

Enhancing Virus-Specific T Cell Persistence: L-Arginine Supplementation Improves the Durability of CD8 + T Cells for Immunotherapy

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Abstract

Virus-specific T cell (VST) therapy offers a promising treatment for life-threatening viral infections following allogeneic hematopoietic stem cell transplantation (HSCT). However, the efficacy of VST therapy remains limited by apoptosis, exhaustion, and loss of stem-like properties with advancing differentiation. In this study, we demonstrate that supplementation of basic culture medium with excess L-arginine significantly enhances the durability and function of VSTs targeting persistent viruses. L-arginine treatment preserved a less differentiated, less exhausted phenotype and conferred resistance to activation-induced cell death (AICD) and freeze-thaw-induced damage. Mechanistically, these effects were associated with increased mitochondrial membrane potential and MCL-1 expression, suggesting enhanced mitochondrial biogenesis and metabolic fitness. L-arginine-treated VSTs initially exhibited reduced cytotoxic activity upon primary stimulation, likely due to the suppression of exhaustion and differentiation; however, they acquired superior polyfunctionality and cytotoxic potential following secondary stimulation. These benefits were achieved without detectable activation of mTORC1 signaling, indicating a favorable metabolic reprogramming independent of effector-skewing pathways. Our findings position L-arginine supplementation as a clinically applicable strategy to improve the persistence and efficacy of human VST therapy and other adoptive T cell therapies requiring *in vitro* expansion and cryopreservation.

Introduction

Hematopoietic stem cell transplantation (HSCT) is one of the curative therapies for patients with refractory or recurrent hematological malignancies and inborn errors of immunity [1–3]. However, recipients of HSCT experience a prolonged period of profound immunosuppression, increasing their susceptibility to the reactivation of persistent viruses, such as human adenovirus (HAdV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), BK virus (BKV), and JC virus [4–7]. These viral infections can be fatal, contributing to 1.6–17% of post-transplant mortality cases [8–10]. Although some antiviral agents (e.g., ganciclovir, cidofovir) are commonly used, they are frequently associated with significant adverse effects and treatment failure [11–13]. Despite considerable progress in transplantation techniques and antiviral therapies over recent decades, the incidence of virus-related mortality following HSCT showed little improvement between 1995 and 2015 [10]. Moreover, with the increasing age of transplant recipients and the expanded use of high-risk transplantation strategies—including haploidentical donor transplants, T cell-depleted HSCT, alemtuzumab conditioning, and umbilical cord blood transplantation—the burden of viral infections is expected to rise further [8, 10, 14, 15]. Although several virus-specific approaches (e.g., EBV-specific T-cell therapy or BKV-neutralizing antibodies [16–18] are emerging, their applicability remains limited. Therefore, there is a pressing need for broadly effective solutions, such as our multi-virus-specific T cell (VST) platform.

Initially, donor-derived virus-specific T cell (VST) therapy was considered a promising approach for controlling viral reactivation [7, 19–21]. However, this approach has considerable limitations, including the time-intensive process required to generate VSTs and the potential for manufacturing failure with

virus-naïve or cord blood donors. To overcome these challenges, VST therapies derived from partially HLA-matched unrelated donors have been proposed as an alternative strategy [6, 7, 22–25]. These VSTs are generated in advance from healthy donor peripheral blood mononuclear cells (PBMCs), cryopreserved, and stored in liquid nitrogen for immediate clinical use [25, 26]. While this approach overcomes challenges related to timeliness and donor limitations, fundamental issues inherent to adoptive T cell therapy—namely apoptosis, exhaustion, and loss of stem-like properties with advancing differentiation—remain unresolved. Collectively, these challenges compromise VST survival, persistence, and antiviral efficacy [27, 28]. Furthermore, the additional stress introduced by the freeze-thaw process exacerbates cellular vulnerability [29], highlighting the increased need to counteract these problems in order to preserve VST viability and functionality after thawing [27, 28, 30]. To this end, interventions such as PD-1 blockade and costimulatory agonists, including 4-1BB or CD28, have been proposed [31–33]. While PD-1 inhibitors show limited efficacy in T cells that have undergone progressive exhaustion due to persistent antigen stimulation *in vivo* [34], costimulatory agonists may offer an alternative means to enhance survival and function. While these immunomodulatory strategies directly target signaling pathways, metabolic modulation has recently gained attention as an alternative approach. In this context, L-arginine, a non-essential amino acid, plays multiple roles in immune metabolism and function [35, 36]. It is transported into T cells via cationic amino acid transporters [37] and metabolized by enzymes such as arginase 1 (ARG1) and nitric oxide synthase 2 (NOS2), generating metabolites that participate in lipid peroxidation, DNA damage repair, protein synthesis, and cell proliferation [36]. Initial studies demonstrated that T cell receptor (TCR)-CD3- ζ complexes are degraded following antigen stimulation and that supplementation with a small amount of L-arginine (150 μ M) in the culture medium can restore their expression [38, 39], even though this concentration is substantially lower than that found in RPMI 1640. Moreover, large amounts of L-arginine are taken up by CD4⁺ naïve T cells following activation, resulting in mTOR-independent anti-apoptotic effects and enhanced cellular stemness through metabolic reprogramming from glycolysis to oxidative phosphorylation (OXPHOS) [40]. However, VSTs targeting persistent viral infections differentiate into central memory-like or effector memory-like T cells as a result of continuous antigen stimulation [27, 41], and their L-arginine metabolism differs from that of naïve T cells [42]. Furthermore, several studies have shown that excessive nitric oxide (NO), a byproduct of L-arginine metabolism, can impair T cell function [36, 43, 44]. In addition, an elevated pH—resulting from excess L-arginine—can suppress cell proliferation, induce apoptosis, and downregulate IL-2 receptor expression [45]. Therefore, it remains unclear whether supplementation with excess L-arginine in culture medium exerts beneficial anti-apoptotic and stemness-promoting effects on VSTs or paradoxically impairs their function due to NO overproduction and pH alterations.

In this study, we investigated whether excess L-arginine supplementation in RPMI 1640 enhances the functional properties of VSTs. Specifically, we examined its effects on T cell exhaustion, cellular stemness, survival after freeze-thawing, resistance to activation-induced cell death (AICD), and both cytotoxicity and polyfunctionality. This strategy may represent a promising approach to improve the efficacy of human adoptive T cell therapies that require *in vitro* expansion and cryopreservation.

Materials and Methods

Study participants

Seven healthy adult donors were recruited for this study. Blood samples were collected after obtaining written informed consent. The study protocol was approved by the Ethics Committee of the Institute of Science Tokyo (approval no. 1452, project title: *Establishment of specific immune cell therapy against opportunistic infections after transplantation*, approval date: March 26, 2013) and the National Institute of Infectious Diseases (approval no. 1347, project title: *Establishment of specific immune cell therapy against opportunistic infections in immunodeficient patients*, approval date: December 27, 2021).

Peptides

Generation of VSTs

PBMCs were isolated using Ficoll density gradient centrifugation. A total of 2×10^6 PBMCs were stimulated with the viral peptides at a concentration of 100 ng/mL each and cultured in RPMI 1640 (Fuji Film, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Nichirei Bioscience, Japan), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, NY, USA), 2 mM glutamine (Sigma-Aldrich, MO, USA), 10 mM HEPES (Sigma-Aldrich), and 1% sodium pyruvate (Gibco), collectively referred to as R10. Cultures were maintained in the presence of 10 ng/mL interleukin (IL)-7, 5 ng/mL IL-15 (Miltenyi Biotec), and 20 ng/mL IL-21 (PeproTech, NJ, USA). The medium was replaced twice per week with R10 supplemented with IL-7 (10 ng/mL) and IL-15 (5 ng/mL), and cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. On day 15, the expanded cells were harvested, analyzed, and cryopreserved for subsequent analysis.

Extra L-arginine (Fuji Film) was added to R10, which already contained 1.15 mM L-arginine, to reach a final concentration of 4.15 mM (baseline 1.15 mM + 3.0 mM supplementation) in the L-arginine-enriched medium. The pH of the culture medium was measured using the SevenCompact S220 pH meter (Mettler Toledo, Switzerland).

Cells obtained on day 15 were restimulated with the viral peptides in the presence of 10 ng/mL IL-7, 5 ng/mL IL-15, 20 ng/mL IL-21, 20 ng/mL IL-12, 50 ng/mL IL-18 (Miltenyi Biotec), and 50 ng/mL of TL1A (PeproTech) [46]. These cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂, with regular supplementation of IL-7 (10 ng/mL) and IL-15 (5 ng/mL) for 14 days. On day 29, the expanded cells were harvested and analyzed for downstream applications, including phenotypic characterization and functional assays.

Cell counts and viability

Cell counts and viability were assessed using acridine orange–propidium iodide (AO/PI) staining and the LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems, South Korea). AO stains live cells, while PI stains dead cells.

Flow cytometric analysis

Antibodies and acquisition

The following antibodies were used: anti-pAkt-AF488, anti-PD-1-BB700, anti-pS6-PE, anti-cleaved caspase-3 (cCasp-3)-V450, anti-CCR7-BV650, anti-IFN- γ -BV786, anti-CD4-BUV496, anti-CD8-BUV563, anti-CD3-BUV615, anti-CD27-BUV661, anti-CD28-BUV737, and anti-CD45RA-BUV805 (BD Biosciences, CA, USA); anti-CD62L-Alexa Fluor[®] 700, anti-TIM-3-APC-Cy[™] 7, anti-CD25-PE-Cy[™] 7, anti-BCL-2-PE, anti-CD4-FITC, anti-CD107a-PE, anti-CD8a-PerCP, anti-IL-2-PE-Cy7, anti-TNF- α -APC, anti-IFN- γ -APC-Cy7, and anti-CD3-Pacific Blue (BioLegend, CA, USA); anti-BCL-xL-PE-Cy[™] 7 and anti-MCL-1-AF647 (Cell Signaling Technology, MA, USA); and anti-Annexin V-FITC (Invitrogen, MA, USA). Flow cytometric analysis was performed using FACSCanto II and FACSymphony flow cytometers (BD Biosciences). Data were analyzed using FlowJo version 10.7.1 (BD Biosciences).

Intracellular cytokine staining

Cells were stimulated with the viral peptides (100 ng/mL) in the presence of anti-CD28 (1 μ g/mL), anti-CD49d (1 μ g/mL), and GolgiStop[™] (BD Biosciences, 1 μ g/mL) for 6 h. After surface antigen staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences), then stained with antibodies against IFN- γ , TNF- α , and IL-2 to evaluate the frequency of cytokine-producing VSTs. Dead cells were stained with the LIVE/DEAD[™] Fixable Aqua Dead Cell Stain Kit (Invitrogen), and Aqua-negative cells were considered viable.

Phenotype, apoptosis, and mTOR signaling pathway

To assess the phenotype, apoptosis, and activation of the mTOR signaling pathway, cells were stimulated for 12 h with the viral peptides in the presence of CD28, CD49d, and GolgiStop[™] at 37°C and 5% CO₂. Following surface antigen staining, cells were washed with Annexin V buffer (Invitrogen) and stained with anti-Annexin V-FITC antibody. Cells were then fixed and permeabilized using Phosflow Fix Buffer and Phosflow Perm/Wash Buffer (BD Biosciences), followed by intracellular staining with antibodies against pAkt, pS6, cCasp-3, BCL-2, BCL-xL, MCL-1, IFN- γ , TNF- α , and IL-2, according to the manufacturer's instructions. Dead cells were stained with the Fixable Viability Stain 575V (BD Biosciences), and FVS575V-negative cells were considered viable.

Mitochondrial membrane potential

Cells were stimulated with or without viral peptides in the presence of CD28, CD49d, and GolgiStop™ for 12 h at 37°C and 5% CO₂. After incubation, cells were stained with MT-1 Dye using the MT-1 MitoMP Detection Kit (Dojindo, Japan), according to the manufacturer's instructions.

Cryopreservation and post-thaw evaluation

The expanded cells were harvested on day 15, cryopreserved, and subsequently thawed for analysis. After thawing, cells were cultured for 12 h in R10 medium. Following this recovery period, cell counts and viability were assessed using AO/PI. Furthermore, Apoptosis analysis was performed as described above to evaluate the anti-apoptotic effect of thawed VSTs.

Statistical analyses

The Wilcoxon matched-pairs signed-rank test was used for comparisons. P-values less than 0.05 were considered statistically significant. Non-significant differences ($p > 0.05$) are indicated as n.s. GraphPad Prism version 10.3.6 (GraphPad Inc., CA, USA) was used for all statistical analyses.

Results

Induction of apoptosis during the generation of VSTs.

PBMCs from healthy donors were stimulated with the aforementioned viral peptides and cultured in R10 basal medium supplemented with IL-7, IL-15 and IL-21 for 24 hours. The cells were then maintained in IL-7 and IL-15 for an additional 14 days, for a total culture period of 15 days. The cells expanded 4–39-fold (median: 8-fold, data not shown) and were predominantly composed of CD8⁺ T cells (Fig. 1a). The VSTs producing IFN- γ were primarily specific for CMV, followed by EBV- and HAdV-specific T cells (Fig. 1b). Two distinct T cell subsets were identified based on CD3 expression levels among the viable cells (Fig. 1c). The median fluorescence intensity of CD8 was significantly higher in cells with higher CD3 expression (CD3^{bright}) than in those with lower CD3 expression (CD3^{dim}) ($p = 0.0156$). Although CD3 expression was transiently downregulated following antigen stimulation [38], the sustained reduction in both CD3 and CD8 expression suggested ongoing apoptotic processes [47]. The CD3^{dim} T cells exhibited significantly higher Annexin V and/or cCasp-3 expression and lower expression of MCL-1 than that of CD3^{bright} T cells (Fig. 1d) ($p = 0.0156$). Consistently, cCasp-3 and MCL-1 expression were mutually exclusive. In contrast, BCL-2 and BCL-xL expression did not show any clear association with cCasp-3 expression. These findings suggest that apoptosis under these conditions may be regulated, at least in part, through MCL-1-associated mechanisms.

Characterization of CD8⁺ T cells expanded under L-arginine-enriched conditions.

To evaluate the effects of supplemented L-arginine in the culture medium during *in vitro* T cell expansion, PBMCs were cultured in either basal medium or L-arginine-enriched medium. The pH of the basal medium prior to incubation was 7.64, whereas that of the L-arginine-enriched medium was slightly more

alkaline at 7.75. There were no significant differences in the cell proliferation rate or differentiation status between the two culture conditions (Fig. 2a, Fig. S1).

The frequencies of viable cells (FVS575V-negative) and CD3^{bright} T cells were significantly higher under the L-arginine-enriched condition than those observed in the control (Fig. 2b) ($p = 0.0156$ and $p = 0.0156$, respectively). The frequencies of non-apoptotic CD8⁺ T cells (cCasp-3⁻ and Annexin V⁻) and MCL-1 expression were also significantly higher under the L-arginine-enriched condition (Fig. 2c) ($p = 0.0156$ and $p = 0.0156$, respectively). Mitochondrial membrane potential (MMP) reflects the electrochemical gradient across the mitochondrial inner membrane, which is essential for ATP production and serves as an indicator of mitochondrial health and early apoptosis [48]. The frequency of MT-1 staining among CD8⁺ T cells was higher across all samples under the L-arginine-enriched condition (Fig. 2d) ($p = 0.0625$), indicating that L-arginine enhances MMP in the CD8⁺ T cells.

Cellular exhaustion and differentiation represent major obstacles in T cell therapy [49]. Exhaustion and differentiation statuses were assessed in the expanded CD8⁺ T cells. Considering a previous report stating that non-specific antibody binding increases as apoptosis progresses [50], Live/Dead Aqua- and cCasp-3-exclusion were applied during phenotypic analysis to avoid misinterpretation due to apoptotic cell artifacts. The non-apoptotic CD8⁺ T cells (cCasp-3⁻ and Annexin V⁻) under the L-arginine-enriched condition exhibited significantly lower PD-1 and TIM-3 expression in the CD8⁺ T cells ($p = 0.0156$ and $p = 0.0469$, respectively), along with higher CD27 and CD62L expression in the CCR7⁻ CD8⁺ T cells (Fig. 2e) ($p = 0.0156$ and $p = 0.0156$, respectively). These findings suggest that L-arginine not only exerts an anti-apoptotic effect but also mitigates cellular exhaustion and preserves a less differentiated phenotype in CD8⁺ T cells.

L-arginine is a key regulator of the mammalian target of rapamycin complex 1 (mTORC1), which promotes cell proliferation and cytokine production but induces exhaustion and differentiation in T cells through glycolysis [51]. In contrast, activation of mTORC2 signaling enhances anti-apoptotic effects and suppresses cellular exhaustion [52]. The frequencies of CD8⁺ T cells with phosphorylated S6 and Akt, indicative of the activation of mTORC1 and mTORC2 signaling, respectively, did not differ between the control and L-arginine-enriched conditions (Fig. 2f). These results suggest that the effects of L-arginine on CD8⁺ T cells may be independent of mTOR signaling.

IL-2 receptors (IL-2Rs) exist in both low- and high-affinity forms [53]. The low-affinity IL-2R is composed of CD122 and CD132. Binding of IL-2 to the low-affinity receptor induces CD25 expression via signal transducers and activators of transcription 5 (STAT5) signaling, leading to the formation of the high-affinity IL-2R. NO from L-arginine suppresses CD25 expression by inhibiting STAT5 phosphorylation [36, 44]. However, CD8⁺ T cells under the L-arginine-enriched condition exhibited a significantly higher frequency of CD25 expression (Fig. 2f) ($p = 0.0156$). This suggests that STAT5 signaling may be upregulated with L-arginine supplementation.

Effects of L-arginine supplementation on VSTs.

Next, we investigated the effect of a high concentration of L-arginine on IFN- γ -producing CD8⁺ T cells, defined as VSTs. Although no significant difference was observed in the overall frequency of IFN- γ -producing CD8⁺ T cells, the proportion of non-apoptotic cells (cCasp-3⁻ and Annexin V⁻) among these cells was significantly higher under the L-arginine-enriched condition (Fig. 3a) ($p = 0.0156$). In addition, MCL-1 expression in IFN- γ -producing CD8⁺ T cells was also significantly increased under the L-arginine-enriched condition (Fig. 3a) ($p = 0.0469$). Moreover, these cells exhibited significantly lower PD-1 expression under the L-arginine-enriched condition (Fig. 3b) ($p = 0.0312$). Collectively, these findings suggest that excess L-arginine exerts anti-apoptotic and anti-exhaustion effects on VSTs, consistent with our observations in the total CD8⁺ T cell population.

L-arginine enhances the resilience of VSTs, enabling survival through cryopreservation and thawing.

To assess the quality of VSTs after cryopreservation and thawing, expanded cells were cryopreserved on day 15 and subsequently thawed for analysis. Cell viability (AO⁺PI⁻) was significantly higher under the L-arginine-enriched condition 12 h post-thawing (Fig. 4a) ($p = 0.0156$). The frequencies of CD3^{bright} T cells and non-apoptotic CD8⁺ T cells were significantly higher ($p = 0.0312$ and $p = 0.0469$, respectively), accompanied by higher MCL-1 expression with L-arginine supplementation (Fig. 4b, c) ($p = 0.0312$). The proportion of non-apoptotic cells among IFN- γ -producing CD8⁺ T cells was also significantly higher ($p = 0.0312$), along with upregulated MCL-1 expression under the L-arginine-enriched condition (Fig. 4d) ($p = 0.0156$). These findings indicate that the anti-apoptotic effects of L-arginine persist even after cryopreservation and thawing, as evidenced by higher viability and MCL-1 expression.

Resilient VSTs acquire cytotoxicity after prolonged culture following restimulation.

To assess survival after antigen restimulation, cells harvested on day 15 were cultured with the viral peptides in the absence of cytokines for 96 h. The number of viable cells (AO⁺PI⁻) was significantly higher under the L-arginine-enriched condition (Fig. 5a) ($p = 0.0312$), suggesting that L-arginine supplementation renders VSTs more resistant to AICD. To evaluate the long-term resilience and cytotoxicity of VSTs following antigen stimulation, the expanded cells were cultured with viral peptides and cytokines for an additional 14 days. Five of seven cases (excluding HC-2 and HC-5) exhibited a higher proliferation rate from day 15 to 29 under the L-arginine-enriched condition (Fig. 5b). On day 29, the frequency of CD3^{bright} T cells was significantly higher in the L-arginine-enriched condition (Fig. 5c) ($p = 0.0156$). Although CD107a expression was initially lower under the L-arginine-enriched condition on day 15 ($p = 0.0156$), it was significantly higher on day 29 (Fig. 5d) ($p = 0.0469$). Interferon- γ and TNF- α production were also higher in all donors except HC-2 under the L-arginine-enriched condition (Fig. 5d). Furthermore, the polyfunctionality analysis revealed a significantly increased frequency of cells simultaneously expressing IFN- γ , TNF- α , and CD107a under the L-arginine-enriched condition on day 29 (Fig. 5e) ($p = 0.0469$). These findings suggest that L-arginine enables VSTs to resist AICD and sustain proliferation, thereby facilitating the acquisition of cytotoxicity and polyfunctionality upon restimulation.

Discussion

L-arginine has been shown to promote mitochondrial biogenesis in bronchial epithelial cells in murine models [54] and to enhance OXPHOS via nicotinamide adenine dinucleotide (NAD⁺) production through serine metabolism in activated naïve CD8⁺ T cells, primarily demonstrated in mice [55]. Its downstream metabolite, spermidine, also supports mitochondrial biogenesis in murine CD8⁺ T cells [56], but has been reported to decrease the survival of activated naïve CD4⁺ T cells in a human study [40]. These prior findings highlight the complex and context-dependent effects of L-arginine metabolism on T cell biology. In this study, L-arginine supplementation improved the survival and functional integrity of VSTs by reducing exhaustion and maintaining a less differentiated phenotype. These benefits were accompanied by increased expression of the anti-apoptotic protein MCL-1 and preservation of mitochondrial integrity, implicating metabolic reprogramming as a key mechanism underlying these effects [28, 57–59]. MCL-1 upregulation was consistently observed in CD8⁺ T cells, including VSTs (Fig. 1d, 2c, 3a), aligning with previous findings in other T cell subsets [60, 61]. Beyond its role in survival, MCL-1 also promotes mitochondrial biogenesis and supports fatty acid β -oxidation (FAO) [62], highlighting its dual function in metabolic and apoptotic regulation. One possible mechanism by which L-arginine upregulates MCL-1 expression is through the enhancement of STAT5 signaling, a well-established transcriptional regulator of MCL-1. Our data showing increased CD25 expression under L-arginine supplementation (Fig. 2f) suggest augmented STAT5 signaling [53]. In addition, L-arginine-mediated improvements in mitochondrial fitness may stabilize MCL-1 protein by reducing cellular stress and proteasomal degradation [60, 62, 63], further investigation is warranted.

Chronic antigen stimulation impairs mitochondrial function and induces T cell exhaustion [64–67]. Consistent with this context, L-arginine treatment downregulated the expression of PD-1 and TIM-3 (Fig. 2e, 3b), markers associated with progressive T cell exhaustion [68]. In parallel, L-arginine preserved CD27 and CD62L expression (Fig. 2e). The maintenance of mitochondrial integrity sustains MCL-1 expression, which in turn reinforces T cell survival, suppresses exhaustion, and preserves stem-like properties [69–73].

In this context, AICD is a major contributor to functional decline in chronically stimulated T cells, particularly during persistent viral infection [30, 74, 75]. Given that CD27-mediated signaling and, conversely, the attenuation of PD-1 signaling have been reported to preserve mitochondrial function [30, 76, 77], these metabolic adaptations during antigen stimulation may contribute to enhanced resistance against AICD (Fig. 5a).

The maintenance of mitochondrial biogenesis is essential for sustaining the functionality of VSTs during *in vitro* expansion and after adoptive transfer *in vivo*. In particular, IL-7 and IL-15 signaling promotes STAT5 phosphorylation, a process dependent on adequate ATP supply from mitochondria [78]. This IL-7/IL-15 signaling cascade supports both MCL-1 expression and the preservation of stem-like properties in CD8⁺ T cells [63, 79–81], and IL-15 further contributes to resistance against T cell exhaustion [33, 82–

84]. Although NO, a downstream metabolite of L-arginine, is generally known to inhibit STAT5 phosphorylation and downregulate CD25 expression [36, 44], CD8⁺ T cells cultured with L-arginine supplementation exhibited higher CD25 expression (Fig. 2f). These observations suggest that the increase in CD25 expression is not a direct effect of L-arginine, but rather reflects sustained STAT5 signaling, which may involve not only the IL-2R pathway but also IL-7R/IL-15R signaling, thereby enhancing mitochondrial biogenesis. Further studies are warranted to clarify these mechanisms.

As T cells differentiate beyond the central memory stage, they progressively undergo a metabolic shift from OXPHOS to glycolysis, which is accompanied by mitochondrial dysfunction and a loss of metabolic plasticity [85, 86]. While the VSTs analyzed in this study exhibited a phenotype consistent with differentiated memory T cells (Fig. 2e), L-arginine supplementation nonetheless conferred both survival and metabolic benefits, even in highly differentiated memory CD8⁺ T cells. Notably, multiple beneficial effects of L-arginine were also observed in donor HC-2, which was enriched for late effector memory T cells (CCR7⁻ CD45RA⁻ CD27⁻ CD28⁻) exhibiting low MMP (Fig. 2d–e). However, no significant improvement in viability after restimulation or in IFN- γ and TNF- α production was observed in donor HC-2 (Fig. 5a, d). These findings suggest that L-arginine remains effective across a broad spectrum of memory T cell differentiation states, although its efficacy may be limited in late effector memory T or T_{EMRA} cells, which rely primarily on glycolysis for ATP production and exhibit irreversible metabolic rigidity [87].

Notably, the L-arginine-treated VSTs exhibited reduced CD107a expression following initial stimulation (Fig. 5d–e), a feature associated with less-differentiated and less-exhausted T cells [88–90]. However, upon secondary stimulation, the L-arginine-treated VSTs regained CD107a expression (Fig. 5d–e). A similar pattern has been reported in naïve CD4⁺ T cells, where L-arginine supplementation enhanced IFN- γ production upon secondary stimulation [40]. The distinct response observed in VSTs may reflect their more advanced differentiation state. CD107a expression and perforin production are more dynamically regulated than IFN- γ production at later stages of differentiation [88]. Although TNF- α production generally decreases as T cells undergo terminal differentiation [88], the L-arginine-treated VSTs exhibited increased TNF- α production upon restimulation in all donors except HC-2 (Fig. 5d). These findings suggest that these L-arginine-supplemented VSTs acquired cytotoxic potential without progressing toward further differentiation. Given the long-term persistence of VSTs *in vivo* following adoptive transfer (e.g., up to 12 weeks) [91], the initial reduction in cytotoxicity observed on day 15 is likely to be reacquired over time *in vivo*, as evidenced by the *in vitro* acquisition observed in this study (Fig. 5d–e).

The phenotypic features of L-arginine-treated VSTs, characterized by increased CD25 expression and reduced exhaustion (Fig. 2e–f), suggest potential for synergistic combination therapies. For instance, PD-1 blockade is more effective in progenitor-exhausted T cells than in terminally exhausted ones and is known to sustain FAO and promote mitochondrial biogenesis [34, 65, 92, 93]. In addition, combining VST therapy with IL-2 cytokine administration may enhance efficacy, as CD25 expression is critical for IL-2-mediated responses *in vivo* [94].

Collectively, our findings indicate that L-arginine supplementation in RPMI 1640 improves the metabolic fitness and functional capacity of VSTs, offering a promising strategy to enhance adoptive T cell therapy targeting persistent viral infections.

Conclusion

Excess L-arginine supplementation in RPMI 1640 enhances the durability of VSTs targeting persistent viruses by exerting anti-apoptotic, anti-exhaustion, and stemness-preserving effects. These changes confer resistance to AICD and facilitate the acquisition of polyfunctionality, including cytotoxic activity, upon restimulation. Although concerns remain regarding NO production and medium alkalization, the overall benefits of L-arginine—particularly its capacity to promote mitochondrial biogenesis—are further enhanced by cytokines such as IL-7 and IL-15. This strategy improves VST survival and function even after antigenic stimulation and freeze–thaw stress and may extend to other T cell therapies requiring *in vitro* expansion and cryopreservation, representing a promising metabolic intervention for clinical translation.

Limitation

This study focuses on apoptosis, exhaustion, and differentiation in memory CD8⁺ T cells. However, potential effects on other cell types and involvement in processes such as autophagy remain unclear. Further research is needed to investigate these additional mechanisms.

Declarations

Author Contribution

T.T. designed and performed the experiments, analyzed the data, and drafted the manuscript. A.K.-T. provided ongoing guidance, supervised data analysis, and revised the manuscript. S.S. contributed critical conceptual insights that substantially shaped the study and offered key perspectives on data interpretation. S.T., H.K., T.K., K.I., and M.T. (as laboratory head) offered academic advice and assisted with manuscript revisions. T.M. provided continuous guidance, supervised the overall project, coordinated resources, and secured funding. All authors reviewed and approved the final manuscript.

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Figures

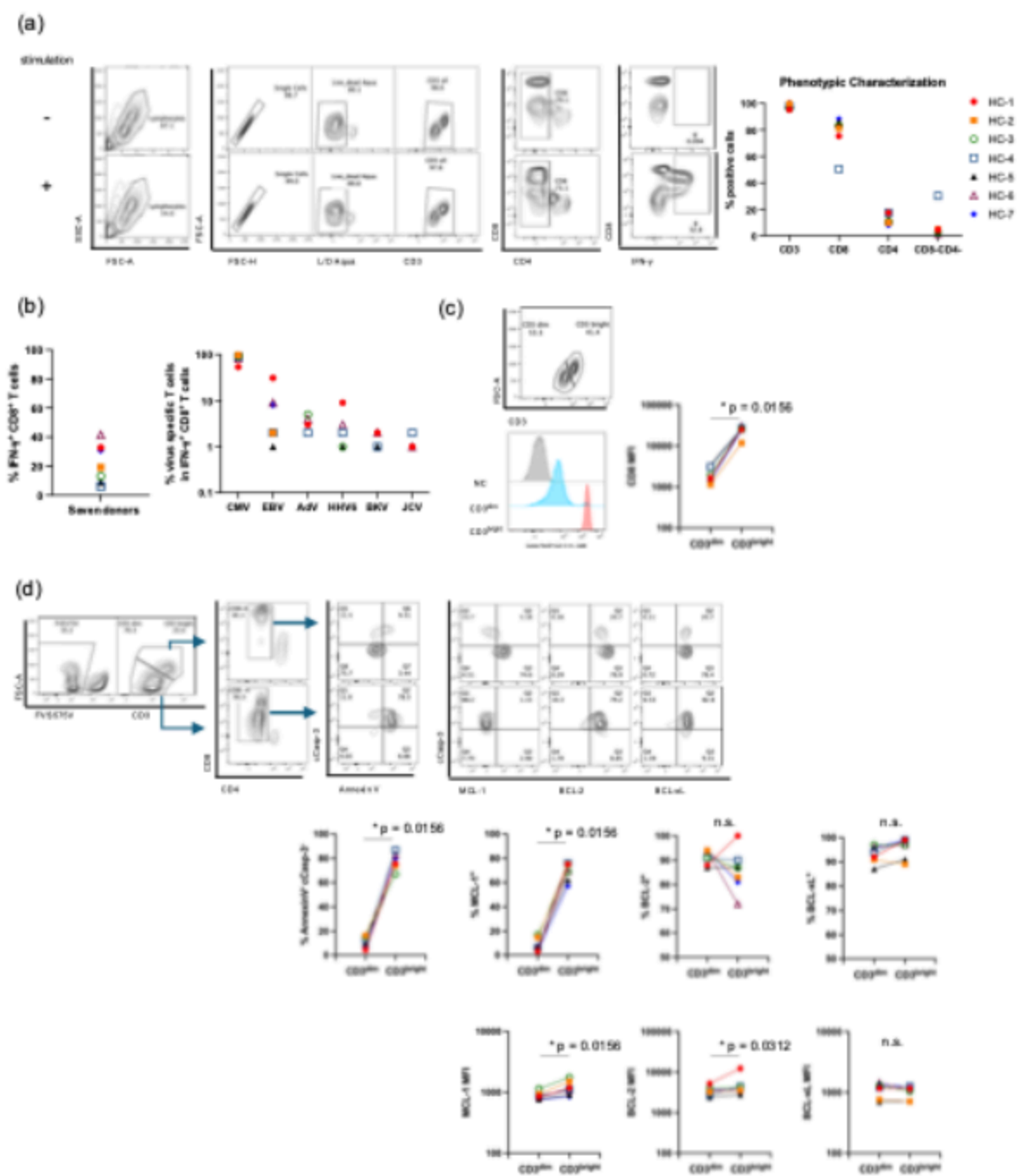


Figure 1

Phenotypic characterization of expanded T cells cultured in basal medium with exogenous interleukin (IL)-7 and IL-15.

(a) Representative flow cytometric plots and phenotypic characterization of expanded cells under the basal condition (n = 7). (b) Frequency of interferon (IFN)- γ -producing CD8⁺ T cells and their virus specificity. The cells were stimulated for 6 h prior to intracellular cytokine staining. (c) CD8 expression in CD3^{bright} T cells and CD3^{dim} T cells. (d) Expression of apoptotic markers (Annexin V and cleaved caspase-3 [cCasp-3]) and anti-apoptotic markers (MCL-1, BCL-2, and BCL-xL).

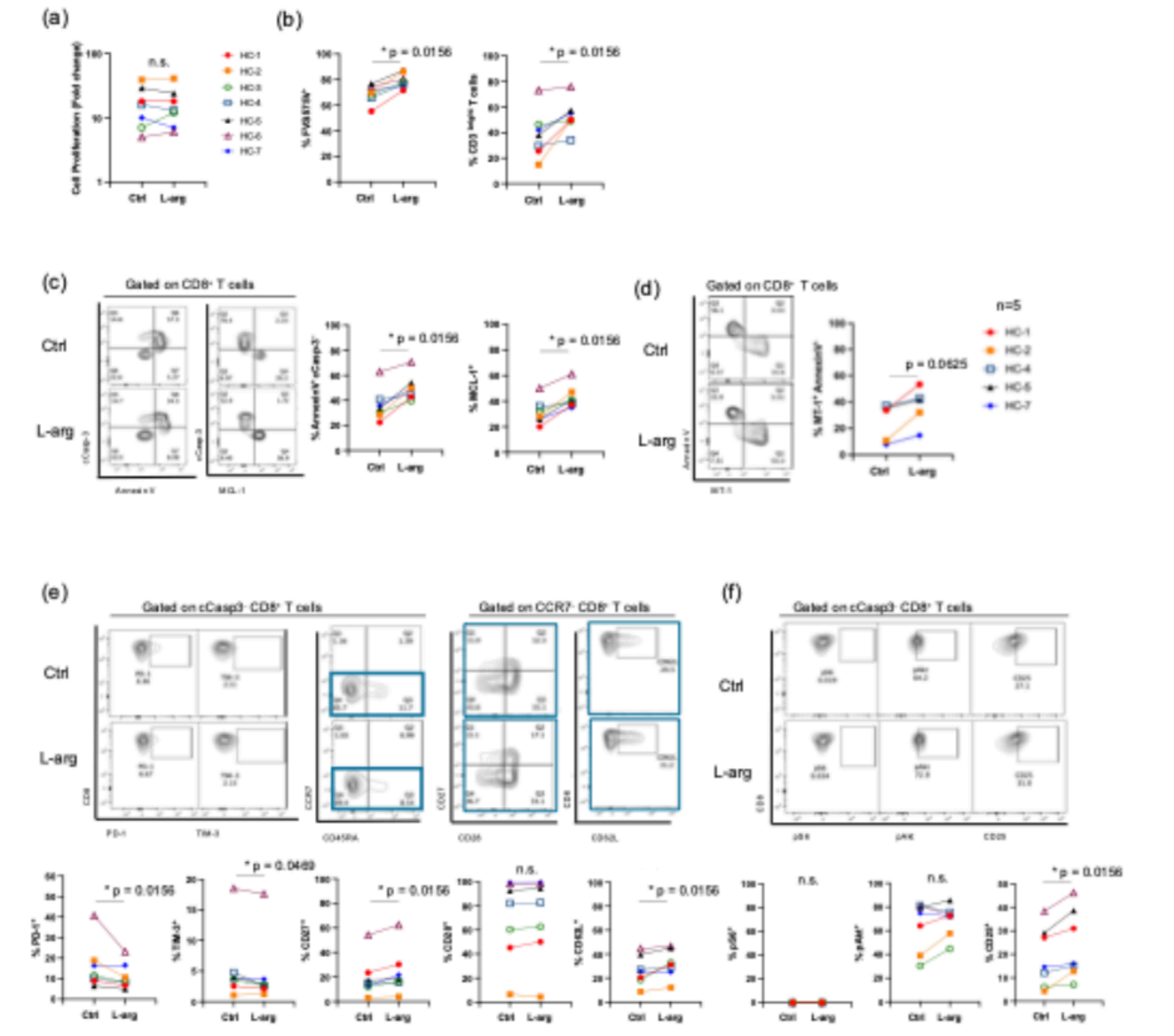


Figure 2

L-arginine reduces apoptosis and exhaustion and modulates differentiation and signaling in total CD8⁺ T cells.

(a) Comparative data showing fold change in cell proliferation between the control and L-arginine-enriched conditions (n = 7), measured using acridine orange–propidium iodide (AO/PI) staining. (b) Frequencies of viable cells (FVS575V-negative) and CD3^{bright} T cells. (c) Frequencies of cells expressing apoptotic markers (Annexin V and cCasp-3) and MCL-1, as well as the median fluorescence intensity (MFI) of MCL-1, in the CD8⁺ T cells under the control and L-arginine-enriched conditions. (d) Mitochondrial membrane potential of total CD8⁺ T cells was monitored using the MT-1 MitoMP Detection Kit after cryopreservation and thawing under the two culture conditions (n = 5). (e) Exhaustion (PD-1 and TIM-3) and differentiation (CCR7, CD45RA, CD27, CD28, and CD62L) marker expression in CD8⁺ T cells under the two conditions (n = 7). Given that apoptotic cells downregulate surface antigen expression, surface marker analysis was performed on cCasp-3⁻ CD8⁺ T cells to evaluate expression in the non-apoptotic population (Fig. S2). Frequencies of CD27, CD28, and CD62L expression represent their proportions within the CCR7⁻ CD8⁺ T cell subset comprising the total CD8⁺ T cell population. (f) Phosphorylation levels of S6 and Akt and CD25 expression in CD8⁺ T cells under control and L-arginine-enriched conditions (n = 7). Phosphorylation and surface marker analyses were performed on cCasp-3⁻ CD8⁺ T cells to assess expression in the non-apoptotic population, for reasons described above.

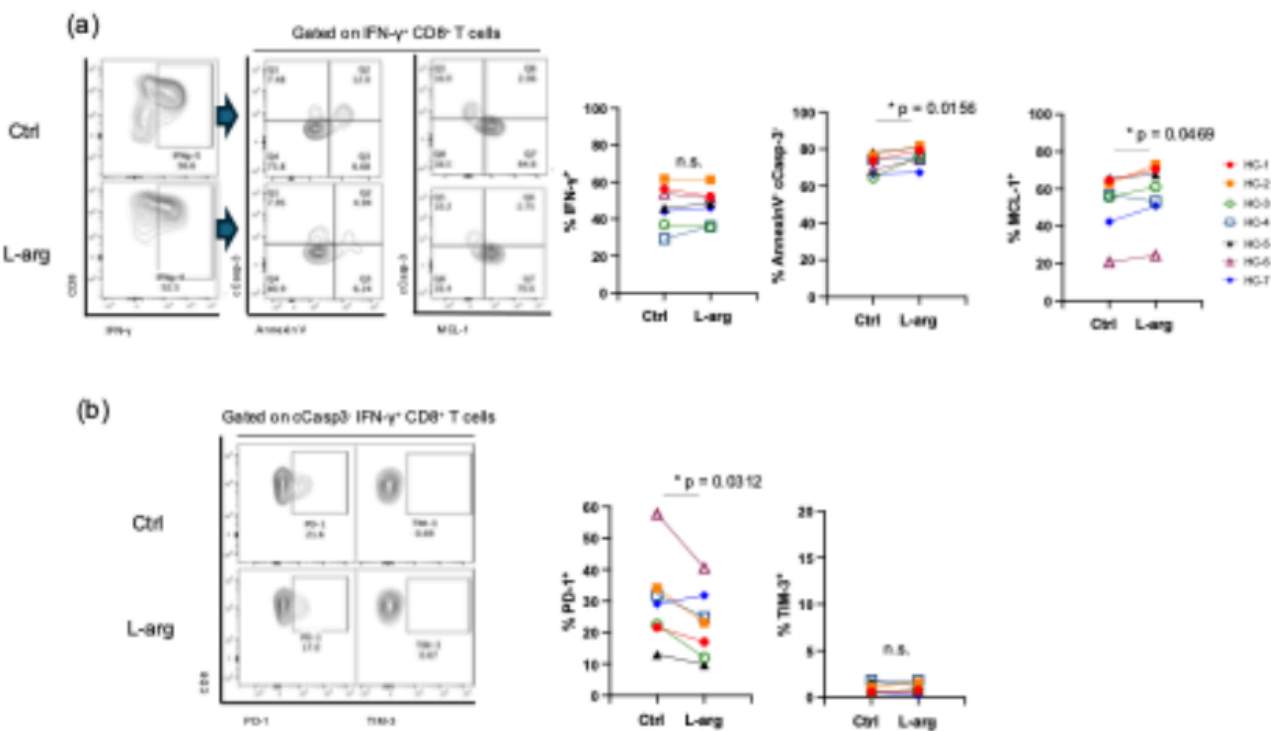


Figure 3

Effects of L-arginine on apoptosis and exhaustion in VSTs for persistent viruses.

(a) Representative flow cytometric plots and comparative data showing the frequencies of cells expressing apoptotic markers (Annexin V and cCasp-3) and MCL-1, as well as the MFI of MCL-1 expression, in IFN- γ -producing CD8⁺ T cells under control and L-arginine-enriched conditions (n = 7). (b) Expression of exhaustion markers PD-1 and TIM-3 in IFN- γ -producing CD8⁺ T cells under control and L-arginine-enriched conditions (n = 7). Phosphorylation and surface marker analyses were performed on cCasp-3⁻ CD8⁺ T cells to evaluate expression in the non-apoptotic population.

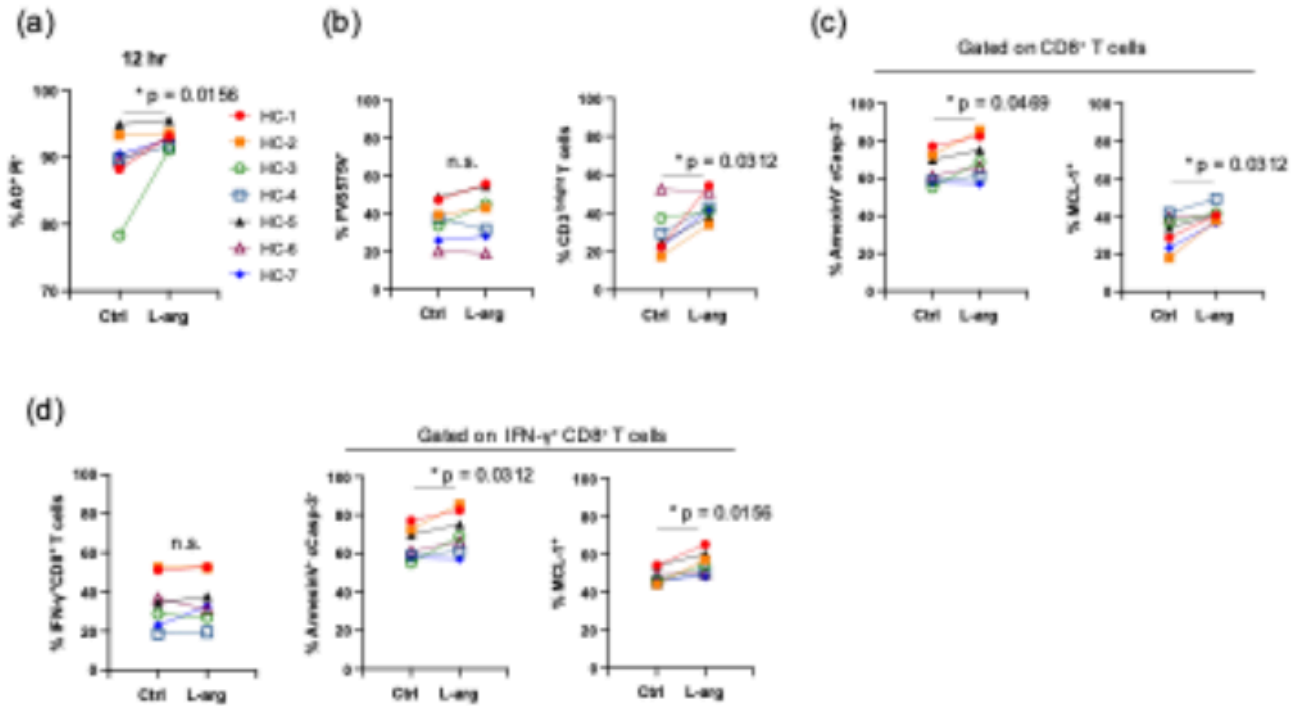


Figure 4

Effects of L-arginine on cell viability and apoptosis in VSTs for persistent viruses after cryopreservation and thawing.

(a) Comparative data showing cell viability at 12 h after thawing, measured using AO/PI staining. Frequencies of live cells (AO⁺PI⁻) are plotted (n = 7). (b) Comparative flow cytometric plots of the frequencies of viable cells (FVS575V-negative) and CD3^{bright} T cells under control and L-arginine-enriched conditions after freeze-thawing (n = 7). (c) Frequencies of cells expressing apoptotic markers (Annexin V and cCasp-3) and MCL-1, as well as the MFI of MCL-1 expression in total CD8⁺ T cells under control and L-arginine-enriched conditions following cryopreservation and thawing (n = 7). (d) Frequencies of cells expressing apoptotic markers (Annexin V and cCasp-3) and MCL-1, as well as the MFI of MCL-1, in IFN- γ -producing CD8⁺ T cells under control and L-arginine-enriched conditions after cryopreservation and thawing (n = 7).

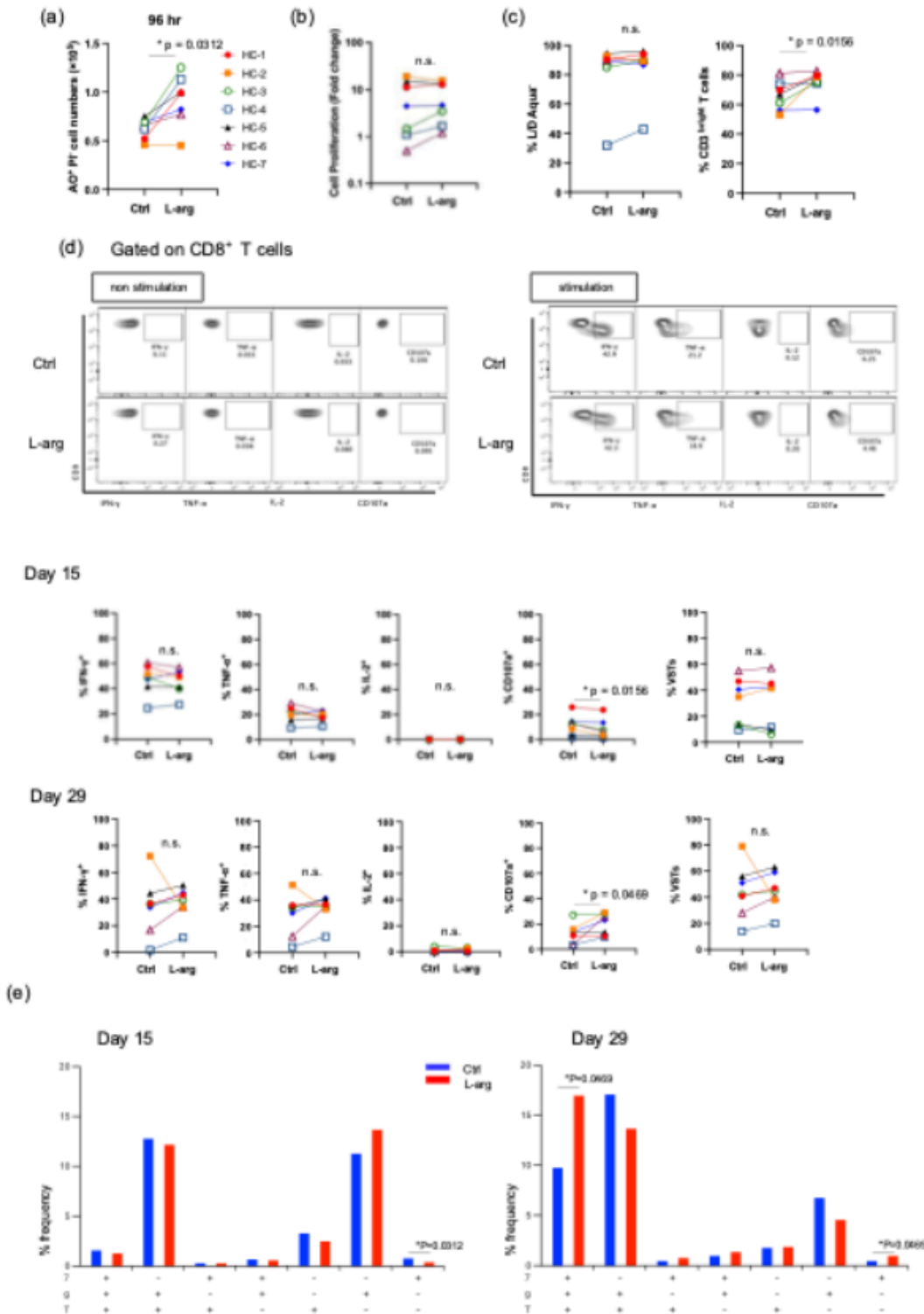


Figure 5

Resistance of VSTs under L-arginine-enriched conditions to activation-induced cell death and acquisition of cytotoxicity upon secondary stimulation.

(a) Differences in survival rates of expanded cells between control and L-arginine-enriched conditions were assessed after stimulation with viral peptides. A total of 1×10^5 expanded cells were stimulated

with the viral peptides at a concentration of 100 ng/mL each and cultured in R10 basal medium. Cell viability was evaluated at 96 h post-stimulation using AO/PI (n = 7). (b) Fold change in cell proliferation from day 15 to 29 following restimulation and cell viability on day 29 under control and L-arginine-enriched conditions, measured using AO/PI (n = 7). Comparative flow cytometric analysis showing frequencies of viable cells (Live/Dead Aqua-negative) and CD3bright T cells on day 29 (n = 7). (c) Frequencies of cells expressing intracellular cytokines (IFN- γ , TNF- α , IL-2) and the degranulation marker CD107a in CD8+ T cells following peptide stimulation on days 15 and 29 under control and the L-arginine-enriched conditions (n = 7). VSTs were defined as antigen-specific CD8+ T cells expressing at least one of the following markers upon stimulation: IFN- γ , TNF- α , IL-2, or CD107a. Cells were stimulated for 6 h prior to intracellular cytokine staining. Polyfunctionality analysis shows median frequencies of CD8+ T cell profiles across seven donors, indicating IFN- γ and TNF- α production and CD107a expression after peptide stimulation on day 15 and on day 29 under both conditions (n = 7). As VSTs exhibited negligible IL-2 production at both time points (Fig. 5d), IL-2 was excluded from polyfunctionality analysis. Polyfunctionality was analyzed using SPICE and PESTLE software [95]. The terms 'g', 'T', and '7' refer to IFN- γ , TNF- α , and CD107a, respectively.

Supplementary Files

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