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The "Preservation & Expansion" Process: A Key Milestone for Enhancing Accessibility of Autologous Tumor-Infiltrating Lymphocyte Therapy by Enabling Long-Term Cryopreservation of Seed Cells

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Background:

Autologous tumor-infiltrating lymphocyte (TIL) therapy has demonstrated promising clinical efficacy in advanced solid tumors, yet its broader application remains constrained by the need for timely tumor resection. Many patients with progressive disease are unable to undergo repeated surgical procedures, resulting in missed treatment opportunities. To overcome this barrier, we developed a “Preservation & Expansion” strategy, wherein TIL seed cells are initially expanded from resected tumor tissue, cryopreserved, and subsequently expanded into final products (FP) on demand. This study aims to validate the feasibility of this approach by evaluating the viability, phenotype, and functional integrity of FPs derived from seed cells cryopreserved for up to two years.

Methods:

- **Seed cell generation:** Tumor tissues were processed and cultured to generate seed cells.
- **Cryopreservation durations:** Seed cells were either expanded immediately (G0) or cryopreserved for 3 days (G3D), 1, 2, 3, 6 months (G1M–G6M), or 1, 2 years (G1Y, G2Y) prior to expansion.
- **Assessments:**
  - Cell viability at seed, intermediate, and final product stages
  - Phenotypic profiling (CD3, CD4, CD8, CD56) via flow cytometry
  - Functional evaluation of final products: IFN-γ secretion and cytotoxicity (real-time cell analysis, RTCA)

Results:

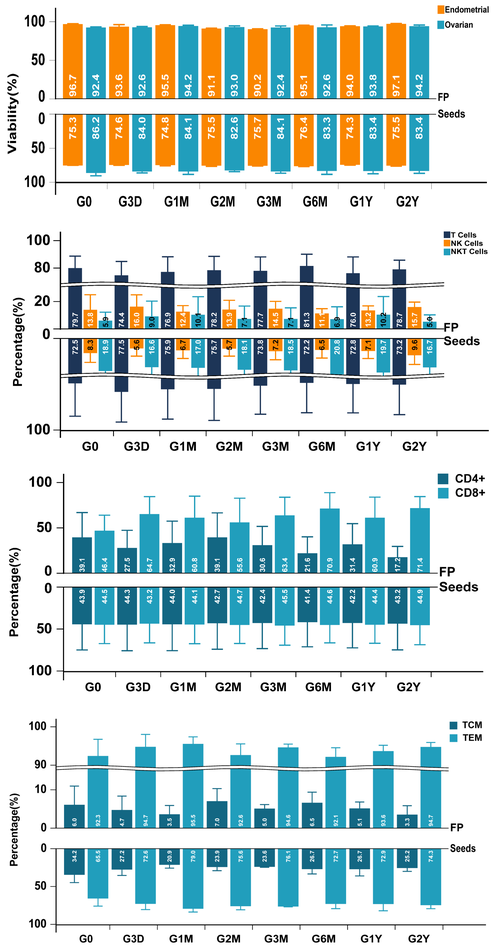

Figure 1. Post-thaw viability of TIL seed cells and final products following different cryopreservation durations.
TIL seed cells and final products derived from endometrial and ovarian cancer tissues were cryopreserved for indicated durations and then thawed for viability assessment by AO-PI staining. Data are presented as mean ± SD. The upper panel shows viability of final products stratified by tumor type, while the lower panel shows viability of seed cells stratified by tumor type. No significant differences in viability were observed across all cryopreservation durations compared to fresh controls within each cell type and tumor origin (all paired P > 0.05). These results indicate that long-term cryopreservation up to two years does not compromise the viability of either TIL seed cells or final products, regardless of tumor origin.

Figure 2. Proportions of T, NK, and NKT cell subsets in TIL seed cells and final products following different cryopreservation durations.
TIL seed cells and final products derived from endometrial and ovarian cancer tissues were cryopreserved for indicated durations and then analyzed by flow cytometry for T cell (CD3⁺CD56⁻), NK cell (CD3⁻CD56⁺), and NKT cell (CD3⁺CD56⁺) proportions within the CD45⁺ lymphocyte population. Data are presented as mean ± SD. The upper panel shows cell subset proportions of final products stratified by tumor type, while the lower panel shows cell subset proportions of seed cells stratified by tumor type. No significant differences in these cell subset proportions were observed across cryopreservation durations compared to fresh controls within each cell type and tumor origin (all paired P > 0.05), indicating that long-term cryopreservation does not alter the T/NK/NKT cell composition of TIL cells.

Figure 3. Proportions of CD4⁺ and CD8⁺ T cells in TIL seed cells and final products following different cryopreservation durations.
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TIL seed cells and final products derived from endometrial and ovarian cancer tissues were cryopreserved for indicated durations and then analyzed by flow cytometry for CD4⁺ and CD8⁺ T cell proportions within the CD3⁺ T cell population. Data are presented as mean ± SD. The upper panel shows CD4/CD8 proportions of final products stratified by tumor type, while the lower panel shows CD4/CD8 proportions of seed cells stratified by tumor type. No significant differences in CD4⁺ and CD8⁺ T cell proportions were observed across cryopreservation durations compared to fresh controls within each cell type and tumor origin (all paired P > 0.05), indicating that long-term cryopreservation does not alter the CD4/CD8 composition of TIL cells.

Figure 4. Proportions of TCM and TEM cells in TIL seed cells and final products following different cryopreservation durations.
TIL seed cells and final products derived from endometrial and ovarian cancer tissues were cryopreserved for indicated durations and then analyzed by flow cytometry for TCM (CD45RO⁺CCR7⁺) and TEM (CD45RO⁺CCR7⁻) cell proportions within the CD3⁺ T cell population. Data are presented as mean ± SD. The upper panel shows TCM/TEM proportions of final products stratified by tumor type, while the lower panel shows TCM/TEM proportions of seed cells stratified by tumor type. No significant differences in TCM and TEM cell proportions were observed across cryopreservation durations compared to fresh controls within each cell type and tumor origin (all paired P > 0.05), indicating that long-term cryopreservation does not alter the memory T cell subset composition of TIL cells.

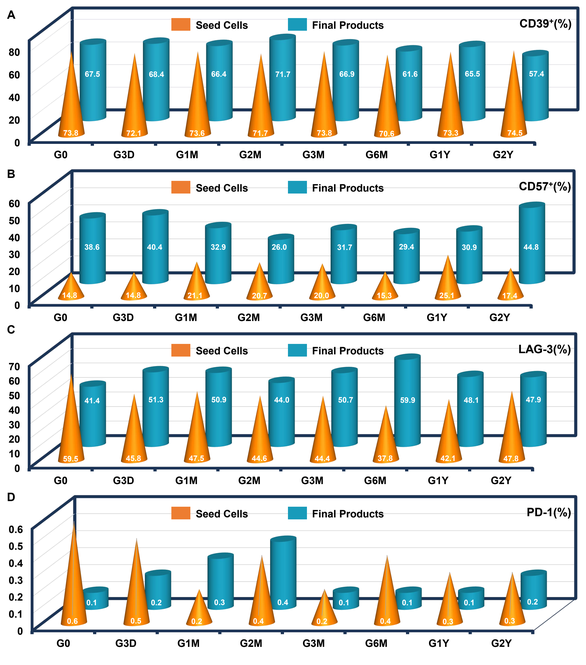

Figure 5. Expression of exhaustion markers in TIL seed cells and final products following different cryopreservation durations.
TIL seed cells and final products derived from endometrial and ovarian cancer tissues were cryopreserved for indicated durations and then analyzed by flow cytometry for the expression of exhaustion-associated markers within the CD3⁺ T cell population. Data are presented as mean ± SD. In each subfigure, cones represent seed cells and cylinders represent final products. (A) CD57⁺, (B) CD39⁺, (C) LAG-3⁺, (D) PD-1⁺. No significant differences in the expression of these exhaustion markers were observed across cryopreservation durations compared to fresh controls for most time points (paired P > 0.05), indicating that long-term cryopreservation does not substantially alter the exhaustion status of TIL cells.

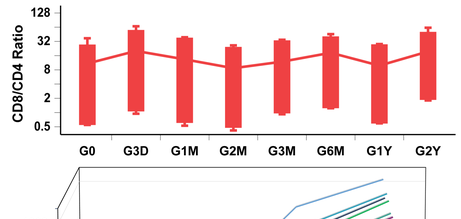

Figure 6. CD8/CD4 ratio in TIL final products following different cryopreservation durations
TIL final products derived from endometrial and ovarian cancer tissues were expanded from seed cells cryopreserved for indicated durations and then analyzed by flow cytometry for CD4⁺ and CD8⁺ T cell proportions within the CD3⁺ T cell population. Data are presented as mean ± SD. No significant differences were observed across cryopreservation durations compared to fresh controls (paired P > 0.05), indicating that long-term cryopreservation does not alter the CD8/CD4 composition of TIL final products. In TIL cell therapy, the CD8/CD4 ratio is not only an important parameter for product characterization but also closely associated with antitumor activity, clinical efficacy, and product consistency. These findings further support the feasibility of the “Preservation & Expansion” process in maintaining TIL product quality

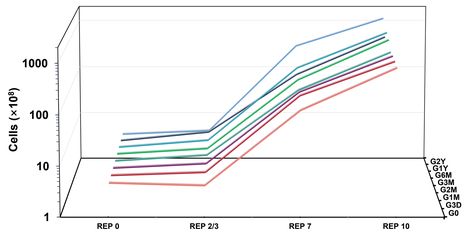

Figure 7. Expansion curves of TIL seed cells following different cryopreservation durations.
TIL seed cells derived from tumor tissues were cryopreserved for indicated durations, thawed, and subjected to rapid expansion protocol (REP) culture. Cumulative cell numbers were monitored at indicated time points. Data are presented as cumulative fold expansion (cell numbers ×10⁸). Comparable expansion kinetics were observed across all cryopreservation durations, with final product cell numbers consistently reaching the hundred-billion scale. These results demonstrate that long-term cryopreservation up to two years does not impair the proliferative capacity of TIL seed cells, supporting the robustness and high-yield consistency of the “Preservation & Expansion” process.

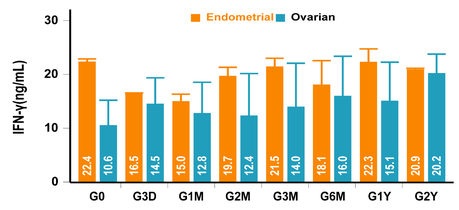

Figure 8. IFN-γ secretion by TIL final products following different cryopreservation durations.
TIL final products derived from endometrial and ovarian cancer tissues were expanded from seed cells cryopreserved for indicated durations and then co-cultured with target cells to assess IFN-γ secretion. Data are presented as mean ± SD. No significant differences in IFN-γ secretion were observed across cryopreservation durations compared to fresh controls for most time points (paired P > 0.05), indicating that long-term cryopreservation does not impair the effector cytokine production capacity of TIL final products.

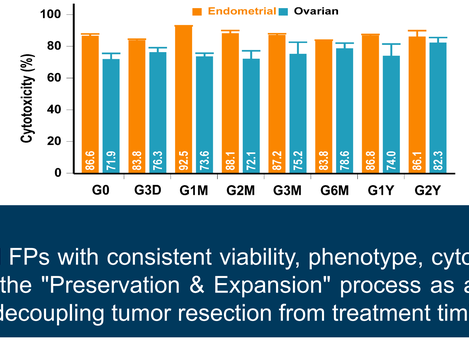

Figure 9. Cytotoxicity of TIL final products following different cryopreservation durations.
TIL final products derived from endometrial and ovarian cancer tissues were expanded from seed cells cryopreserved for indicated durations and then evaluated for cytotoxicity against target cells at an effector-to-target ratio of 4:1 using real-time cell analysis (RTCA). Data are presented as mean ± SD. No significant differences in cytotoxicity were observed across cryopreservation durations compared to fresh controls for most time points (paired P > 0.05), indicating that long-term cryopreservation does not compromise the tumor-killing ability of TIL final products.

Conclusion:

TIL seed cells can be effectively expanded into functional FPs with consistent viability, phenotype, cytokine secretion, and cytotoxicity after long-term cryopreservation up to two years. These results validate the "Preservation & Expansion" process as a robust and feasible approach to improve the accessibility and practicality of autologous TIL therapy by decoupling tumor resection from treatment timing.

Abbreviation
FP, final product; G0, immediate expansion group; G1M, 1-month cryopreservation group; G1Y, 1-year cryopreservation group; G2M, 2-month cryopreservation group; G2Y, 2-year cryopreservation group; G3D, 3-day cryopreservation group; G3M, 3-month cryopreservation group; G6M, 6-month cryopreservation group; IFN-γ, interferon-gamma; RTCA, real-time cell analysis; TCM, central memory T cell; TEM, effector memory T cell; TIL, tumor-infiltrating lymphocyte.

Disclosures
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