specific T cell lines without prolonged culture and repetitive stimulation. Since it has been demonstrated that adoptive transfer of interferon-gamma (IFNγ)-producing memory T cells can provide long-lived functional memory T cell reconstitution and that the presence of IFNγ producing CMV-specific T cells is associated with protection from CMV reactivation isolation of CMV-specific memory CD8+ T based on their IFNγ production is an attractive strategy for generating effective CMV-specific CD8+ T cell lines [4,6,7,9,15,18,19]. Although clinical studies have shown that adoptive transfer of CMV-specific T cells is safe en effective, in

these studies the infusion of CMV specific CD8+ T cells could not always be directly correlated to a clinical effect.
In this study, a phase I/II clinical protocol for the adoptive transfer of donor or patient derived CMV pp65-specific CD8+ T cell lines for patients with refractory CMV reactivation after alloSCT was conducted. CMV-specific T cells were isolated using a IFNγ-based isolation technique and cultured for 1 to 2 weeks. Five patients with CMV reactivation failing antiviral therapy were treated with these CMV pp65-specific CD8+ T cell lines. In all five patients CMV DNA load turned negative and CMV-specific T cells could be detected in the peripheral blood. In two patients a direct relation was shown between the administration of CMV-specific T cells, the appearance of CMV-specific T cells in the peripheral blood and the clearance of CMV reactivation.

Materials and Methods

Study design
Adult and pediatric patients with refractory CMV reactivation after alloSCT were eligible. Patients were weekly monitored for CMV reactivation after receiving a stem cell transplant. Refractory CMV reactivation was defined as high CMV load for more than two weeks while receiving pharmacotherapy or as early relapse after therapy. Since only peptides binding HLA-A*0201 or HLA-B*0702 were available for clinical use and therefore only T cells specific for these epitopes could be isolated, only HLA-A*0201 and/or HLA-B*0702 positive patients were eligible. When the donor was CMV seropositive a donor-derived T cell line was generated, when the donor was CMV seronegative a patient-derived T cell line was generated. When the CMV pp65-specific T cell line met the release criteria and CMV load was still positive the T cell line was administered to the patient. The procedure could be repeated two times in case of stable or progressive disease and no severe toxicity.

Collection of peripheral blood cells
After informed consent at least $2x10^9$ peripheral blood mononuclear cells (PBMC) were harvested by leukopheresis from each donor or patient. Red blood cells were lysed using a NH_4Cl (8.4 g/l) and $KHCO_3$ (1 g/l) buffer (pH=7.4) (Leiden University Medical Center, Department of Clinical Pharmacy, Leiden, The Netherlands). PBMC were directly used or cryopreserved in the vapor phase of liquid nitrogen until further use. Cells were cryopreserved in Iscove's modified Dulbecco's medium (IMDM) (Lonza, Basel, Switzerland) containing 20 g/L human albumin (CeAlb, Sanquin, Amsterdam, The Netherlands) and 10% dimethyl sulphoxide (DMSO) (Leiden University Medical Center, Department of Clinical Pharmacy, Leiden, The Netherlands).

Generation and administration of clinical grade CMV-specific CD8+ T cell lines
Donor or patient PBMC were resuspended in medium consisting of IMDM supplemented with 3 mmol/L L-glutamine (Lonza) and 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza) plus 10% heat-inactivated pooled prescreened AB serum from healthy blood bank donors. Cells were cultured at a cell concentration of $10x10^6$ cells/ml and 1 µg/ml of peptide was added. Peptides used were the HLA-A*0201 binding peptide NLVPMVATV (NLV) and the HLA-B*0702 binding peptide TPRVTGGGAM (TPR) both purchased from Bachem (Bubendorf, Switzerland).
After overnight stimulation IFNγ secreting cells were isolated using the CliniMACS cytokine capture system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. After washing the positive fraction was resuspended in culture medium containing 10 IU/ml IL-2 (Proleukin, Novartis Pharma B.V., Arnhem, The Netherlands). Cells were cultured at a 1:10 ratio with irradiated (30 kGy) feeder cells, which were obtained from the negative fraction after isolation. Cultures were refreshed every 3 to 4 days and after 8 to 14 days the T cell lines were analyzed. Cultures were evaluated for the presence of CMV NLV- and/or TPR-specific CD8+ T cells by tetramer staining and quality control was performed. Quality assurance specifications included: (1) no microbiological contamination, (2) confirmation of the origin of the material (donor or patient), (3) the presence of minimally 50% of CMV-specific CD8+ T cells within the CD3+ population, (4) the presence of maximally 10% CD19+ B-cells and (5) functionality in a ^{51}Cr release assay defined as minimally 20% lysis of positive target cells and maximally 10% lysis of negative target cells. When released for administration, the CMV-specific T cell lines were resuspended in 100 ml NaCl 0.9% (LUMC Pharmacy) supplemented with 2% human albumin (Sanquin) in a 500 ml Cryocyte Freezing Container (Baxter, Deerfield, IL) .

Phenotypic analysis
To determine the composition and phenotype of the starting material, the fractions after isolation and the T cell line, cells were stained using FITC-labeled TCRαβ, CD14, CD4 and HLA-DR (BD Biosciences, San Jose, CA), PE-labeled TCRγδ, CD56, CD25 (BD) and IFNγ (Miltenyi Biotec), PerCP-labeled CD8 and CD3 (BD), APC labeled CD19, CD33 (BD) and CD4 (Beckman Coulter, Fullerton, CA) and PE- or APC-labeled tetramers of the NLVPMVATV bound to HLA-A*0201 or TPRVTGGGAM bound to HLA-B*0702, which

were prepared as described previously [20]. After labeling cells were analyzed on a FACSCalibur (BD).

Cytotoxicity assay

To determine the cytotoxicity of the T cell lines standard ^{51}Chromium (^{51}Cr) release assays were performed as described previously [21]. Target cells (donor and patient phytohemagglutinin (PHA) blasts) were loaded with 100 μCi of $Na^{51}Cr_2O_4$ (Amersham, Roosendaal, The Netherlands) for 1 hour and when necessary 1μM of peptide (NLV or TPR) was also added. Effector/Target (E/T) ratios ranged from 30:1 to 1:1 and after 4 hours of incubation ^{51}Cr release was determined.

Ex vivo identification of CMV-specific CD8+ T cells

To identify donor or recipient origin of CMV-specific T cells reconstituting in peripheral blood after infusion of the CMV pp65-specific CD8+ T cell lines, PBMC from the patient were stained using HLA class I tetramers specific for NLV or TPR. Subsequently, the-HLA tetramer-positive CD8+ T cells were selected by flow cytometric sorting using a FACSDiva and Cellquest software (BD). The DNA profile of the sorted cells was established by chimerism analysis based on short tandem repeats polymorphism as previously described [22].

Results

Generation of clinical grade CMV specific CD8+ T cell lines

A total of 11 donor-derived and 3 patient-derived CMV-specific T cell lines specific for the HLA-A*0201 restricted NLV epitope and/or for the HLA-B*0702 restricted TPR epitope were generated for patients failing antiviral therapy after alloSCT. Donor-derived T cell lines were generated when both donor and patient were CMV seropositive, whereas patient derived T cell lines were generated when the donor was CMV seronegative and the patient CMV seropositive. Figure 1 shows the data of a representative donor-derived (1A - 1D) and patient-derived (1E – 1H) CMV specific T cell line. The precursor frequencies of the NLV-specific T cells in the PBMC starting material were 0.4% in the donor material (Figure 1A) and 1.45% in the patient material (Figure 1E). After overnight stimulation with the HLA-A*0201 binding NLV peptide, IFNγ producing cells were isolated using the Cytokine Capture System. The positive fraction contained between 20 – 25% IFNγ positive CD8+ T cells within the T cell fraction (Figure 1B and Figure 1F). Other T cells present in the positive fraction were CD4+ T cells and IFNγ negative CD8+ T cells. After 10 days and 7

days of culture respectively (Figure 1C and 1G), specific proliferation of the NLV-specific CD8+ T cells resulted in the donor-derived T cell line containing 83% NLV-specific CD8+ T cells and the patient-derived T cell line containing 66% NLV-specific CD8+ T cells.

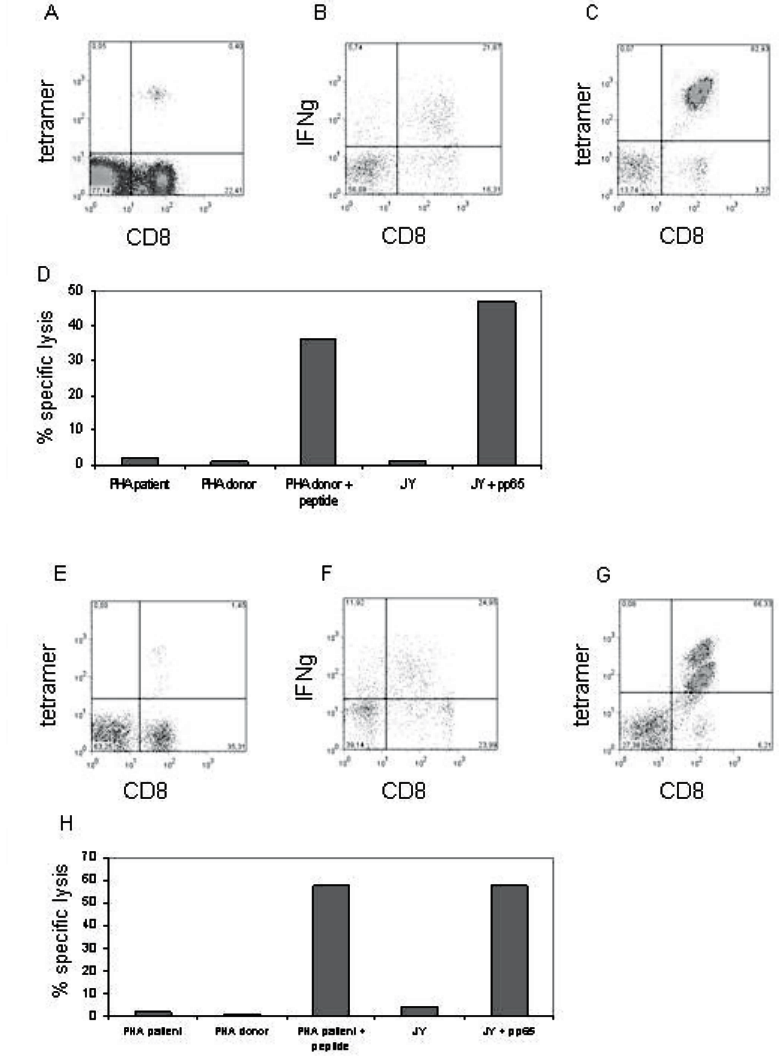


Figure 1. Composition and functionality of donor derived (A-D) and patient derived (E-H) CMV-specific T cell lines. **(A, E)** the percentage of NLV (A) and TPR (E) -specific T cells in the starting material (day 0); **(B, F)** the percentage of IFNγ positive cells in the positive fraction directly after isolation; **(C, G)** the percentage of NLV (C) or TPR (G) in the T cell line after culture (day 7); **(D, H)** functionality of the T cell lines in a ^{51}Cr release assay. Dot plots are shown and events shown are gated on lymfogate and PI-negative gate. Percentage lysis after 4 hours in a ^{51}Cr release assay at an E:T ratio of 3:1 is depicted.

Less than 1% of CD19+ B-cells were detected in the T cell lines (data not shown). As is shown in Figure 1D and 1H both T cell lines showed reactivity against NLV- or TPR peptide-loaded target cells and no reactivity against donor or patient cells. Furthermore, both T cell lines also showed recognition of endogenously presented antigen using pp65 transduced EBV-LCL.

Table 1 shows the results of all the CMV-specific T cell lines generated. Precursor frequencies of NLV- and/or TPR-specific CD8+ T cells in the donor or patient starting material used for the generation of the T cell lines ranged from 0.02 – 1.45% of total PBMC. Cell numbers obtained direct after isolation ranged from 1.0 to 14.4x10^6 cells and contained between 3 to 45 % IFNγ positive CD8+ T cells within the T cell population. Total cell numbers obtained after culture ranged from 1.0 to 17.1x10^6 cells. During culture no increase in total cell numbers was observed, however, enrichment of CMV-specific T cells was observed, since the T cell lines contained between 54- 96% of NLV-and/or TPR-specific T cells.

Table1. Characteristics of the CMV-specific T cell lines

T cell line	Speci-ficity	Frequency in PBMC	# cells (x10^6)[2]	Phenotype (% positive cells)[2]			# cells (x10^6)[2]	Cytotoxicity[2,3]	
								PHA Donor	PHA patient
				CD4	CD8	CD8+ Tet+	CD8+ Tet+	- peptide	+ peptide
P1-a	TPR	0.28	3.4	26	71	68	2.3	1	53	4
P1-b	TPR	0.28	3.8	10	88	72	2.7	2	70	0
P2-a	TPR	0.3	7.5	9	91	81	6.1	2	56	2
P3-a	TPR	0.02	5.0	20	80	72	3.6	1	41	4
P4-a	NLV/TPR	<0.01 / 0.25	5.9	11	85	5 / 69	3.8	3	66	0
P4-b	NLV/TPR	<0.01 / 0.25	17.1	5	73	14 / 50	12.7	1	39	0
P5-a	TPR	0.14	4.8	4	95	95	3.1	0	26	1
P7-a	NLV/TPR	0.04 / 0.52	10	7	96	4 / 92	9.6	9	65	0
P8-a[1]	TPR	0.7	3.0	11	77	66	2.1	1	62	1
P9-a	NLV	0.48	1.0	22	77	65	0.7	1	80[4]	0
P9-b	NLV	0.98	3.3	7	93	83	2.7	2	30	1
P10-a[1]	NLV	1.45	6.0	8	90	79	4.7	1	58	2
P11-a[1]	NLV	1.1	6.7	27	61	54	3.6	2	25	4
P12-a	NLV	0.4	16.8	9	85	82	13.8	1	36	2

[1]patient-derived T cell line;
[2] after 7-14 days culture;
[3] percentage lysis after 4 hours in a ^{51}Cr release assay, E:T ratio of 3:1;
[4] measured in a CFSE based cytotoxicity assay [27]; Tet = tetramer; PHA = phytohemagglutinin blast.

All 14 donor and patient derived CMV specific T cell lines generated met the quality control criteria. No microbiological contamination was detected in any of the T cell lines and chimerism analysis showed the correct origin of the material. As is shown in Table 1 all T cell lines contained more than 50% of NLV- and/or TPR- specific T cells within the CD3+ population and all T cell lines showed recognition of peptide-loaded PHA blasts and no reactivity against donor or patient derived PHA blasts.

Administration of CMV-specific CD8+ T cell lines

In a phase I/II study the toxicity and the potential antiviral effect of treatment with CMV pp65-specific T cell lines for refractory CMV reactivation following allogeneic SCT was investigated. Five patients with refractory CMV reactivation following alloSCT received donor derived CMV pp65-specific T cell line(s). Three patients (patient 2, 3 and 4) received one CMV pp65-specific T cell line and two patients (patient 1 and 9) received two CMV pp65-specific T cell lines. The other T cell lines generated were not administrated, since patients were CMV load negative (n=4), had relapsed (n=1) or deceased due to CMV disease (n=2) at the time the production of the CMV pp65-specific T cell line was completed.

Despite treatment with antiviral agents all five patients who received a CMV pp65-specific T cell line had positive CMV DNA loads (log 2.5 to log 3.2) at the time of administration of the CMV specific T cell lines. The patients received a total cell dose ranging from 1.0 to 7.5x10^6 cells, corresponding to 0.6 to 6.1x10^6 NLV- and/or TPR-specific T cells. None of the five patients developed any complications during infusion of the T cell lines and all patients cleared CMV reactivation within weeks after administration of the CMV pp65-specific T cell line.

Reconstitution of CMV-specific T cells after administration of the CMV-specific CD8+ T cell lines

In all patients CMV-specific NLV- and/or TPR-specific CD8+ T cells could be detected after administration of the T cell lines. To determine the correlation between the infusion of the CMV-specific T cell lines and the appearance of CMV-specific T cells in peripheral blood of the patients, the presence of CMV-specific T cells was analyzed at different time points before and after administration of the T cell lines. Prior to infusion of the CMV-specific T cell lines in 3 of the 5 patients CMV pp65-specific T cells with the same specificity as the T cell line could already be detected in peripheral blood. In two of these patients a rise in CMV-specific T cells in peripheral blood was observed after infusion of the T cell line. In the other patient the numbers of CMV-specific T cells remained stable after infusion, however, in this

patient a very high level of CMV-specific T cells was already observed prior to infusion of the T cell line. In 2 of the 5 patients (patient 4 and 9) no CMV-specific T cells were detected prior to infusion of the T cell line, and a direct relation was observed between infusion of the CMV pp65-specific T cell line, the appearance of CMV-pp65 specific T cells in peripheral blood, and the clinical effect.

Patient 4 developed CMV reactivation within the first month after alloSCT which was treated with antiviral drugs (Figure 2). The rise in CMV coincided with a rise in CMV TPR-specific T cells. Four months after alloSCT the patient received unmanipulated donor lymphocyte infusion (DLI), and six months after alloSCT the patient developed GvHD for which he was treated. The CMV-specific T cells disappeared, and the patient developed refractory CMV reactivation. A CMV TPR-specific T cell line was administered 8 months after alloSCT ($1x10^5$ T cells/kg) and within 2 weeks TPR-specific $CD8^+$ T cells appeared in peripheral blood and the CMV load turned negative and remained negative (follow up >4 years). To asses why no persistence of the TPR-specific T cells from the first peak response was observed and to determine whether the TPR-specific T cells appearing in the second peak response were the same as the T cells in the first peak response, the patient or donor origin of the CMV-specific T cells from both peak responses (day 33 and day 289 after alloSCT respectively) was determined. The TPR-specific T cells were isolated from both peak responses by FACS sorting and DNA profiling showed that the CMV-specific T cells during the first response (day 33) were patient derived, and that the CMV-specific T cells that appeared after administration of the T cell line (day 289) were completely donor derived, illustrating the correlation between the infusion of the TPR-specific T cell line and the in vivo appearance of the donor derived TPR-specific T cells.

Patient 9 developed refractory CMV reactivation post alloSCT in the absence of CMV-specific T cells (Figure 3). A CMV NLV- specific T cell line was administrated (day 72), but no CMV NLV-specific T cells appeared in peripheral blood and the CMV load remained positive. The quality of this T cell line was very poor due to poor quality of the starting material and this might explain that there was non in vivo persistence of the infused T cells. A second CMV NLV-specific T cell line was generated, from new starting material, that was administered at day 116 after alloSCT ($3.1x10^5$ T cells/kg) and after two weeks CMV NLV-specific T cells appeared in peripheral blood, which was coincided with a disappearance of CMV DNA load (follow up >2 years).

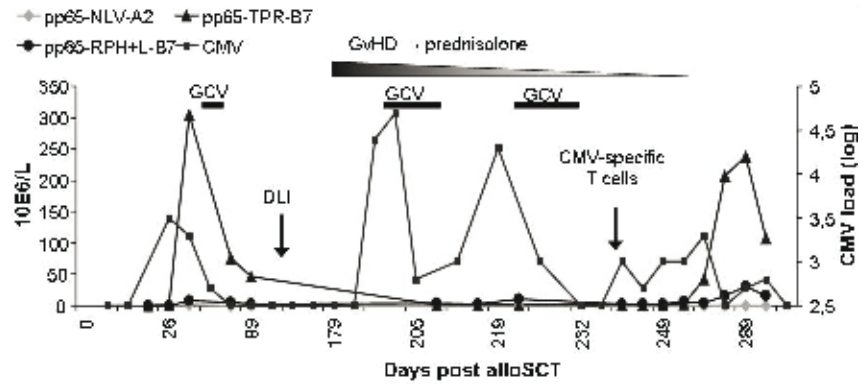


Figure 2. CMV pp65-specific T cells in peripheral blood from patient 4 in relation to the CMV DNA load in peripheral blood. CMV-specific T cells (CD8+/tetramer+) are expressed as absolute numbers per liter in peripheral blood (left axis). ▲, TPR-specific T cells; ◆, NLV-specific T cells; ●, RPH-specific T cells. CMV DNA load (■) is depicted as log CMV DNA load in peripheral blood (right axis).The lower level of detection is log 2.5.

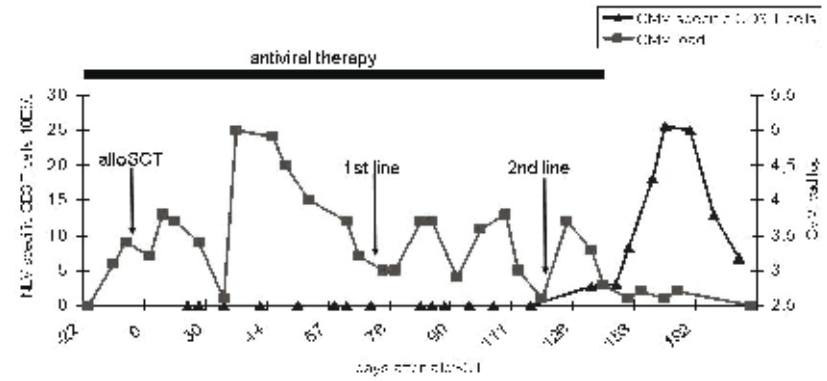


Figure 3. CMV pp65 (NLV)-specific T cells in peripheral blood from patient 9 in relation to the CMV DNA load in peripheral blood. CMV-specific T cells (▲, CD8+/NLV-tetramer+) are expressed as absolute numbers per liter in peripheral blood (left axis) and CMV DNA load (■) is depicted as log CMV DNA load in peripheral blood (right axis). The lower level of detection is log 2.5.

Discussion

In this study, a phase I/II clinical protocol for the adoptive transfer of donor- or patient-derived CMV pp65-specific CD8+ T cell lines for patients with refractory CMV reactivation after alloSCT was conducted. No toxicity was observed after administration of the T cell lines and all patients who received a CMV-specific T cell line cleared CMV reactivation. A direct relation between administration of donor derived CMV pp65-specific T cell lines and clinical effect could be demonstrated.

Both donor- and patient-derived CMV pp65-specific CD8+ T cell lines could be reproducible generated, even when precursor frequencies of CMV pp65-specific CD8+ T cells were very low (<0.01%). Directly after isolation the purity of the positive fraction (% CD8+/IFNγ+ T cells) appeared to be low for some isolations, which was also observed in other studies using the IFNγ based isolation method [18,23]. This can be due to nonspecific co-isolation of other cells and may also be due to the fact that the antibody on the microbeads used for isolation and the antibody used for analysis of the population recognize the same epitope. Preferential expansion of the CMV-specific CD8+ T cells during the subsequent culture period led to T cell lines with a high purity of CMV-specific CD8+ T cells, reducing the risk of the induction of GvHD after administration.

In the current study five patients with refractory CMV reactivation received one or two donor-derived CMV-specific CD8+ T cell line(s). Administration was safe, no toxicity was observed after infusion, and all patients cleared CMV reactivation after treatment with the CMV-specific CD8+ T cell lines. In three patients, T cells with the same reactivity as the T cell line could already be detected in peripheral blood of the patients prior to administration of the T cell lines. Although it is likely that the infused T cells contributed to the clearance of CMV reactivation in these patients, direct evidence for this can not be provided. However, in two of the five patients a direct correlation between the administration of donor derived CMV pp65-specific T cell lines, the appearance of CMV-specific CD8+ T cells and clearance of CMV reactivation was demonstrated. No CMV-specific T cells were present prior to the administration of the T cell lines, and within two weeks after administration of the T cell line increasing numbers of NLV- or TPR-specific CD8+ T cells could be detected in peripheral blood of the patients, illustrating persistence and in vivo proliferation of the infused T cells. The appearance of the CMV-specific CD8+ T cells co-incided with the clearance of CMV reactivation. Although CMV-specific CD8+ T cells have been detected in peripheral blood after the adoptive transfer of CD8+ T cell lines in previous studies, this study shows a direct correlation between the administration of a CD8+ T cell line and the clinical effect [10-17].

Two patients eligible for the study deceased due to CMV disease. CMV-specific T cell lines could be generated for these patients, however both patients deceased before the T cell line could be administered. Decreasing the time interval between the appearance of the clinical need and administration of the T cell product by direct administration of the isolated CMV pp65-specific T cells may improve the efficacy. Although in vivo proliferation is also likely to be necessary in these patients to allow a clinical effect, it has been shown that direct administration of CMV-specific T cells isolated based on their IFNγ production is effective and does not lead to GvHD induction [18].

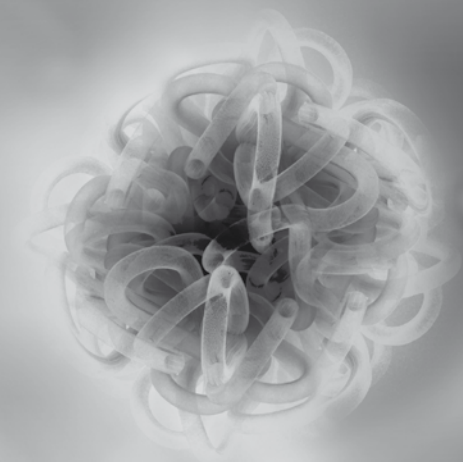
In the current protocol T cell lines only could be generated for patients who were HLA-A*0201 and HLA-B*0702 positive. To increase the potential clinical use of CMV specific T cells a CMV pp65 whole protein overlapping 15 mer (11 mer overlapping) peptide pool may be used for the generation of CMV pp65-specific T cell lines. It has been shown by us and others that using this peptide pool CMV pp65-specific T cells can be isolated irrespective of HLA restriction of the donor and patient [24-26]. Furthermore, using these peptide pools also CMV-specific CD4+ T cells will be isolated and studies have indicated that adoptively transferred CMV-specific CD4+ T cells may promote development of a CMV-specific CD8+ T cell response in vivo and that adoptively transferred CMV-specific CD8+ T cells show better persistence in the presence of CMV-specific CD4+ T cells [10, 12, 14-16, 26].

In conclusion, we have shown that the clinical grade generation of donor and patient derived CMV pp65-specific CD8+ T cell lines is feasible, and that administration of CMV-specific T cell lines to patients with refractory CMV reactivation after alloSCT was safe. A direct relation between infusion of CMV specific CD8+ T cells and clinical effect could be demonstrated.

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8

Summary

Summary

In recipients of allogeneic stem cell transplantation (alloSCT), virus-specific T cells are eradicated or impaired by the conditioning regimen and immune suppression. Since reconstitution of antiviral immunity can be considerably delayed and is often incomplete, patients are exposed to a prolonged period of high risk of viral infections [1]. The major viral pathogens causing serious morbidity and mortality after alloSCT are the common viruses cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human adenovirus (HAdV) [2-6]. Since pharmacological treatment is limited by significant toxicity and is often not sufficient for long-term control of viral infections, other treatment options are needed [7-9]. It has been shown that reconstitution of T cells specific for CMV, EBV, and HAdV is essential for sustained protection from viral disease after alloSCT [10-17]. Therefore, the adoptive transfer of donor-derived virus-specific T cells to alloSCT recipients may be an effective strategy for prophylaxis or treatment of viral infections or reactivations. Since administration of donor T cells with unknown specificity increases the risk of development of graft-versus-host disease (GvHD), treatment with virus-specific T cells isolated from donor peripheral blood mononuclear cells (PBMC) is needed. In this thesis, methods were investigated for efficient activation of virus-specific CD8+ and CD4+ T cells in PBMC to allow detection and selection of the specific cells from PBMC. Using these methods, we studied the role of T cells in protection from HAdV disease after alloST, to support the rationale for HAdV-specific T cell therapy. Furthermore, strategies were developed for the efficient isolation of activated virus-specific T cells from PBMC. The clinical grade T cell lines with high specificity against multiple viral antigens, which can be generated using these strategies, may be used to establish the clinical benefit of adoptive immunotherapy for viral infections after alloSCT.

In **chapter 2**, the IFNγ response of CMV-specific CD8+ T cells was studied to exploit the full potential of IFNγ-based isolation. Investigation of the kinetics of the T cell response after CMV peptide stimulation showed a rapid downregulation of the T cell receptor (TCR) which coincided with the induction of IFNγ production. The production of IFNγ was maximal after 4 hours of stimulation, and rapidly decreased thereafter, despite the continued presence of specific peptide. By varying the strength of stimulation, we observed that overstimulation can result in profound TCR downregulation, more rapid decline of IFNγ production, and reduced expansion of the specific cells. Based on these findings, we defined optimal conditions for IFNγ-based isolation of CMV-specific CD8+ T cells with maximal potential for clinical application. IFNγ-based isolation of CMV-specific CD8+ T cells

was shown to be most efficient after 4 hours of stimulation with 10^{-7} M CMV peptide. The median frequency of CMV peptide-specific CD8+ T cells obtained in the T cell lines of the IFNγ-enriched fractions was 91% after 9-11 days of culture, while very low numbers of specific T cells were retained in the IFNγ-depleted fractions. The isolated CMV-specific CD8+ T cells produced both IFNγ and TNFα upon restimulation, and showed production of perforin and granzyme B, which was similar to the functional profile of CMV-specific CD8+ T cells in donor PBMC directly ex vivo. The data illustrated the importance of analyses of the kinetics of cytokine production for isolation of T cells specific for other infectious or malignant antigens to exploit the full potential of cytokine capture isolation of antigen-specific T cells.

The adoptive transfer of coordinated CD8+ and CD4+ T cell responses directed against multiple CMV epitopes may be more effective for protection from CMV disease after alloSCT, compared to CD8+ T cell lines specific for a single CMV epitope as described in chapter 2. In **chapter 3**, we showed that CD8+ and CD4+ T cells specific for CMV pp65 epitopes were detected in all healthy CMV seropositive donors, while T cells specific for CMV IE1 epitopes were detected directly ex vivo in PBMC of 45% of healthy CMV seropositive donors. Subsequently, we developed a method for the simultaneous isolation of CMV pp65- and IE1-specific CD8+ and CD4+ T cells from PBMC of healthy CMV seropositive donors, irrespective of their HLA type. Stimulation of donor PBMC with a CMV pp65 protein-spanning pool of 15-mer peptides efficiently induced activation of CD8+ and CD4+ T cells specific for multiple CMV pp65 epitopes. In contrast, stimulation with the full-length CMV pp65 protein resulted in efficient activation of pp65-specific CD4+ T cells, but only part of the pp65-specific CD8+ T cells. The kinetics of IFNγ production by CMV-specific CD8+ and CD4+ T cells after stimulation with 15-mer peptide pools was shown to be similar to IFNγ kinetics after stimulation with minimal MHC I or II binding peptides, with maximal IFNγ production after 4 hours of stimulation, which decreased thereafter. Based on these data, we developed a clinical grade protocol for generation of combined CD8+ and CD4+ T cell lines specific for CMV pp65 and IE1. Isolation of IFNγ-secreting cells was performed by IFNγ capture assay during the peak of IFNγ production after 4 hours of stimulation with CMV 15-mer peptide pools. Using this method, T cell lines containing a median of 91% of T cells specific for a broad repertoire of CMV CD8+ and CD4+ epitopes were reproducibly generated from all donors. The composition of the T cell lines correlated with the frequencies of pp65 and IE1-specific CD8+ and CD4+ T cells in donor PBMC, illustrating that the repertoire of CMV-specific T cells in donor PBMC was represented in the T cell lines. The phenotype and function of CMV-specific T cells in generated T cell lines

was comparable to CMV-specific T cell lines in donor PBMC. This study provided a feasible strategy for the rapid generation of clinical grade CD8+ and CD4+ T cell lines with high specificity for multiple CMV pp65 and IE1 epitopes.

Although the appearance of HAdV-specific T cells has been previously shown to confer protection from HAdV disease after alloSCT, the epitope specificity of these responses has not been characterized, and the relative contribution of CD8+ and CD4+ T cells in protection from HAdV was not clear. In **chapter 4**, we investigated the development of HAdV-specific T cell responses in peripheral blood from pediatric and adult alloSCT recipients who showed spontaneous resolution of disseminated HAdV disease. We demonstrated that clearance of HAdV viremia after alloSCT coincided with emergence of a coordinated CD8+ and CD4+ T cell response against HAdV hexon epitopes, providing a rationale for HAdV hexon-specific adoptive immunotherapy. Therefore, we developed a clinical grade method for rapid generation of T cell lines with high and defined specificity for HAdV hexon epitopes for adoptive transfer. Activation of HAdV hexon-specific CD8+ and CD4+ T cells in peripheral blood with a hexon protein-spanning pool of synthetic 15-mer peptides followed by IFNγ-based isolation allowed rapid expansion of highly specific T cell lines from healthy adults, including donors without detectable frequencies of HAdV hexon-specific T cells. HAdV-specific T cell lines recognized multiple MHC class I and II restricted epitopes, including known and novel epitopes, and efficiently lysed HAdV-infected target cells. This study provided a rationale and strategy for the adoptive transfer of donor-derived HAdV hexon-specific CD8+ and CD4+ T cells for treatment of disseminated HAdV infection after alloSCT.

In **chapter 5**, we developed a single and feasible method for the simultaneous isolation of coordinated CD8+ and CD4+ T cell responses against epitopes from multiple viral proteins. To ensure that the method was applicable to a wide variety of virus-specific T cells that may differ in phenotypic and functional properties, we focused on T cells specific for the persistent viruses CMV and EBV, as well as T cells specific for HAdV and influenza (FLU), which are not repetitively activated in vivo after initial viral clearance. Although IFNγ-based isolation of antigen-specific T cells was shown to be feasible, it was unknown whether all virus-specific T cell populations showed similar kinetics of IFNγ production upon activation, which might allow simultaneous IFNγ-based isolation. Furthermore, the IFNγ-based isolation procedure is technically demanding, which is a major drawback for wide clinical application. Therefore, we also explored the use of the activation-induced expression of CD137 as isolation marker. Following activation, the virus-specific T cells produced IFNγ

and TNFα, and expressed CD137, while the populations varied in production of IL-2, degranulation, and expression of phenotypic markers. Different kinetics of IFNγ production were observed between CMV/EBV-specific T cells and HAdV/FLU-specific T cells. However, after stimulation of donor peripheral blood with viral protein-spanning peptide pools, the activated virus-specific CD8+ and CD4+ T cells could be simultaneously isolated by either IFNγ-based or CD137-based enrichment. The simple CD137 labeling procedure is a major practical advantage over the laborious IFNγ labeling procedure, which may allow more feasible and cost-effective production of virus-specific T cell lines. However, only IFNγ-based isolation is currently available for the generation of clinical grade virus-specific T cell lines. This study provided an efficient and widely applicable method for the simultaneous isolation of both CD8+ and CD4+ T cells specific for a broad repertoire of antigens from multiple viral pathogens from donor peripheral blood.

In chapter 3-5, in vitro stimulation of peripheral blood with pools of synthetic overlapping 15-mer peptides spanning viral proteins was shown to efficiently induce activation of both CD8+ and CD4+ virus-specific T cells. Furthermore, synthetic long peptides that contain immunogenic T cell epitopes have been previously shown to efficiently activate antigen-specific CD8+ T cells in vivo following peptide vaccination. However, the mechanisms and efficiency of presentation of exogenous long peptides in MHC class I are unknown. In **chapter 6**, we demonstrated that the efficiency of antigen-specific CD8+ T cell activation using extended peptide variants of common viral epitopes can be very variable. Although long peptides were shown to bind to the relevant MHC class I molecules, peptide trimming was likely to be required for MHC class I presentation and T cell activation. Since processing and MHC class I presentation of the long peptides was not dependent on the proteasome and TAP, it is very likely that peptide trimming was mediated by peptidases, which may be located either extracellularly, at the cell surface, or in endosomal compartments. Furthermore, the results suggested that processing of the correct minimal peptides was facilitated by binding in MHC class I molecules. The results illustrate the need of a more rational design and extensive evaluation of the proper dose of synthetic long peptides to optimize activation of CD8+ T cells in approaches of immune monitoring, adoptive transfer, and vaccination.

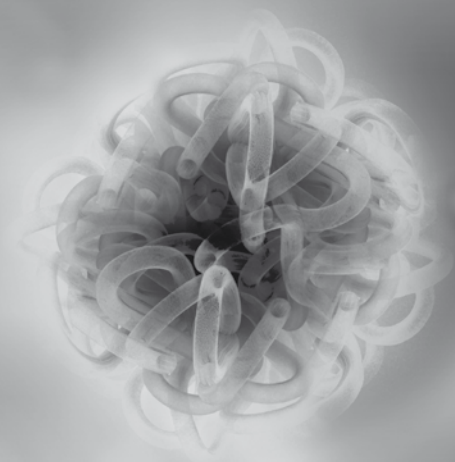
In **chapter 7**, the clinical results were presented of a phase I/II study for treatment of refractory CMV reactivation after alloSCT by adoptive transfer of donor- or patient-derived CMV pp65-specific CD8+ T cells. Refractory CMV reactivation was defined by a high CMV DNA load in peripheral blood for more than 2 weeks under antiviral therapy or an early

viral relapse after antiviral pharmacotherapy. PBMC from CMV seropositive donors or patients were stimulated with HLA-A2/HLA-B7 restricted CMV pp65 peptides, followed by IFNγ-based enrichment. Eleven donor-derived CMV-specific T cell lines were generated of which 7 were administered to patients with refractory CMV reactivation. The T cell lines generated contained high frequencies of CMV-specific CD8+ T cells and showed CMV-specific lysis of target cells. After administration no adverse events were observed, CMV peptide-specific CD8+ T cells could be detected, and CMV load decreased and remained undetectable. One pediatric patient, who remained lymphopenic for months after alloSCT and maintained a high CMV load under antiviral therapy during this period, received 2 CMV-specific T cell lines. Within weeks after administration of the second CMV specific T cell line, CMV-specific T cells appeared and the CMV load turned negative. Patient-derived T cell lines were generated for 3 patients, but were not administered due to changes in patient status. This study demonstrated that administration of CMV-specific CD8+ T cell lines appears to be safe, feasible, and effective.

In conclusion, the rationale for adoptive immunotherapy for viral disease after alloSCT was supported in this thesis. Furthermore, we developed efficient and feasible strategies for the rapid generation of clinical grade CD8+ and CD4+ T cell lines with high specificity for multiple viral antigens. These methods are widely applicable, and allow evaluation of the clinical benefit of adoptive immunotherapy for viral infections after alloSCT in future clinical trials.

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9

General discussion

General discussion

Previously, several methods have been developed to isolate virus-specific T cells from PBMC to provide antiviral immune reconstitution after alloSCT, with a minimal risk of GvHD. However, these methods have only been performed in small exploratory clinical trials by a limited number of groups in the past decade. Some methods can not be implemented for clinical use, due to regulatory restrictions and the absence of clinical grade reagents. Other methods can be used for clinical treatment, but have not been optimized to consistently provide T cell lines with high specificity for viral antigens. In this thesis, we developed efficient and feasible strategies for the rapid generation of clinical grade CD8+ and CD4+ T cell lines with high specificity for viral antigens. Overlapping pools of 15-mer peptides spanning viral proteins were demonstrated to efficiently induce the simultaneous activation of virus-specific CD8+ and CD4+ T cells. Both IFNγ-based and CD137-based isolation were shown to result in efficient enrichment of CD8+ and CD4+ T cells specific for multiple viral epitopes from PBMC, but the simple CD137 labeling procedure has an advantage over the more laborious IFNγ labeling procedure. This method is widely applicable, and may be used in future clinical trials to establish the clinical benefit of adoptive immunotherapy for viral infections after alloSCT.

In the clinical study presented in chapter 7, large scale IFNγ-based enrichment of CD8+ T cells specific for a single CMV pp65 peptide was shown to be feasible. The infusion of 7 CMV pp65-specific CD8+ T cell lines without adverse events indicated that this treatment was safe. Furthermore, the adoptive transfer of CMV pp65-specific CD8+ T cells seemed to be effective, since the specific CD8+ T cells could be detected in vivo, and the CMV load decreased and remained undetectable. The potential effectiveness of this strategy was clearly illustrated in two patients with a high CMV DNA load, who rapidly cleared the CMV infection after appearance of CMV pp65-specific CD8+ T cells within weeks after adoptive transfer. However, in the other patients who received CMV-specific T cell infusions, low frequencies of CD8+ T cells recognizing the specific CMV pp65 epitope had already been detected in PBMC before infusion. It is possible that at least part of the specific CD8+ T cells detected after infusion was derived from the CD8+ T cells which had already been present, and that the CMV load would also have been cleared without adoptive transfer. Therefore, despite the promising results, non-controlled studies can not prove that adoptive transfer of virus-specific T cells is effective.

In this same study, a total number of 14 clinical grade CMV-specific CD8+ T cell lines were generated, which all complied with the release criteria, but only 7 CMV-specific T cell lines were administered. Reasons not to administer the T cell lines were clearance of the CMV

load (4 T cell lines), which could either be spontaneous or due to standard antiviral pharmacotherapy, relapse of the malignancy (1 T cell line), or CMV-related death (2 T cell lines). From the moment of decision to generate a T cell line, on average 10 days were required to receive donor or patient cells, and 7 to 14 days were required to generate and expand the T cell line, perform quality controls, and release the product. This period of 2 to 4 weeks can be too long for patients with rapid progression of viral disease and for patients developing complications which result in study exclusion. Direct infusion of isolated virus-specific T cells would allow a more rapid treatment, and simplify the generation procedure. In addition, direct infusion may prevent the potentially negative effects of culture in vitro. Although direct infusion and in vitro expansion of isolated antigen-specific T cells have not been directly compared in animal models or clinical trials yet, the repetitive in vitro stimulation and culture of CMV-specific T cells has been shown to negatively affect their efficacy after adoptive transfer in mice [1]. Likewise, some human studies have demonstrated that in vitro expanded CMV-specific, but also HIV-specific and melanoma-specific CD8+ T cells did not persist long-term in vivo [2-5]. In vivo efficacy and survival may be hampered by abrupt withdrawal from high concentrations of cytokines, or exhaustion, and functional changes developing during prolonged culture [6]. No functional alterations or decreased proliferative capacity were detected in the virus-specific T cell lines described in this thesis. In vitro, the virus-specific T cells kept proliferating in culture for many weeks in the presence of IL-2 and IL-15. However, the effect of in vitro expansion of the virus-specific T cells on their in vivo survival and efficacy remains uncertain. To allow a more rapid treatment, simplify the generation procedure, and exclude the effect of in vitro expansion, direct infusion of isolated virus-specific T cells is preferable. Since only limited information about product composition will be available before administration using this approach, the procedures of generation should be validated. Retrospective testing of an in vitro cultured fraction can provide additional information about the cellular product infused afterwards. For high risk patients, donor or patient cells should be stored as potential starting material before development of viral disease. Patient cells may be stored before transplantation for all patients transplanted from a CMV seronegative donor, and donor cells may be stored after transplantation when patients do not show spontaneous reconstitution of CMV-specific T cells. Subsequently, the procedure of thawing cells, viral peptide stimulation, and CD137 isolation can be rapidly performed, allowing direct infusion of virus-specific T cells within 24 hours.

The strategy of activating virus-specific T cells by incubation of PBMC with synthetic peptides followed by CD137-based isolation permits a wide choice of viral peptides to be used as antigen. The CD8+ T cell lines described in chapter 7, which were likely to have

contributed to reconstitution of CMV-specific T cells and clearance of the CMV load, were specific for a single CMV pp65 epitope. Previous results of adoptive transfer of CMV-specific CD8+ T cells directed against a single CMV epitope have also shown reconstitution and proliferation of the infused T cells in vivo, which was associated with clearance of CMV viremia [7]. Incubation with a single peptide allows a simple analysis of the specificity of the T cell lines generated and of the follow-up of the T cells after administration. However, both in healthy individuals and in immunocompromised patients, spontaneous antiviral immunity is mediated by polyclonal CD8+ and CD4+ T cell responses directed against a broad repertoire of epitopes derived from multiple viral proteins [8-13]. Although the incubation with pools of overlapping peptides spanning entire proteins can be performed to activate and isolate CD8+ and CD4+ T cells simultaneously, the analysis of specificity and follow-up in vivo will be more complicated. However, peptide pools can be used for all donors and patients irrespective of their HLA, and will result in the isolation of larger absolute numbers of both CD8+ and CD4+ T cells specific for a broad repertoire of viral epitopes. Therefore, the use of viral peptide pools for generation of T cell lines is not only more convenient, but also likely to result in more rapid and effective immune reconstitution. As shown in chapter 6, the efficiency of antigen-specific CD8+ T cell activation using long synthetic peptides can be very variable, and is significantly reduced compared with minimal peptides. The competition between endogenous peptides presented in MHC class I and exogenously added long peptides, which are likely to require peptide trimming by peptidase activity, may result in physiological levels of presentation of the exogenous peptides in MHC class I and proper CD8+ T cell activation. In contrast, when using minimal peptides for in vitro stimulation, the optimal dose of minimal peptide should be determined empirically to prevent high levels of peptide presented in MHC class I, which may result in overstimulation and activation-induced cell death. Due to the large variability in efficiency of CD8+ T cell activation using exogenous long peptides, protein-spanning overlapping peptide pools should be composed of many different overlapping peptide variants to increase the repertoire of specific CD8+ T cells that can be activated properly.

As shown in chapter 3, the peripheral blood of all healthy CMV-seropositive adults tested contained high frequencies of CD8+ and CD4+ T cells specific for multiple epitopes derived from the immunodominant CMV pp65 protein. For treatment of CMV disease, it is therefore preferable to incubate PBMC with CMV pp65 peptide pool followed by CD137-based isolation, which is likely to allow generation of combined CD8+ and CD4+ T cell lines specific for multiple CMV pp65 epitopes from all healthy CMV seropositive donors. Since T cells specific for CMV pp65 only compose a small fraction of the complete CMV-specific T cell response [9], the inclusion of peptide pools derived from other immunogenic CMV

proteins, such as IE1, may be considered. The addition of other CMV peptide pools will further increase the absolute numbers of CMV-specific T cells isolated, and further extend the repertoire of epitopes recognized. However, it is likely that adoptive transfer of CD8+ and CD4+ T cells specific for CMV pp65 only will be sufficient for control of CMV disease.

In chapter 4, as a rationale for adoptive immunotherapy for treatment of HAdV disease in patients after alloSCT, resolution of disseminated HAdV infection was shown to be associated with development of coordinated CD8+ and CD4+ T cell responses against HAdV hexon in alloSCT recipients. Furthermore, although previous studies had only shown very low frequencies of predominantly CD4+ T cells specific for HAdV in healthy individuals, we demonstrated that coordinated CD8+ and CD4+ HAdV hexon-specific T cell lines could be generated from all healthy adults. These data indicated that both CD8+ and CD4+ T cells specific for HAdV hexon contribute to control of HAdV infection, and persist as memory T cell afterwards, which is similar to T cell responses described against most other viral infection. Although the use of HAdV penton peptide pool and peptide pools derived from other HAdV proteins may be considered to activate and isolate additional HAdV-specific T cells, the adoptive transfer of HAdV hexon-specific T cells is likely to be effective for treatment of HAdV disease.

The strategy of using specific viral peptide pools for stimulation of PBMC followed by CD137-based isolation and direct infusion of the virus-specific T cells can provide rapid and effective treatment of viral disease in alloSCT recipients. However, it will require significant expenses and efforts to maintain the appropriate facilities, reagents, personnel, and knowledge, for treatment of a limited number of patients. The availability of this treatment modality can also be exploited to isolate virus-specific T cells for a larger number of alloSCT recipients as prophylaxis of viral disease. Prophylactic administration of virus-specific T cells after alloSCT may be followed by in vivo activation of the virus-specific T cells during viral reactivations and de novo viral infections, resulting in adequate antiviral T cell responses preventing the development of viral disease. For this purpose, it would be preferable to stimulate PBMC with peptide pools derived from multiple viruses followed by CD137-based isolation. When performing T cell depleted alloSCT, the best moment to infuse the isolated virus-specific T cells will be soon after T cell depleting drugs have disappeared to provide most effective immune reconstitution due to homeostatic proliferation. The use of immune suppression as part of the alloSCT protocol or for treatment of GvHD may hamper T cell proliferation and function, but is not a contra-indication for adoptive transfer of virus-specific T cells. Although expenses will be significant to apply this treatment prophylactically to large numbers of patients, the increased turnover will reduce the costs of the reagents per treatment. Furthermore, it is likely that the prevention of viral morbidity

will result in a reduction of total expenses for treatment of viral morbidity, and in improvement of the quality of life of alloSCT recipients. An additional advantage of prophylaxis would be that peptide pools derived from other pathogens that cause serious morbidity after alloSCT, such as varicella-zoster virus, human polyomaviruses, candida albicans, and aspergillus, may be included at low costs in the same prophylactic procedure. Due to the large number of eligible patients and predetermined moment of infusion, the safety and effectiveness of prophylactic administration of virus-specific T cells will be more feasible to assess in randomized clinical trials compared with therapeutic administration.

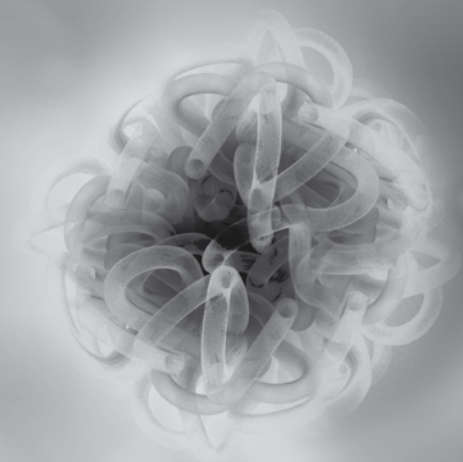
In this thesis and previous studies, the development of adoptive immunotherapy using virus-specific T cells has focused on the generation of virus-specific T cell lines from healthy seropositive donors for adoptive transfer to their alloSCT recipients. However, virus-specific T cell lines can not be generated readily from seronegative alloSCT donors. Although no licensed CMV vaccine has become available yet, a recent case report suggested that current experimental CMV vaccines can be sufficient to generate CMV-specific memory T cells in CMV-seronegative donors that can be transferred adoptively [14]. An alternative approach for patients transplanted from a CMV-seronegative donor would be the generation of CMV-specific T cell lines from PBMC that are harvested from the patient before transplantation, as has already been shown to be feasible in chapter 7. The risk of graft rejection by patient T cells and the risk of introducing malignant patient cells should be carefully weighed against the risk and severity of viral disease. The specific isolation of virus-specific T cells from PBMC will be even more important in this setting, to minimize the infusion of potentially donor-reactive patient T cells. An additional group of patients that could benefit from cellular immunotherapy for viral disease are solid organ transplant (SOT) recipients. A recent study has shown that CMV-specific T cell lines could be generated from patients after SOT who received immune suppression [15]. In this population, immunotherapy using expanded patient-derived virus-specific T cells can be considered for refractory viral disease.

To ensure maximal safety, effectiveness, and feasibility of clinical application of virus-specific T cell products, release criteria should be carefully defined. The release criteria as defined in the clinical study in chapter 7 are suitable, including DNA profiling of the starting material, absence of microbiological contamination, detection of a high frequency of virus-specific T cells, absence of significant numbers of B cells and non-virus-specific T cells, and functional responses against virus-loaded patient cells, but not unloaded patient cells. Direct infusion with limited pre-release quality control and retrospective testing of an in vitro cultured fraction afterwards can be warranted when using high quality reagents and validated procedures in centers with experienced technicians and staff. Since the adoption of

the European Directive 2003/63/EC and the Advanced Therapy Medicinal Product (ATMP) regulation, the generation and infusion of virus-specific T cell lines should comply with all relevant regulations for investigational medicinal products for human use, including GMP and GCP guidelines. Although these regulations require a lot of effort in academic centers, they will contribute to increased quality of cellular products and are necessary to establish adoptive immunotherapy as standard clinical practice. To continue translation of promising cellular therapies towards clinical benefit, the cooperation between clinicians, researchers, pharmacists, and biotechnology companies will even become more crucial.

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Nederlandse Samenvatting
List of publications
List of abbreviations
Nawoord
Curriculum Vitae

Nederlandse samenvatting

Cellulaire afweer tegen virussen

In gezonde mensen worden virale infecties onder controle gehouden door virus-specifieke antilichamen en virus-specifieke T cellen. Virus-specifieke antilichamen binden aan virusdeeltjes die in het lichaam circuleren, en voorkomen dat deze virusdeeltjes gezonde cellen kunnen infecteren. Indien antilichamen de infectie van cellen niet volledig kunnen voorkomen, kunnen virusdeeltjes na infectie van cellen zich in die cellen gaan vermenigvuldigen, waardoor nieuwe virusdeeltjes gevormd worden en de cel kapot gaat. Dit proces kan zich uiten in ziekteverschijnselen die karakteristiek zijn voor het betreffende virus. De tweede vorm van specifieke afweer tegen virale infecties zijn virus-specifieke T cellen, een type witte bloedcellen, die viraal geïnfecteerde cellen kunnen herkennen en vernietigen. De T cellen herkennen door middel van hun T cel receptor een specifiek stukje van het virus, een viraal peptide van ongeveer 9 tot 15 aminozuren, dat wordt gepresenteerd door major histocompatibiliteit complex (MHC) moleculen op het oppervlak van de geïnfecteerde cellen. Over het algemeen is de T cel receptor van een enkele T cel specifiek voor een bepaalde combinatie van een viraal peptide en een MHC molecuul. Er wordt onderscheid gemaakt tussen CD8+ T cellen die peptiden herkennen in MHC klasse I moleculen, en CD4+ T cellen die peptiden herkennen in MHC klasse II moleculen. Tijdens een eerste virale infectie worden naïeve virus-specifieke T cellen geactiveerd. Deze ontwikkelen zich tot functionele effector T cellen, die zich kunnen vermenigvuldigen en de virale infectie onder controle kunnen brengen. Vervolgens gaat een groot gedeelte virus-specifieke effector T cellen dood, en zullen een aantal effector T cellen differentiëren naar *memory* T cellen, die in kleine aantallen langdurig aanwezig blijven om bescherming te bieden tegen volgende infecties met hetzelfde virus. Na herkenning van viraal antigeen kunnen virus-specifieke *memory* T cellen snel een immuunrespons veroorzaken door vermenigvuldiging, productie van ontstekingsmediatoren of cytokines, zoals interferon-gamma (IFNg), tumor-necrosis factor alpha (TNFa) and interleukin-2 (IL-2), en directe vernietiging van de geïnfecteerde cellen. CD8+ T cellen hebben vooral het vermogen om geïnfecteerde cellen te vernietigen en CD4+ T cellen hebben vooral een ondersteunende rol bij de ontwikkeling van specifieke CD8+ T cellen en antilichamen door productie van cytokines of ontstekingsmediatoren, hoewel ze ook direct geïnfecteerde cellen kunnen vernietigen.

Niet alle virussen worden na een acute infectie volledig uit de patiënt verwijderd. Door evolutionaire interactie tussen virale pathogenese en humane cellulaire afweerreacties hebben sommige virussen eigenschappen ontwikkeld om te kunnen persisteren. Persistentie kan resulteren in chronische infecties, waarbij actieve virusdeeltjes voortdurend blijven delen in geïnfecteerde cellen, of in latente infecties, waarbij het virale genoom latent aanwezig blijft in cellen zonder zich te vermenigvuldigen. Vanuit deze latente vorm kan het virus reactiveren en opnieuw gaan delen.
Cellulaire afweer tegen virussen kan verstoord zijn door genetische afwijkingen in het immuunsysteem. Daarnaast kan cellulaire afweer geremd worden door immuunsuppressieve geneesmiddelen, die nodig zijn ter voorkoming van afstoting in ontvangers van een getransplanteerd orgaan of stamcellen van een gezonde donor, of ter behandeling van auto-immuunziekten. In immuundeficiënte patiënten kan een gebrek aan antivirale cellulaire afweer leiden tot ernstige ziekte en mortaliteit door nieuwe virale infecties of reactivering van latente virale infecties.

Allogene stamceltransplantatie

Het transplanteren van stamcellen van een gezonde donor, allogene stamceltransplantatie (alloSCT), is een potentieel genezende behandeling van hematologische maligniteiten en aangeboren hematopoïetische ziekten.. Patiënten met hematologische maligniteiten die in aanmerking komen voor alloSCT worden voor transplantatie geconditioneerd met hoge dosis chemotherapie, bestraling, en immuunsuppressie om de overgebleven maligne cellen te vernietigen, en om het hematopoïetische systeem van de patiënt te vernietigen en te onderdrukken en zo plaats te maken voor het hematopoïetische systeem van de gezonde donor . Traditioneel werden sterke myelo-ablatieve conditioneringen toegepast die gepaard gingen met forse toxiciteit, waardoor de behandeling alleen bij jongere patiënten toegepast kon worden. Inmiddels zijn conditioneringen met gereduceerde intensiteit ontwikkeld om alloSCT voor oudere patiënten of patiënten met bijkomende ziekten mogelijk te maken. Vervolgens worden donor hematopoïetische stamcellen toegediend, die zich kunnen vermenigvuldigen en ontwikkelen tot rijpe bloedcellen en het hematopoïetische systeem van de patiënt kunnen vullen met donor bloedcellen. De hematopoïetische stamcellen kunnen direct uit het beenmerg van de donor geoogst worden, maar worden tegenwoordig meestal uit het perifere bloed geoogst na mobilisatie vanuit het beenmerg door toediening van *granulocyte colony stimulating factor* (G-CSF).
Indien in het stamceltransplantaat naast stamcellen ook donor T cellen aanwezig zijn, kunnen deze T cellen zowel gunstige als ongunstige klinische effecten veroorzaken. Virus-

specifieke donor T cellen kunnen direct bescherming bieden tegen virale infecties. Hoewel patiënt en donor *gematched* worden voor MHC moleculen, kan donor afweer ook gericht zijn tegen patiënt cellen. Genetische verschillen in eiwitten tussen patiënt en donor kunnen leiden tot herkenning van patiënt antigenen door donor T cellen. Herkenning van overgebleven maligne patiënt cellen door donor T cellen kan leiden tot een langdurige afweerreactie tegen de maligne cellen, het zogenaamde *graft-versus-leukemia* effect, en daardoor genezing van de ziekte. Herkenning van gezonde patiënt cellen door donor T cellen kan echter leiden tot ernstige schade aan met name huid, darmen, en lever, en wordt *graft-versus-host* ziekte genoemd. Om de frequentie en ernst van *graft-versus-host* ziekte te verminderen kunnen donor T cellen uit het transplantaat verwijderd worden. Echter, de afwezigheid van virus-specifieke donor T cellen vergroot de kans op virale complicaties, en de vermindering van het *graft-versus-leukemia* effect vergroot de kans op persistentie van maligne cellen. Het toedienen van donor T cellen kan daarom worden overwogen om virale infecties of terugkeer van de maligniteit te voorkomen of te behandelen.

Virale infecties na allogene stamceltransplantatie

Het langzame herstel van virus-specifieke T cellen na alloSCT gaat gepaard met een lange periode waarin ernstige virale infecties kunnen optreden. De belangrijkste virale pathogenen die ernstige ziekte en mortaliteit veroorzaken na alloSCT zijn de veel voorkomende virussen cytomegalovirus, Epstein-Barr virus, en humaan adenovirus. Daarnaast kan varicella-zoster virus reactiveren in alloSCT ontvangers wat kan leiden tot herpes zoster, met gelokaliseerde plekken op de huid en zenuwpijn. Ook andere herpesvirussen en humaan polyomavirus kunnen reactiveren vanuit latente infectie en leiden tot ernstige ziekte. In dit proefschrift is de aandacht gericht op cytomegalovirus, Epstein-Barr virus, en humaan adenovirus, omdat deze virussen een duidelijke invloed hebben op de mortaliteit na alloSCT.
Cytomegalovirus (CMV) kan na een eerste infectie persisteren in latente vorm in endotheelcellen en myeloïde cellen. Afhankelijk van het geografische gebied is 40-90% van de bevolking CMV seropositief. In gezonde personen wordt vermenigvuldiging van CMV voorkomen door CD8+ en CD4+ *memory* T cellen, die antigene peptiden herkennen van vele CMV eiwitten, waaronder de eiwitten CMV pp65 en CMV IE1. In alloSCT ontvangers kan de verstoorde controle door CMV-specifieke T cellen leiden tot CMV reactivering en ziekte, die zich vooral uit in de longen en darmen, en ook in de lever, ogen en hersenen. In afwezigheid van adequate cellulaire CMV-specifieke afweer kan CMV reactivering zich ontwikkelen tot ernstige ziekte, die geassocieerd is met hoge mortaliteit. Behandeling met

geneesmiddelen als ganciclovir en foscarnet kan effectief zijn, maar gaat gepaard met hematologische en renale toxiciteit, en is vaak onvoldoende voor langdurige controle van CMV. Herstel van CMV-specifieke T cellen kan wel langdurige bescherming tegen CMV ziekte geven na alloSCT.
Epstein-Barr virus (EBV) is latent aanwezig bij meer dan 90% van de bevolking, en wordt in gezonde personen onder controle gehouden door EBV-specifieke CD8+ en CD4+ T cellen. EBV-specifieke T cellen herkennen virale peptiden, zowel afkomstig van eiwitten die tot expressie komen tijdens de latente fase als van eiwitten die tot expressie komen tijdens vermenigvuldiging van het virus. In geïnfecteerde B cellen kan EBV de regulatie van celdeling beïnvloeden, wat uiteindelijk kan leiden tot de ontwikkeling van kanker. Door gebrek aan controle van EBV door EBV-specifieke T cellen in patiënten na alloSCT kan EBV reactivering leiden tot maligne ontaarding van geïnfecteerde B cellen. Deze EBV-geassocieerde maligniteiten kunnen vervolgens leiden tot ernstige ziekte en mortaliteit. EBV-geassocieerde maligniteiten kunnen behandeld worden met antilichamen tegen CD20, dat op het oppervlak van maligne B cellen zit. Deze behandeling is echter niet altijd effectief.
Humaan adenovirus (HAdV), waarvan 51 verschillende types bekend zijn, veroorzaakt vooral infecties in kinderen tussen 6 maanden en 5 jaar, met symptomen van de luchtwegen, darmen en huid. Het is niet helemaal duidelijk of HAdV latent aanwezig blijft in de luchtwegen en darmen, of dat de ziekte altijd veroorzaakt wordt door een nieuwe infectie. In gezonde personen zijn lage frequenties van vooral CD4+ T cellen gemeten, die specifiek waren voor met name het HAdV hexon eiwit. Vooral bij kinderen kan HAdV infectie na alloSCT leiden tot ernstige ziekte en mortaliteit. De effectiviteit van antivirale geneesmiddelen zoals ribavirine en cidofovir voor HAdV infectie is niet aangetoond. Herstel van HAdV-specifieke T cellen kan wel zorgen voor langdurige controle van HAdV infectie. Het is nog niet bekend welke eiwitten herkend kunnen worden door deze HAdV-specifieke T cellen. Daarnaast is het onduidelijk of zowel CD8+ als CD4+ T cellen belangrijk zijn voor de bescherming tegen HAdV ziekte na alloSCT.

Behandeling van virale infecties met cellulaire immuuntherapie

Omdat het herstel van virus-specifieke T cellen gepaard gaat met langdurige bescherming tegen virale ziekte na alloSCT, is het overbrengen van virus-specifieke donor T cellen naar de ontvanger een aantrekkelijke strategie ter voorkoming of ter behandeling van virale ziekten. Bij toediening van niet-geselecteerde donor T cellen kunnen ook T cellen worden overgebracht die gezond weefsel herkennen en daardoor *graft-versus-host* ziekte veroorzaken. Om herstel van virus-specifieke T cellen te bereiken met een minimaal risico

op *graft-versus-host* ziekte zijn technieken ontwikkeld om virus-specifieke T cellen uit donor perifeer bloed te selecteren.
De selectie van virus-specifieke T cellen is mogelijk na stimulatie van perifeer bloed met virale antigenen, waardoor de geactiveerde virus-specifieke T cellen cytokines uitscheiden en moleculen op het oppervlak van de cel tot expressie brengen. Om gelijktijdig CD8+ en CD4+ T cellen te activeren die specifiek zijn voor verschillende virale peptiden, kunnen virale eiwitten gebruikt worden om cellen in perifeer bloed te stimuleren. Er zijn aanwijzingen dat het toevoegen van volledige virale eiwitten vooral leidt tot presentatie van virale peptiden in MHC klasse II en activering van virus-specifieke CD4+ T cellen, en minder van CD8+ T cellen. Echter, als meerdere synthetische lange peptiden die elkaar gedeeltelijk overlappen en zo de hele sequentie van het virale eiwit omvatten gebruikt worden kan dat leiden tot presentatie van virale peptiden in zowel MHC klasse I en II en activering van virus-specifieke CD8+ en CD4+ T cellen. Het is nog niet duidelijk op welke manier deze lange peptiden uiteindelijk in de MHC klasse I moleculen gepresenteerd worden.
Na activering kunnen virus-specifieke T cellen geïsoleerd worden op basis van IFNg productie. Het uitgescheiden IFNg wordt hierbij door een antilichaam aan het celmembraan gebonden, en vervolgens wordt een tweede IFNg-specifiek antilichaam met een magnetisch deeltje gebruikt om de aldus gelabelde cellen met behulp van een magneet te isoleren. Deze techniek is succesvol toegepast voor isolatie van T cellen specifiek voor CMV, EBV, en HAdV. De kinetiek van IFNg productie na activering van virus-specifieke T cellen is echter nog niet onderzocht, en het is onduidelijk of alle virus-specifieke T cellen op hetzelfde moment na activering IFNg produceren en dus ook gelijktijdig op basis van IFNg geïsoleerd kunnen worden.
Omdat isolatie op basis van IFNg technisch lastig uitvoerbaar is, is men gaan onderzoeken of het ook mogelijk is T cellen te isoleren op basis van moleculen die na activering op het oppervlak van virus-specifieke T cellen verschijnen. Het molecuul CD137 speelt een rol bij de stimulatie van T cellen en verschijnt ongeveer 24 uur na activatie op het oppervlak van CD8+ T cellen specifiek voor CMV en EBV. Op vergelijkbare wijze als bij isolatie op basis van IFNg productie kon een magnetisch gelabeld CD137-specifiek antilichaam worden gebruikt om virus-specifieke T cellen te isoleren. Echter, het is nog onduidelijk of CD137 op CD8+ T cellen specifiek voor andere virussen en op virus-specifieke CD4+ T cellen ook tot expressie komt, en hoe de kinetiek van CD137 expressie precies verloopt.
Inmiddels is een aantal exploratieve klinische studies uitgevoerd waarin patiënten met virale infecties na alloSCT zijn behandeld met virus-specifieke donor T cellen. In deze studies leek de behandeling met virus-specifieke T cellen veilig en niet te leiden tot

ontwikkeling van *graft-versus-host* ziekte. Daarnaast werden na toediening toegenomen frequenties virus-specifieke T cellen in perifeer bloed waargenomen, die zich vervolgens ook in het lichaam konden vermenigvuldigen en langdurig aanwezig bleven. Omdat de studies niet gerandomiseerd waren is het lastig om conclusies te trekken over de effectiviteit van de behandelingen. In de meeste studies werd wel een associatie gezien tussen het toedienen van virus-specifieke T cellen en een afname van virusdeeltjes in bloed of langdurige controle van de virale infecties. Op basis hiervan lijkt antivirale cellulaire immuuntherapie effectief te zijn. De methoden in deze studies waren erg verschillend wat betreft de virale antigenen die gebruikt werden, de isolatietechniek, de kweekperiode, en het aantal virus-specifieke T cellen dat werd toegediend. Daarnaast waren de patiënten erg verschillend wat betreft hun conditionering, transplantieprotocol, virale infecties, en het moment van toediening van virus-specifieke T cellen. Sommige methoden die ontwikkeld zijn om virus-specifieke T cellen te isoleren kunnen klinisch (nog) niet toegepast worden vanwege de huidige regelgeving of de afwezigheid van de juiste kwaliteit van de reagentia. Andere methoden kunnen wel klinisch toegepast worden, maar zijn nog niet geoptimaliseerd om consistente productie van T cel lijnen met hoge specificiteit voor virale antigenen mogelijk te maken. De optimale strategie voor cellulaire immuuntherapie voor virale infecties na alloSCT en de klinische waarde van deze behandeling zijn daarom nog niet duidelijk.

Dit proefschrift

In dit proefschrift zijn methoden onderzocht om op een efficiënte wijze virus-specifieke CD8+ en CD4+ T cellen in perifeer bloed te activeren om detectie en isolatie van deze specifieke cellen mogelijk te maken. Gebruik makende van deze methoden, is de rol van HAdV-specifieke T cellen onderzocht in de bescherming tegen HAdV ziekte na alloSCT, om op basis hiervan een HAdV-specifieke cellulaire immuuntherapie te ontwikkelen. Daarnaast zijn methoden ontwikkeld voor efficiënte isolatie van geactiveerde virus-specifieke T cellen uit perifeer bloed. De klinisch toepasbare T cel lijnen met hoge specificiteit tegen meerdere virale antigenen, die met deze methoden geproduceerd kunnen worden, kunnen gebruikt worden om de klinische waarde van cellulaire immuuntherapie voor virale infecties na alloSCT vast te stellen.

Cytomegalovirus-specifieke T cellen als cellulaire immuuntherapie

In **hoofdstuk 2** is de IFNg respons van CMV-specifieke CD8+ T cellen onderzocht om deze cellen op het juiste moment te kunnen isoleren op basis van IFNg productie. De productie van IFNg was maximaal na 4 uur stimulatie en nam daarna snel af, ondanks de voortdurende aanwezigheid van het specifieke CMV peptide. Door de sterkte van stimulatie te variëren werd duidelijk dat een te sterke stimulatie kan leiden tot een nog snellere afname van de IFNg productie en een verminderde vermenigvuldiging. Op basis van deze gegevens werd een optimaal schema voor isolatie van CMV-specifieke CD8+ T cellen gekozen. Met deze methode konden T cel lijnen geproduceerd worden met een mediaan van 91% CMV-specifieke T cellen na 9-11 dagen, en nauwelijks specifieke cellen in de achtergebleven fracties. De functionaliteit van de CMV-specifieke T cellen was na kweken in vitro vergelijkbaar met die van de CMV-specifieke T cellen in het uitgangsmateriaal.
Het toedienen van zowel CD8+ als CD4+ T cellen specifiek voor meerdere CMV peptiden is waarschijnlijk effectiever voor bescherming tegen CMV ziekte na alloSCT. In **hoofdstuk 3** is beschreven dat in alle gezonde volwassenen zowel CD8+ als CD4+ T cellen specifiek voor het CMV pp65 eiwit aangetoond konden worden, terwijl T cellen specifiek voor het CMV IE1 eiwit in slechts 45% van de gezonde personen gedetecteerd konden worden. Vervolgens werden twee verschillende CMV antigenen getest om gelijktijdig zowel CD8+ als CD4+ CMV-specifieke T cellen te activeren en te isoleren. Stimulatie met pools van overlappende peptiden van 15 aminozuren in lengte resulteerde in efficiënte activering van zowel CD8+ als CD4+ specifieke T cellen, terwijl stimulatie met een volledig eiwit resulteerde in efficiënte activering van specifieke CD4+ T cellen, maar slechts een deel van de specifieke

CD8+ T cellen. De kinetiek van IFNg productie na stimulatie met de peptide pools was vergelijkbaar met die na stimulatie met de losse peptiden, en hetzelfde voor CD8+ als CD4+ T cellen. Op basis van deze gegevens konden T cel lijnen geproduceerd worden met hoge frequenties CD8+ en CD4+ T cellen specifiek voor meerdere CMV peptiden, die gebruikt kunnen worden voor behandeling van CMV ziekte na alloSCT.

Humaan adenovirus-specifieke T cellen als cellulaire immuuntherapie

Hoewel al eerder was aangetoond dat de ontwikkeling van HAdV-specifieke T cellen geassocieerd was met bescherming tegen HAdV ziekte na alloSCT, was het niet duidelijk welke HAdV eiwitten hierbij herkend werden, en of zowel CD8+ als CD4+ T cellen hierbij belangrijk waren. In **hoofdstuk 4** is daarom de ontwikkeling van HAdV-specifieke T cellen bestudeerd in perifeer bloed van kinderen en volwassenen na alloSCT die spontaan herstelden van gedissemineerde HAdV ziekte. Klaring van HAdV na alloSCT bleek gepaard te gaan met het verschijnen van zowel CD8+ als CD4+ T cellen specifiek voor HAdV hexon peptiden. Daarom werd een methode ontwikkeld om T cel lijnen te produceren met hoge specificiteit tegen deze peptiden. Stimulatie van perifeer bloed van gezonde volwassenen met HAdV hexon peptide pool gevolgd door IFNg isolatie resulteerde in gecombineerde CD8+ en CD4+ HAdV hexon-specifieke T cel lijnen. Deze HAdV-specifieke T cel lijnen herkenden meerdere HAdV hexon peptiden, waaronder zowel bekende als nieuwe antigene peptiden, en herkenden ook HAdV geïnfecteerde cellen. Deze gegevens bieden een onderbouwing en strategie voor de behandeling van HAdV ziekte na alloSCT met HAdV hexon-specifieke donor T cellen.

Gelijktijdige isolatie van T cellen specifiek voor meerdere virussen

In **hoofdstuk 5** werd een methode ontwikkeld om CD8+ en CD4+ T cellen specifiek voor meerdere virussen tegelijk te kunnen isoleren. Om ervoor te zorgen dat de methode bruikbaar was voor verschillende virussen had het onderzoek betrekking op T cellen specifiek voor CMV, EBV, HAdV en influenza. Daarnaast werd zowel isolatie op basis van IFNg productie als isolatie op basis van CD137 expressie onderzocht. Na activering produceerden de virus-specifieke T cellen IFNg en TNFa, en brachten ze gelijktijdig CD137 tot expressie op het oppervlak, terwijl slechts een gedeelte van de cellen IL-2 produceerde. Ondanks verschillen in kinetiek van IFNg productie tussen de virus-specifieke T cel populaties konden de geactiveerde CD8+ en CD4+ virus-specifieke T cellen gelijktijdig efficiënt geïsoleerd worden op basis van zowel IFNg als CD137. Isolatie op basis van CD137

expressie is eenvoudiger uitvoerbaar dan isolatie op basis van IFNg productie. Echter, op dit moment is alleen de IFNg isolatie methode beschikbaar voor klinische toepassing. De in deze studie ontwikkelde methode voor gelijktijdige isolatie van CD8+ en CD4+ T cellen specifiek voor meerdere peptiden van verschillende virussen bleek efficiënt en is breed toepasbaar.

Gebruik van synthetische lange peptiden als viraal antigeen

De gegevens uit hoofdstuk 3-5 laten zien dat in vitro stimulatie van perifeer bloed met synthetische lange peptiden resulteert in efficiënte activering van zowel CD8+ als CD4+ virus-specifieke T cellen. Daarnaast is eerder aangetoond dat wanneer synthetische lange peptiden gebruikt worden voor vaccinatie dit ook resulteert in een efficiënte activering van specifieke CD8+ T cellen in vivo. Echter, normaal gesproken binden kortere peptiden van 9-11 aminozuren in MHC klasse I moleculen, en de mechanismen die leiden tot MHC klasse I presentatie van lange peptiden zijn onbekend. In **hoofdstuk 6** werd aangetoond dat de efficiëntie van activering van specifieke CD8+ T cellen met lange peptiden erg variabel is. Het knippen van lange peptiden bleek niet te verlopen volgens de klassieke route van MHC klasse I presentatie. Hoewel lange peptiden wel direct konden binden in MHC klasse I moleculen, is het zeer waarschijnlijk dat ze geknipt moeten worden voor MHC klasse I presentatie en activering van CD8+ T cellen. Omdat de klassieke route niet nodig bleek, zijn waarschijnlijk peptidase enzymen nodig, die buiten de cel, op het oppervlak van de cel, of binnen de cel in endosomen aanwezig kunnen zijn. Daarnaast leek het erop dat het knippen tot de juiste peptiden werd gefaciliteerd door de binding van de lange peptiden in MHC klasse I moleculen. Dit mechanisme kan belangrijk zijn voor het activeren van specifieke CD8+ T cellen met synthetische lange peptiden bij cellulaire immuuntherapie of bij vaccinatie.

Klinische resultaten van adoptieve immuuntherapie voor cytomegalovirus ziekte

In **hoofdstuk 7** werden de resultaten gepresenteerd van een klinische studie voor de behandeling van CMV ziekte na alloSCT door middel van cellulaire immuuntherapie met CMV-specifieke CD8+ T cellen. De geïncludeerde patiënten reageerden onvoldoende op de standaard behandeling met antivirale geneesmiddelen. CMV-specifieke CD8+ T cellen werden geïsoleerd uit perifeer bloed op basis van IFNg productie na stimulatie met één of twee MHC klasse I gerestricteerde CMV pp65 peptide(n). Er werden 14 T cel lijnen geproduceerd met hoge frequenties CMV-specifieke CD8+ T cellen, waarvan er 7 zijn

toegediend aan 5 patiënten. Na toediening werden geen bijwerkingen waargenomen, werden CMV-specifieke CD8+ T cellen gedetecteerd in perifeer bloed, en de CMV infecties kwamen onder controle. In 2 patiënten was er een duidelijke relatie tussen het moment van toedienen en de opkomst van CMV-specifieke CD8+ T cellen in perifeer bloed en controle van de infectie. Deze studie heeft laten zien dat het toedienen van CMV-specifieke CD8+ T cellen uitvoerbaar, veilig, en effectief kan zijn.

De resultaten beschreven in dit proefschrift onderbouwen de rationale voor het toepassen van cellulaire immuuntherapie voor virale infecties na alloSCT. Daarnaast zijn efficiënte en bruikbare methoden ontwikkeld om gecombineerde CD8+ en CD4+ T cel lijnen te produceren met hoge specificiteit voor meerdere peptiden van verschillende virale eiwitten. Deze methoden zijn breed toepasbaar en maken het mogelijk om de klinische waarde van cellulaire immuuntherapie voor behandeling van virale infecties na alloSCT in klinische studies te onderzoeken.

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Zandvliet ML, Falkenburg JHF, Oostendorp J, Guchelaar H-J, Meij P. Cellular immunotherapy for viral infections after allogeneic stem cell transplantation. Submitted

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List of abbreviations

AlloSCT	Allogeneic stem cell transplantation
APC	Antigen-presenting cell
ATMP	Advanced Therapy Medicinal Product
BFA	Brefeldin A
CCR7	CC-chemokine receptor 7
CD40L	CD40 ligand or CD154
CD62L	CD62 ligand
cDNA	Complementary deoxyribonucleic acid
CDR3	Complementarity-determining region 3
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
EBV	Epstein-Barr virus
EBV-LCL	Epstein-Barr virus-transformed B cell lines
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FLU	Influenza virus
GCP	Good clinical practice
G-CSF	Granulocyte colony stimulating factor
GCV	Ganciclovir
GMP	Good manufacturing practice
GvHD	Graft-versus-host disease
HAdV	Human adenovirus
HLA	Human leukocyte antigen
IFNγ	Interferon-gamma
IL-2	Interleukin-2
IL-15	Interleukin-15
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cells
PTLD	Post-transplant lymphoproliferative disease
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TNFα	Tumor-necrosis factor alpha

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Curriculum Vitae

Maarten L. Zandvliet is geboren op 11 november 1979 te Alkmaar. Na het behalen van het VWO diploma aan het Gymnasium Beekvliet te St. Michielsgestel in 1998, begon hij in datzelfde jaar met de studie farmacie aan de Universiteit Utrecht. Na een wetenschappelijke stage bij de afdeling Hypertensie en Nefrologie van het Universitair Medisch Centrum Utrecht (Dr. G.B. Braam) werd de doctoraalfase farmacie in 2003 cum laude afgerond. Vervolgens werd gestart met de postdoctorale apothekersopleiding aan de Universiteit Utrecht. Na een wetenschappelijke stage bij de Faculty of Pharmacy van de University of Montréal te Canada (Dr. P. Moreau) behaalde hij zijn apothekersdiploma en ontving hij de Studentenprijs van de Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie in 2005. In april van dat jaar begon hij als apotheker-onderzoeker met het in dit proefschrift beschreven onderzoek op het laboratorium voor Experimentele Hematologie van de afdeling Hematologie in samenwerking met de afdeling Klinische Farmacie en Toxicologie van het Leids Universitair Medisch Centrum. Het onderzoek werd begeleid door Dr. P. Meij, Prof. Dr. J.H.F. Falkenburg, en Prof. Dr. H.-J. Guchelaar. In maart 2009 is hij gestart met de opleiding tot ziekenhuisapotheker (opleider Prof. Dr. A.G. Vulto) bij de Apotheek van het Erasmus MC te Rotterdam.