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

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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 IFN\(\gamma\) during the isolation procedure can be isolated using the IFN\(\gamma\) capture assay. Murine virus-specific T cells have shown to constantly produce IFN\(\gamma\) between 1 and 24 hours after activation in the continued presence of antigen [34-36]. Production of IFN\(\gamma\) 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 IFN\(\gamma\) 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 IFN\(\gamma\) 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 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.

In this study, we performed detailed analysis of the IFN\(\gamma\) response of CMV-specific CD8+ T cells and defined optimal conditions for activation and isolation of CMV-specific CD8+ T cells based on IFN\(\gamma\) secretion. Kinetic studies showed that the rapid induction of IFN\(\gamma\) 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 IFN\(\gamma\) 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\(\text{2}\), and stimulated with 10^3 to 10^11 M CMV pp65-derived peptide YSEHPTFTSQY (YSE/HLA-A*0101), NLPVMVATV (NLP/HLA-A*0201), TPRVTGGGAM (TPR/HLA-B*0702), RPHERNQFTVL (RPH/HLA-B*0702), or IPSINVHHY (IPS/HLA-B*3501).

Flow cytometric analyses

Cells were stained with FITC-labeled CD3, CD14, CD22 (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 IFN\(\gamma\) mAb or PE-labeled TNF\(\alpha\) 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 ELISAArrayTM kit (SA Biosciences, Frederick, MD, USA), performed according to the manufacturer’s instructions.

Detection, isolation, and culture of IFN\(\gamma\)-secreting cells

Cells were harvested, thoroughly washed in PBS, and IFN\(\gamma\)-secreting cells were stained by the IFN\(\gamma\) capture assay (Milenyi Biotech, 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 1x10⁶ cells/ml in culture medium, containing 50 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) with 10x10⁶ 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 10x10⁶ 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 10x10⁶ cells/ml with 10⁻⁶ to 10⁻⁹ M CMV peptide and 50 UI/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](image_url)
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 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 (R²=0.994) is further illustrated in Figure 2B.

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 IFN-γ production by CMV-specific CD8+ T cells. Real-time intracellular IFN-γ 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 IFN-γ-producing cells after stimulation with 10^{-10} to 10^{-5} M HLA-B7 restricted TPR peptide over time. C) Percentage of IFN-γ-producing cells after stimulation with 10^{-5} to 10^{-4} M HLA-B35 restricted IPS peptide over time.

To investigate whether the different peptide concentrations required for maximal IFN-γ 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 IFN-γ 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 IFN-γ produced by activated peptide-specific cells were determined by the peptide concentration. A maximal IFN-γ 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 IFN-γ 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 IFN-γ, 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^{-7} 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 of CMV-specific T cells. 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.
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 7. Intracellular staining of IFN-γ 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 IFN-γ 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.

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References


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