Preferential Expansion of Human Virus-Specific Multifunctional Central Memory T Cells by Partial Targeting of the IL-2 Receptor Signaling Pathway: The Key Role of CD4+ T Cells

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Preferential Expansion of Human Virus-Specific Multifunctional Central Memory T Cells by Partial Targeting of the IL-2 Receptor Signaling Pathway: The Key Role of CD4+ T Cells

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Effecter memory T cells are effective in controlling acute infections, but central memory T cells play a key role in long-lasting protection against viruses and tumors. In vivo/in vitro challenge by Ag commonly supports the generation of effector memory T cells with limited longevity. To our knowledge, this study demonstrates for the first time in the human system and under rechallenge conditions that targeting IL-2R by partial mammalian target of rapamycin inhibition or blocking IL-2Rα enriches human CD4+/CD8+ central memory T cells within the virus-specific T cell product associated with enhanced functionality (i.e., multicytokine secretors, including IL-2; enhanced CD137 and CD107a expression on CD8+ and CD4+ T cells, respectively; and killing infected target cells). Remarkably, the effects on CD8+ T cells are mainly mediated via the enhancement of CD4+ T cell function. The data reveal new insights into the role of CD4+ T cell support for the quality of CD8+ T cell memory, even under rechallenge conditions. Moreover, our method offers a new approach to improve the long-lasting efficacy of adoptive T cell therapy in patients. The Journal of Immunology, 2012, 188: 5189–5198.
before/after expansion revealed very few, if any, central memory T cells \( (T_{CM}) \), whereas a variable, but detectable, number of virus-specific \( T_{CM} \) contributed to the EBV-specific response \( (1, 10) \). According to these and other experimental data, the relapse might be due to the late-differentiation phenotype of the infused T cells and, therefore, their insufficient longevity in vivo \( (11) \). Late-differentiated effector memory T cells \( (T_{EM}) \) and terminally differentiated effector T cells \( (T_{EMRA}) \) express powerful effector function but are not able to establish long-lasting protective memory because of diminished proliferative potential and brief survival following antegenic rechallenge \( (12) \). Several preclinical studies indicated the superior lasting engraftment of \( T_{CM} \)-derived T cells. The persistence of transferred cells correlates with therapeutic efficacy in animals \( (13) \). Balancing engraftment fitness with a strong Ag-specific effector function might lead to the best therapeutic outcome.

The mammalian target of rapamycin (mTOR) has been described as a major regulator of memory CD8\(^+\) T cell differentiation \( (14, 15) \). Several studies described that mTOR inhibition by rapamycin treatment during the expansion and contraction phases improved both the quality and quantity of memory T cells after primary immunization in preclinical models \( (14–17) \). Rapamycin seems to prolong the survival of Ag-specific T cells \( (18, 19) \). It paradoxically enhances the Ag-specific CD8\(^+\) T cell response and accelerates memory T cell formation in vivo \( (15) \). Thus, mTOR inhibition enhances the magnitude and quality of an Ag-specific T cell response \( (14, 15) \). However, it remains unclear whether mTOR inhibition might also support the targeted composition of T cell products for improved adoptive T cell therapy in patients, particularly because, in contrast to the preclinical models, the memory response was already established in vivo.

In this article, we demonstrate a novel method that enriched human virus-specific \( T_{CM} \) in the T cell product, expanded to clinically useful numbers under GMP-conform conditions by modifying IL-2R signaling. This study reveals a potent strategy to generate Ag-specific T cells with a high functional capacity and, presumably, with a robust long-term persistence and functionality in vivo.

### Materials and Methods

#### Cell culture

All cell cultures and assays were performed in complete media (RPMI 1640 supplemented with 2 mM t-glutamine, penicillin [100 U/ml], and streptomycin, all from Biochrom), containing 10% FBS (PAA) in humidified incubators at 37°C and 5% CO\(_2\).

#### Blood samples

For all experiments, blood samples were collected from healthy volunteers and PBMC were isolated by lymphopreparation density-gradient centrifugation (PAA). The Charité University Medicine Berlin ethics committee (Institutional Review Board) approved the study protocol, and all blood donors provided written informed consent.

#### Enrichment and expansion of Ag-specific T cells

HCMV-specific T cells were enriched and expanded using a previously described technique \( (1, 7, 20) \). Briefly, \( 4 \times 10^6 \) autologous PBMC were stimulated for 6 h with overlapping HCMV pp65/IE-1 peptide pools (JPT Peptide Technologies) at 1 \( \mu \)g/ml each peptide. Pools consisted of 15-mer peptides overlapped by 11 aa and were reconstituted in DMSO. IFN-\( \gamma \)-producing cells underwent positive selection using the IFN-\( \gamma \)-secretion capture MACS system, according to the manufacturer’s instructions (Miltenyi Biotec). Enriched IFN-\( \gamma \)-secreting cells were cultured for 18 d in 24-well plates with irradiated (30 Gy) autologous feeder cells (derived from capture assay-negative fraction) at a 1:100 ratio in culture media supplemented with both 50 IU/ml recombinant human (r)IL-2 (Chiron) and 10 ng/ml rIL-7 (Cellgenix) or either one alone, when indicated. The medium was changed every 3–4 d during culture. Where indicated, a very low dose (ND; 2 nm) or a low dose (LD; 20 nm) of mTOR inhibitor (rapamycin; Sigma-Aldrich) or IL-2R antagonist (20 \( \mu \)g/ml basiliximab, Simulect; Novartis) was added every 2 d, beginning on day 1, together with subsequent media supplementation. Where indicated, CD4\(^+\) and CD8\(^+\) T cells were MACS purified, according to the manufacturer’s instructions (Miltenyi Biotec).

#### Allocation of APCs

Expanded HCMV-specific T cells were analyzed for effector functions by their ability to recognize and eliminate peptide-loaded or virus-incubated target cells which consisted of autologous lymphoblastoid B cell lines (LCL) transformed with B95-8 virus and autologous monocytes. LCL were generated as described previously \( (7) \). CD14\(^+\) monocytes were enriched from PBMC by magnetic isolation (MACS; Miltenyi Biotec). Subsequently, CD14\(^+\) cells were cultured for 6 d in 1000 IU/ml rhGM-CSF (Cellgenix) and 400 IU/ml rhIL-4 (Cellgenix). NEWT or fibroblasts (passaged control cell line) were added to monocyte-differentiation cultures on day 4.

#### Assessment of cytotoxic activity

A modified vital assay was used for cytotoxicity testing, as described previously \( (21) \). Briefly, monocytes incubated with HCMV-related laboratory wild-type strain NEWT were used as targets \( (22) \). In addition, autologous LCL were pulsed with 1 \( \mu \)g/ml overlapping HCMV pp65/IE-1 peptide pools, whereas unpulsed LCL were used as control targets. Targets were labeled with 10 \( \mu \)M CFDA (Molecular Probes). As controls, unpulsed LCL and monocytes coinubated with fibroblasts were labeled with 5 \( \mu \)M dimethylodoclane oxide-succinimidyl ester (Far Red; Invitrogen). Cells were cocultured for 16 h at a T cell/target cell ratio of 1:1 and 10:1. Probes were analyzed as triplicates using an LSRII flow cytometer. Samples without T cells, containing only APC (pulsed/NEWT or unpulsed) were used as internal control. Analysis was gated on LIVE/DEAD dichrome staining dye-negative cells (near-IR fluorescent re-active dye; Invitrogen). The mean percentage survival of pulsed or NEWT-incubated targets was calculated relative to Ag-unpulsed controls. Percentage lytic activity of target lysis was calculated as follows: mean percentage survival of targets in cultures containing defined numbers of effector T cells in comparison with control cells without T cells.

#### Intracellular cytokine staining

Following Ag-specific stimulation, cytokine secretion (IFN-\( \gamma \), TNF-\( \alpha \), and IL-2) and activation marker expression (CD154 and CD137) were determined by intracellular fluorescence staining. All Abs were purchased from Becton Dickinson, except when otherwise indicated. Virus-incubated monocytes and peptide pool-loaded LCL or monocytes (1 \( \mu \)g/ml IE-1/pp65) were added to T cells in a diluting ratio of 1:10. Unpulsed LCL or monocytes (DMSO incubated) were used as unstimulated controls. For effector cytokine detection, cultured T cells were restimulated for 18 h in the presence of 1 \( \mu \)g/ml brefeldin A (Sigma-Aldrich). For CD107a (H4A3) detection, 2\( \mu M \) monensin (GolgI Stop; Becton Dickinson) was added together with brefeldin A. Cells were then harvested, and the phenotype was stained with mAbs for surface markers CD3 (UCH7; eBioscience), CD8 (3B5; Invitrogen), and CD4 (SK3). To define memory phenotypes, T cells were extracellularly stained for CCR7 (#150503; R&D Systems), CD62L (DREG56; Beckman Coulter), CD45RA (2H4LHD11LLDB9; Beckman Coulter), and CD127 (hiL-7R-M21). To exclude dead cells, samples without T cells, containing only APC (pulsed/NEWT or unpulsed) were used as internal control. Analysis was gated on LIVE/DEAD dichrome staining dye-negative cells (near-IR fluorescent re-active dye; Invitrogen). The mean percentage survival of pulsed or NEWT-incubated targets was calculated relative to Ag-unpulsed controls. Percentage lytic activity of target lysis was calculated as follows: mean percentage survival of targets in cultures containing defined numbers of effector T cells in comparison with control cells without T cells.

### Statistical analysis and calculations

The Kolmogorov–Smirnov test was used to determine whether the values followed a Gaussian distribution. Normally distributed data were analyzed by the nonparametric Wilcoxon matched-pairs test. Probability \( p \) values \( \leq 0.05 \) were considered statistically significant.

CD4\(^+\)/CD8\(^+\) T cell expansion was calculated by comparing CD4\(^+\)/CD8\(^+\) T cell frequency (assessed by FACS) with the cell number of the whole culture. Fold expansion was calculated by dividing the cell number on day 18 by the cell number on day 1. T cells exhibiting a TCM phenotype were defined by CCR7 expression on CD4\(^+\)/CD8\(^+\) T cells on day 18. Based on
T<sub>CM</sub> phenotype on day 1, the fold expansion of T<sub>CM</sub> was calculated based on overall CD4<sup>+</sup>/CD8<sup>+</sup> fold expansion and T<sub>CM</sub> proportion on day 18.

**Results**

Partial mTOR inhibition results in increased numbers in early memory T cells and leads to superior specific T cell response

HCMV<sub>pp65/IE1</sub>-specific CD4<sup>+</sup>/CD8<sup>+</sup> T cells from PBMC were isolated using an immunomagnetic IFN-γ–secretion assay following stimulation by HCMV<sub>pp65/IE1</sub> peptide pools (Fig. (1) (23)). Based on CCR7 and CD45RA expression, human memory T cells can be segregated into T<sub>EMRA</sub>, T<sub>CM</sub>, and T<sub>EM</sub> (12). HCMV-specific CD4<sup>+</sup>/CD8<sup>+</sup> T cells before/after expansion were predominantly enriched for T<sub>EM</sub> and T<sub>EMRA</sub>/T<sub>CM</sub>, respectively (1).

We wondered whether mTOR inhibition during the culture might support T<sub>CM</sub> expansion. Although an intermediate dose of rapamycin (50–100 nM, commonly used for supporting natural regulatory T cell [nTreg] expansion) attenuated T cell proliferation (data not shown), the expansion was only marginally diminished at VLD and LD concentrations (Fig. 2A). Interestingly, VLD/LD rapamycin treatment resulted in a greater CD4<sup>+</sup> T cell yield compared with the control cell line after expansion (Fig. 2B, 2C). As expected, CD4<sup>+</sup> T cells, which exhibited enhanced expansion in the presence of LD/VLD rapamycin, did not express nTreg markers Helios and Foxp3 (data not shown).

Human T<sub>CM</sub> uniformly express CD62L; however, T<sub>EM</sub> are heterogeneous for CD62L expression. Moreover, T<sub>CM</sub> coexpress CCR7 and characteristically lose CD45RA during naive → effector transition (12). CCR7 expression is rapidly downregulated upon antigenic restimulation, along with the differentiation of T<sub>CM</sub> → T<sub>EM</sub> (12). Remarkably, rapamycin-treated HCMV-specific T cell lines showed a significantly greater proportion and absolute number of CCR7<sup>+</sup>/CD62L<sup>-</sup> (CD45RA<sup>-</sup>) T<sub>CM</sub> (Fig. 2D–G). To assess their effect on expansion, we evaluated the response following specific restimulation. Rapamycin-treated HCMV-specific CD8<sup>+</sup> T cells showed enhanced CD137 expression, whereas CD137 and CD154 expression on CD4<sup>+</sup> T cells was not significantly influenced (Fig. 2H, 2I). Furthermore, irrespective of a significantly greater proportion of T<sub>CM</sub>, rapamycin treatment had little or no effect on effector cytokine secretion (e.g., IFN-γ and TNF-α) (Fig. 2J). Gating on CD62L<sup>+</sup> (T<sub>CM</sub>) and CD62L<sup>-</sup> T cells (T<sub>EM</sub>) revealed no differences in the cytokine-secretion pattern between treated and untreated samples (Supplemental Fig. 1A). One specific feature of T<sub>CM</sub> is the ability to secrete IL-2 in response to Ag re-encounter (12). Furthermore, multicytokine secretion of CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses correlates with protective antiviral immunity (24). Interestingly, compared with untreated controls, VLD/LD rapamycin-treated T cell lines showed significantly greater IL-2 secretion after specific restimulation and most of the IL-2-secreting T cells were multicytokine producers (Fig. 2J, 2K).

**Blocking IL-2Rα fosters memory formation and enhances specific T cell response**

The mTOR pathway is involved in many signaling cascades. One mechanism is the inhibition of IL-2–mediated cell cycle G1/S phase progression (25). To address whether the effect on T<sub>CM</sub> expansion is due to targeting the IL-2R, we partially inhibited IL-2R signaling during the expansion culture by targeting the IL-2Rα–chain using an antagonistic anti-CD25 mAb. At 20 μg/ml anti-CD25 mAb, the expansion was only marginally diminished (Fig. 3A). However, CD4<sup>+</sup> T cells paradoxically expanded even more, whereas CD8<sup>+</sup> T cell proliferation was attenuated, resulting in a significantly greater CD4<sup>+</sup> T cell yield compared with the control cell line after expansion (Fig. 3B, 3C). T<sub>CM</sub> formation was enhanced, defined by the expression of CCR7 (CD45RA<sup>-</sup>) and CD62L (Fig. 3D–G). Correspondingly, expansion of CD4<sup>+</sup>/CD8<sup>+</sup> T<sub>CM</sub> and multicytokine secretion were significantly greater in the anti-CD25 mAb-treated samples (Fig. 3H, 3I). Similarly, restimulated CD4<sup>+</sup> T cells showed enhanced CD137 expression (Fig. 3J, 3K), whereas CD137 and CD154 expression on restimulated CD4<sup>+</sup> T cells was unchanged. Of note, similarly to rapamycin treatment, IFN-γ and TNF-α effector cytokine secretion was not diminished after treatment, despite a decrease in the T<sub>EMRA</sub>/T<sub>EM</sub> proportion (Fig. 3J).

**Targeting the IL-2R pathway does not diminish specific cytolytic capacity of T cells**

Despite a greater proportion of T<sub>CM</sub>, rapamycin/anti-CD25 mAb-treated HCMV-specific T cells showed similar specific killing of peptide-loaded target cells (Fig. 3L). The expression of the degranulation marker CD107a on CD8<sup>+</sup> T cells remained constant; however, CD4<sup>+</sup> T cells exhibited increased CD107a surface expression (Supplemental Fig. 1B, 1C).

**CD4<sup>+</sup> T cell help improves antiviral CD8<sup>+</sup> T cell response**

Because both approaches of partial IL-2R targeting supported the expansion of CD4<sup>+</sup> T cells (Figs. 2C, 3C), we wondered whether the maintenance of CD8<sup>+</sup> effector functions might be related to the enforcement of CD4<sup>+</sup> T cell help (26). To test the impact of CD4<sup>+</sup> T cell quantity on CD8<sup>+</sup> function, we isolated HCMV-specific T cells from PBMC and cultured, in parallel, bulk T cells, sorted CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells. CD8<sup>+</sup> T cells alone showed slightly poorer proliferation, whereas CD4<sup>+</sup> T cell expansion was similar to bulk culture (data not shown). Of note, HCMV-specific restimulation of purified CD8<sup>+</sup> T cells revealed nearly complete inhibition of cytokine/activation marker responsiveness (Fig. 4A), but neither the memory phenotype nor degranulation was changed (data not shown). Next, we wondered whether CD8<sup>+</sup> T cell functionality could be enhanced by supplementing CD4<sup>+</sup> T cells just before specific restimulation but not during the expansion phase. Surprisingly, CD8<sup>+</sup> T cell effector cytokine secretion and activation marker expression following specific restimulation proportionally increased with the presence of CD4<sup>+</sup> T cells, up to a CD4/CD8 ratio of 0.2 (Fig. 4A–C). However, CD4<sup>+</sup> T cell supplementation had no impact on the expression of CD107a on CD8<sup>+</sup> T cells (Supplemental Fig. 2A).

Next, we separately cultured isolated HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells with rapamycin/IL-2Rα antagonist. Interestingly, when cultured alone, neither CD4<sup>+</sup> T cells nor CD8<sup>+</sup> T cells exhibited any changes with regard to cytokine secretion or acti-

![FIGURE 1](http://www.jimmunol.org/) Enrichment of HCMV-specific T cells. Specifically stimulated T cells underwent positive selection by the IFN-γ–secretion capture assay technique. Flow cytometric analysis of IFN-γ<sup>+</sup> T cells following 6 h of HCMV<sub>pp65/IE1</sub> peptide pool stimulation (upper panel, before isolation; lower panel, IFN-γ–secretion capture assay positive fraction → enrichment of specific T cells for CD4<sup>+</sup> and CD8<sup>+</sup> T cells.)
FIGURE 2. Partial mTOR inhibition by VLD (2 nM) or LD (20 nM) rapamycin during expansion culture for 18 d increased the number of early memory cells and led to superior specific antiviral T cell response. Frequencies were assessed by flow cytometry. Respective inhibitor treatment is indicated. [(A) and (B); summarized in (C)] Rapamycin increases the proportion of CD4+ HCMV-specific T cells within bulk culture without affecting fold expansion, as shown in (A) (absolute cell counts ranged from 3.2 \times 10^5 to 6.2 \times 10^5 cells before expansion, from 2.6 \times 10^7 to 8.3 \times 10^7 cells [w/o], from 2 \times 10^7 to 7.8 \times 10^7 cells [VLD], and from 1.45 \times 10^7 to 7.6 \times 10^7 cells [LD] after expansion). [(D); summarized in (E) and (F)] Rapamycin preserves CCR7 and CD62L expression within CD45RA− T cells [(D); upper panel; TCM = CCR7+/CD45RA−] and CD62L [(D); lower panel]. (G) Preferential expansion of TCM (refer to fold expansion of CD4+/CD8+ T cells). CD137 and CD154 activation marker expression [(H); summarized in (I)] and IFN-γ, TNF-α, and IL-2 cytokine secretion [(K); summarized in (J)] were determined by intracellular staining. Cells were stimulated for 18 h with HCMVpp65/IE1 peptide pool-pulsed LCL at a ratio of 10:1. Cells were permeabilized and stained for IFN-γ, TNF-α, IL-2, CD3, CD4, CD8, CD137, and CD154. Summary of six independent experiments showing enhanced polyfunctionality of both CD4+/CD8+ T cells [(J); IFN-γ*, TNF-α*, IL-2*] and activation marker expression of CD8+ T cells [(K); CD137*, CD154+] in response to HCMVpp65/IE1 peptide pool-pulsed LCL. Box plots with median and interquartile range. Mean levels are shown in (A), (C), (E)–(G), (I), and (J). Representative examples are shown in (B), (D), (H), and (K). *p < 0.05, Wilcoxon matched-pairs test.
Rapamycin/IL-2Ra antagonist-incubated CD8+ T cell bulk cultures, as well as IL-2R pathway-targeted CD4+ T cells, showed increased CD62L expression (Fig. 4D). In contrast, CD62L expression on purified CD8+ T cells could not be enhanced. Hence, CD8+ T cell effector function and memory phenotype in Ag-specific T cell cultures can be improved in the presence of CD4+ T cells. Furthermore, CD4+ T cells, when cultured alone, elicited direct antiviral cytolytic function apart from helper mechanisms by means of cytokine secretion (Supplemental Fig. 2H/K).

**FIGURE 3.** IL-2Ra antagonistic engagement (aIL-2Ra; 20 μg/ml) during 18 d-expansion culture fosters memory formation and enhances antiviral T cell responses. Frequencies were assessed by flow cytometry. Inhibitor treatment is indicated. ([A] and [B]; summarized in [C]) aIL-2Ra increases the proportion of CD4+ HCMV-specific T cells within bulk culture without affecting total fold expansion, as shown in (A) (absolute cell counts ranging from 1.2 × 10^5 to 3.2 × 10^5 cells before expansion, from 3.3 × 10^7 to 9.3 × 10^7 cells [w/o], and from 2.9 × 10^7 to 6.5 × 10^7 [aIL-2Ra] cells after expansion). ([D]; summarized in [E] and [F]) Differentiation phenotype of the expanded HCMV-specific T cells according to CCR7, CD45RA (upper panel, TCM = CCR7+/CD45RA−) and CD62L (lower panel). (G) Mean fold expansion of TCM (refer to fold expansion and CD4+/CD8+ T cells). IFN-γ, TNF-α, and IL-2 cytokine secretion ([H]; summarized in [I]) and CD137 and CD154 activation marker expression ([K]; summarized in [J]) were determined, as described in Fig. 2H/K. Summary of six independent experiments showing enhanced polyfunctionality ([H]: IFN-γ+, TNF-α+, IL-2+) and activation marker expression ([J]: CD137+, CD154+) in response to HCMVpp65/IE1 peptide pool-pulsed LCL. (L) Assessment of cytotoxic activity by flow cytometric vital assay. Similar cytolytic activity of rapamycin- and IL-2Ra antagonist-treated cultures. Box plots with median and interquartile range. Mean levels are shown in (A), (C), (E)–(G), (I), and (K). Representative examples are shown in (B), (D), (H), and (J). *p < 0.05, Wilcoxon matched-pairs test.

In vitro generation of TCM is growth factor dependent

Our expansion protocol is based on standard IL-2/IL-7 growth factor supplementation. Because both approaches of partial IL-2R targeting interfere with cytokine/cytokine receptor interaction,
particularly of IL-2/IL-2R, we wondered whether removal of one or both growth factors would have similar effects on the T cell product composition. T cells expanded with IL-2 alone showed strong proliferation and good cytokine-secretion capacity; however, the majority of the T cells displayed a late differentiated TEM phenotype (Fig. 5). IL-7 alone showed remarkably reduced T cell proliferation but somewhat preserved CCR7/CD62L expression compared with supplementation with IL-2 or IL-2/IL-7. Addition of rapamycin/IL-2Rα antagonist with either IL-2 or IL-7 promotes TCM formation by maintaining cytotoxicity. In the presence of rapamycin/anti-CD25 mAb, IL-2 alone was less effective in preserving the TCM phenotype, but more potent in cell expansion and inducing effector functionality than treated with IL-7 alone (Fig. 5). Most importantly, the best results with regard to the expansion rate and preserving the TCM phenotype with full functionality were seen after cultivation with both IL-2 and IL-7 in the presence of rapamycin/IL-2Rα antagonist. Interestingly, rapamycin/IL-2Rα antagonist generated T cell lines showed prolonged (7 d) expression of IL-7Rα (CD127) compared with controls (Supplemental Fig. 2D, 2E).

Modifying IL-2R signaling enables superior protective CD4-mediated CD8+ T cell immunity

In a prevailing HCMV infection, bone marrow-derived myeloid progenitors are latently HCMV infected (27). Following reactivation, HCMV early Ags can be presented by circulating monocytes and their derivatives. This can be simulated by infecting monocyte-derived immature dendritic cells (iDC) using HCMV wild-type strain NEWT (22). The lower epitope density on infected cells compared with peptide-loaded cells is a challenge requiring highly effective T cell recognition.

Compared with peptide pool-pulsed iDC, the frequency of cytokine-secreting and CD137-expressing conventionally expanded CD8+ T cells responding to NEWT-infected iDC was markedly reduced (Fig. 6A–D), whereas specific lysis was unchanged (data not shown). However, CD8+ T cells derived from IL-2R pathway-targeted cultures revealed a much greater IFN-γ response to NEWT-infected iDC, whereas CD137 expression was only marginally upregulated (Fig. 6A–D). Interestingly, CD8+ T cells secreted substantial amounts of IFN-γ, whereas TNF-α/IFN-γ double positivity was reduced in response to NEWT-infected iDC (Fig. 6A, 6B). Expression of the degranulation marker CD107a at CD8+ T cells remained constant at a low level compared with peptide-loaded iDC. Yet again, CD8+ T cells tended to increase CD107a surface expression (Fig. 6E).

To test the impact of CD4+ T cell quantity on CD8+ function in this model, we isolated HCMV-specific T cells from PBMC and cultured bulk T cells, sorted CD4+, and CD8+ T cells in parallel. Following specific restimulation with NEWT-infected iDC, CD8+ T cells cultured in bulk showed marginally increased IFN-γ ef-
fectory cytokine secretion, whereas CD8+ T cells cultured in bulk, derived from IL-2- or IL-7-targeted cultures, exhibited a much greater response (Fig. 6F). CD8+ T cells cultured without IL-2 or IL-7 showed almost completely inhibited IFN-γ cytokine secretion (Fig. 6F). Of note, CD8+ T cell functionality, defined by IFN-γ secretion, could be enhanced by supplementing equal numbers of CD4+ T cells just before specific restimulation (Fig. 6F). Correspondingly, CD8+ T cells cultured alone revealed decreased dose-dependent–specific killing of NEWT-infected iDC, whereas specific lysis of the bulk culture was unchanged, despite being treated or not with IL-2- or IL-7-targeted reagents (data not shown).

The living infection model requires effective T cell recognition, because the presentation of epitopes is reduced compared with peptide-loaded cells. To define the range of T cell recognition, we stimulated the T cells with decreasing peptide concentrations. The IFN-γ T cell response decreased proportionally with declining peptide concentrations (Fig. 6G). However, compared with T cells derived from IL-2- or IL-7-targeted cultures, untreated T cells showed a greater decrease in IFN-γ cytokine secretion with declining peptide concentrations (Fig. 6G).

Discussion
Adoptive T cell therapy is a promising new option for combating severe infections and tumors. The longevity of adoptively transferred T cells is one challenge for long-lasting success. Conventional in vitro expansion strategies of human Ag-reactive T cells preferentially support T_{EM}/T_{EMRA} cells with high effector potency but limited in vivo survival. This is even more true for T cells generated from patients suffering from chronic (relapsing) Ag exposure (5, 6). In this article, we describe a novel simple and GMP-feasible approach to generate Ag-specific long-lived memory T cells with enhanced functionality compared with conventionally generated T cell products by partially blocking IL-2R signaling using an IL-2-R antagonist or VLD/LD mTOR inhibitor. IL-7 or rapamycin/anti-CD25 mAb alone, respectively, preserve the T_{CM} phenotype; however, the expansion and effector functionality of resulting T cells is strongly reduced compared with expansion in the presence of IL-2, which results in a dominant T_{EM} phenotype. However, in the presence of rapamycin/anti-CD25 mAbs, the positive effects of IL-2 on expansion and functionality can be preserved without losing the supportive effects of IL-7 on T_{CM} generation, indicating the usefulness of partial IL-2R signaling (via β/γ-chain). The narrow range of the optimal rapamycin dose supports this view.

Targeting the IL-2R signaling pathway during extensive cell expansion revealed virus-specific CD4+/CD8+ T cells with particular properties: a dominant T_{CM} phenotype, powerful specific effector functions, and enhanced functionality (i.e., multicytokine secretors, including IL-2; enhanced CD137 and CD107a expression on CD8+ and CD4+ T cells, respectively; and effective killing of infected target cells). Gating on T_{CM} and T_{EM} revealed no differences in the cytokine pattern between untreated and IL-2R pathway-targeted cultures, speculating that improved T cell functionality correlates with the quantity of T_{CM} in the T cell product. Remarkably, CD8+ T_{CM} formation was strongly dependent on the increased proportion and functionality of CD4+ T cells within those T cell products. These data might explain why CD4 help is a key factor for establishing long-lasting CD8 memory; obviously, CD4+ T cells support the formation and expansion of long-lived CD8+ T cells by preventing the shift of all Ag-activated early memory CD8+ T cells into the terminally differentiated phenotype. Moreover, our studies show that there is differing sensitivity to mTOR inhibition in distinct T cell subsets, with the least dependency in nTreg, intermediate dependency in T_{CM}, and the greatest dependency on mTOR activity in T_{EM}/T_{EMRA}.

Our data are in line with the findings of Araki et al. (14, 15), who reported that rapamycin increases the quantity and quality of memory T cell differentiation in mice. The positive effects of rapamycin were dissected in nonhuman primates following vaccinia virus vaccination (16). Rapamycin augmented the magnitude, duration, and quality of vaccine-specific T cell responses. However, rapamycin was applied prior to vaccination and was continuously administered for >90 d. Most studies analyzed the impact of rapamycin on the differentiation of memory T cells from naive T cells (14, 28). Rapamycin promotes the generation of memory precursor effector cells and accelerates the effector → memory T cell transition during the contraction phase (15). Rapamycin treatment during both the expansion and contraction phases improves the quality and quantity of memory T cells (15). However, the absolute number of memory T cells was not increased during the contraction phase. Our approach differs from those studies, because we boosted a pre-existing memory response by isolating and expanding virus-specific T cells from seropositive donors. IL-2R targeting in our approach improved the quality, as well as enhanced the growth factor-supported quantity, of early memory T cells.

Studies in mice using mTOR inhibitors described reduced effector cytokine secretion at early stages of differentiation (29, 30). A key finding of our study was that the generated HCMV-specific T_{CM} are not diminished in effector functions compared with the control lines, with a significantly higher proportion of the T_{EM}/T_{EMRA} counterpart. Moreover, our T cell products even showed enrichment of polyfunctionality. This contradicts another report, in which early differentiated T_{EM} cultures were incapable of early effector function (31). This might be related to the supplementation by growth factors, particularly IL-2, in our system. Rapamycin mediates several effects, including inhibition of the T cell response to IL-2 (25). We showed in this study that reducing the binding capacity of IL-2 to the IL-2R complex by IL-2Rα an-

![FIGURE 5. In vitro generation of T_{CM} cells is growth factor dependent. HCMV-specific T cell lines were generated as described. Summary of four independent experiments showing differentiation, proliferation, and effector function of all possible permutations of the variables: IL-2, IL-7, rapamycin (LD), and IL-2R antagonist (aIL-2Rα).](http://www.jimmunol.org/DownloadedFrom/5195)
FIGURE 6. Modifying IL-2 signaling by targeting IL-2R binding or mTOR pathway enables superior protective CD4-mediated CD8+ T cell immunity. HCMV-specific T cell lines were generated, as described. Frequencies were assessed by flow cytometry. Inhibitor treatment is indicated. Assessment of IFN-γ and TNF-α cytokine secretion [(A); summarized in (B)], CD137 and CD154 activation marker expression [(C); summarized in (D)], and CD107a degranulation capacity [(E)] following specific stimulation with 1 μg/ml HCMVpp65/IE1 peptide pool-pulsed monocytes and NEWT-incubated monocytes for 18 h. (F) CD8+ T cell function can be induced by supplementing equal numbers of CD4+ T cells just before specific restimulation. Shown is IFN-γ secretion of CD8+ T cells within bulk culture or IFN-γ secretion of CD8+ T cells that were cultured alone. CD8+ T cells were cultured separately with rapamycin (LD) or all-2Rα, as indicated. (G) IL-2R pathway-targeted cultures exhibit enhanced T cell recognition. T cells were restimulated for 18 h with peptide-loaded autologous LCL at declining peptide concentrations of 1, 0.1, or 0.01 μg/ml pp65/IE-1 (crescendo bar denotes increasing peptide concentrations). Cells were permeabilized and stained with the respective Abs. In all plots, one representative of three experiments is shown.
agonist gave similar results. The mode of action of our effects is mediated by modifying IL-2R signaling. Certainly, T_{CM} might be more resistant to survive IL-2 withdrawal. This is consistent with observations in murine models of viral infections, in which rapamycin-treated T cells showed an enhanced ability to survive growth factor withdrawal as the result of a hybrid metabolism induced by rapamycin, upregulating both oxidative phosphorylation and glycolysis (29). Thus, the generated T_{CM} have a greater ability to resist growth factor withdrawal, in contrast to the T_{EM} counterpart. Consequently, inadequate IL-2 signaling preferentially supports less-sensitive T_{CM} expansion.

At culture initiation, the targeted T cell lines show prolonged IL-7Rα (CD127) expression, which is essential for memory T cell maintenance. However, after 18 d of expansion, a distinct population of CD127+ T cells could not be seen. This could be due to the supplementation of IL-7 during culture and, thereby, downregulation of the receptor. Interestingly, a previous study showed that in vitro IL-2R antagonist treatment augmented the T cell responsiveness to IL-7 caused by prolonged binding to the CD127/CD132 complex (32). These results indicate that IL-2R antagonist can favor response to IL-7; however, IL-7 alone was less sufficient than was the combination of IL-2/IL-7 in supporting expansion.

We demonstrated that CD8+ T cell effector function and memory phenotype in Ag-specific T cell cultures proportionally improve with the presence of CD4+ T cells. In agreement, Sun et al. (33) demonstrated that the generation of memory CD8+ T cells in response to an acute infection is independent of CD4 help; however, during memory maintenance, CD4+ T cells provide essential support for memory CD8+ T cell survival. In our studies, CD8+ T cells showed prolonged CD62L expression only when CD4+T cells were present; this effect could be further augmented by the addition of rapamycin/IL-2R antagonist during culture. This exemplifies the importance of CD4+ T cells for memory CD8+ T cells during a rechallenge response.

Remarkably, we showed that HCMV-specific CD8+ T cells expanded with rapamycin/IL-2Rα antagonist responded superiorly to NEWT-infected autologous monocytes/iDC. Moreover, CD8+ T cells only responded when CD4+ T cells were present. Hence, it is likely that virus-specific CD8 T cell responses are partially Ag unspecific and are caused through bystander activation (34). Consistent with other studies, our results demonstrated that CD4+ T cells elicited antiviral lytic T cell response apart from helper mechanisms (35) that is essential for maintaining long-term immune control (36, 37). Cytolytic CD4+ T cells are important in many chronic infections, such as EBV, HIV, and HCMV (38). In addition, the CD4+ T cells elicited a profound response to NEWT-infected iDC. Reasonably, HCMV-infected cells downregulate MHC class I expression and, thereby, decrease Ag presentation (39). Potentially, insufficient infection of iDC, resulting in an inadequate display of intracellular viral proteins through MHC class I, might lead to preferential processing of MHC class II.

In comparison with the unlimited MHC loading by incubation of target cells with HCMV-derived peptide pools, the living infection model requires an effective T cell recognition because of reduced specific epitope density on the target cells. By stimulating the differentially generated T cell lines with decreasing peptide concentrations, we were able to show that IL-2R pathway-targeted cultures exhibit enhanced T cell recognition. The advantages of T cells generated by targeting IL-2R signaling compared with control T cells in this clinically relevant target model further underscore their functional superiority.

In summary, this study provided evidence of the functional efficacy of human Ag-specific T_{CM}. These findings identify a novel method of cell expansion that possesses important implications for T_{CM} generation and subsequent selection of adoptive immunotherapy for in vivo clinical use.

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Disclosures
The authors have no financial conflicts of interest.

References


