Genetically engineered tumor-infiltrating lymphocytes (cytoTIL15) exhibit IL2-independent persistence and anti-tumor efficacy against melanoma *in vivo*

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Abstract 166

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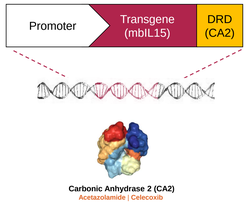
Background

Adoptive cell therapy with tumor-infiltrating lymphocytes (TILs) has demonstrated tremendous promise in clinical trials for patients with solid tumors. However, current TIL therapy requires systemic administration of IL2 to promote TIL survival, and IL2-associated toxicities greatly limit patient eligibility and reduce the long-term clinical benefit of TIL therapy. Unlike IL2, which promotes T cell exhaustion, IL15 maintains antigen-independent TIL persistence through homeostatic proliferation of memory T-cells and supports CD8+ T cell anti-tumor activity without stimulating regulatory T cells. We designed genetically engineered TILs to express a regulated form of membrane-bound IL15 (mbIL15) for tunable long-term persistence, leading to enhanced persistence and efficacy in vitro and in PDX tumor models.

Rationale and Methods

Our Solution: cytoTIL™ with Regulated IL15

- Increase **ORR** in **post-PD1 setting**
  - Promote antigen-independent TIL expansion and persistence
  - Drive adjacent T and NK cell activation and expansion
  - Drive phenotype towards CD8+ & memory T-cells
  - Eliminates concerns of Treg expansion or capillary leak syndrome-associated toxicity
- Eliminate toxic **high-dose IL2 regimen**
  - Reduce toxicity and cost-of-care
  - Expand patient and site eligibility



Obsidian's cytoDRiVE® platform includes small human protein sequences called drug responsive domains (DRD)s that enable regulated expression of a fused target protein under control of FDA-approved, bioavailable small molecule ligands. cytoTIL15 contains TILs engineered with mbIL15 under the control of a carbonic-anhydrase-2 DRD, controlled by the ligand acetazolamide (ACZ), an approved oral diuretic with good safety and tolerability. After isolation from tumors, TILs were transduced and expanded *in vitro* through a proprietary TIL expansion process using an engineered feeder cell line. cytoTIL15 were immunophenotyped and assessed for *in vitro* antigen-independent survival and co-cultured with tumor cells to assess polyfunctionality and cytotoxicity. *In vivo* TIL persistence and anti-tumor efficacy was evaluated through adoptive transfer of TILs into immunodeficient NSG mice, either naïve or implanted with subcutaneous patient-derived-xenograft (PDX) tumors. cytoTIL15 were compared to IL2-derived. conventional TILs isolated from the same patients.

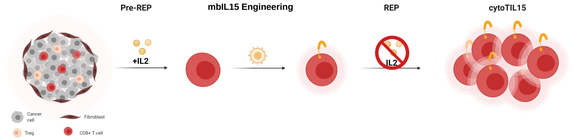


Figure 1. Schema of cytoTIL15 generation. *Left to Right:* The pre-REP expansion includes dissociation of the tumor followed by culture with IL2. Following pre-REP, extracted TILs are engineered with regulatable mbIL15 using viral vectors; with a consistent transduction efficiency of over 50-80% mbIL15 from a range of donors at our site. Once transduced, these cytoTIL15 cells are initiated in a novel Rapid Expansion Process (REP) which uses engineered feeder cells to provide co-stimulation and growth signals to cytoTIL15, allowing expansion of mbIL15 expressing TILs. Uniquely, this REP process is entirely IL2 independent. These expanded cytoTIL15 cells are phenotypically primed and ready for downstream applications as a preclinical product.

Beneficial features of cytoTIL15

cytoTIL15 exhibit IL15-dependent expansion and maintenance of memory T-cell phenotype

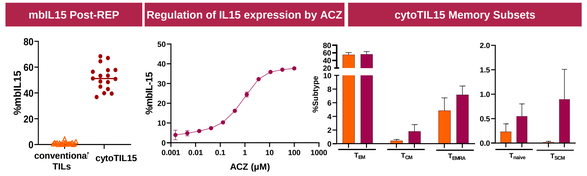


Figure 2. Expression of engineered membrane-bound IL15 (mbIL15) in cytoTIL15 is regulated by ACZ in a dose-dependent fashion. *Left:* cytoTIL15 and un-engineered TILs (conventional TILs) were expanded in REP with feeder cells +/- IL2 for 14-days, after which cells were assessed for expression of mbIL15 by flow cytometry. *Middle:* Cryopreserved cytoTIL15 were thawed and rested in ACZ-free culture media for 24 hours, then cultured overnight in increasing concentrations of ACZ. The next day, cytoTIL15 were analyzed for IL15 expression by flow cytometry. *Right:* After expansion in REP, conventional TILs and cytoTIL15 we assessed by flow cytometry to characterize T cell memory subsets. Conventional TILs are indicated by the orange icons and bars, and cytoTIL15 are indicated by the maroon icons and bars. (n=15)

cytoTIL15 are polyfunctional and maintain TCR Vβ diversity

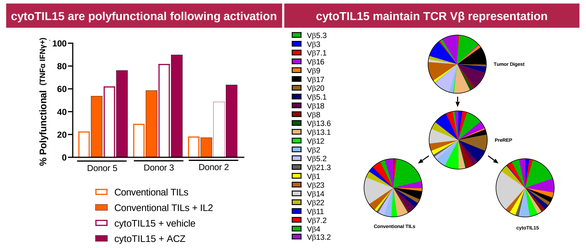


Figure 3. cytoTIL15 are more polyfunctional than conventional TILs and maintain TCR Vβ diversity. *Left:* Post-REP TILs from 3 TIL donors were rested for 24 hours in cytokine or ACZ-free culture media. Next, conventional TILs were cultured with high dose IL2 and cytoTIL15 were cultured overnight with vehicle or ACZ as marked. The next day, TILs were stimulated with PMA + ionomycin for 6 hours in the presence of protein transport inhibitors, then assayed by flow cytometry for the frequencies of TNFα and IFNγ expressing cells (double positive cells identified as polyfunctional) within total CD3+ TILs. *Right:* TILs (CD3+ cells) were assessed at the point of tumor digest, at the end of Pre-REP, and at the end of REP (from engineered cytoTIL15 as well as conventional TIL processes) using a flow cytometry-based multiparametric assay to evaluate frequencies of TCR Vβ sub-families. Evaluation of 24 different TCR Vβ sub-families (covering 70% normal human Vβ repertoire) revealed maintenance of a diverse TCR Vβ repertoire through the manufacturing process (Pre-REP and REP) for both conventional TILs and cytoTIL15. μM

cytoTIL15 demonstrate improved IL2 independent survival *in vitro*

cytoTIL15 exhibit enhanced persistence in vitro in the absence of IL2

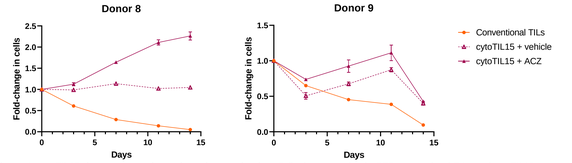


Figure 4. cytoTIL15 demonstrate enhanced persistence *in vitro*. Following expansion in REP, conventional TILs and cytoTIL15 were rested overnight in the absence of IL2 or ACZ. After rest, cells were plated at controlled cell densities and cultured in a 48-well plate over 14 days with cytokine and ACZ support. Cell count and assessment of mbIL15 expression was examined by flow cytometry on Days 0, 3, 7, 11, and 14 in culture. Conventional TILs are indicated by the orange icons and lines, and cytoTIL15 are indicated by the maroon icons and lines (n=2 donors in duplicate).

cytoTIL15 demonstrate superior persistence *in vivo* compared to conventional TILs + IL2

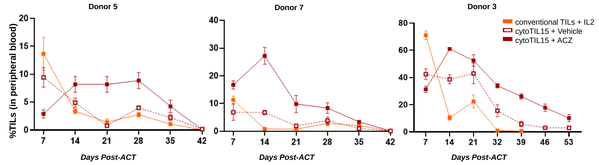
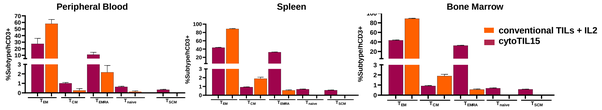


Figure 5. cytoTIL15 exhibit enhanced persistence *in vivo*. Un-engineered conventional TILs and cytoTIL15 generated from three different donors (2 melanoma & 1 head & neck) were injected intravenously in NSG (NOD-SCID-γc-/-) at 10e6 TIL/mouse. Mice dosed with conventional TILs received high dose IL2 twice daily for the first 4 days post-adoptive cell transfer, while mice dosed with cytoTIL15 received 200mg/kg ACZ or vehicle every day post-adoptive cell transfer. At weekly intervals, submandibular blood was collected, processed to lyse red blood cells, and resultant single cell suspension was stained with a panel including murine CD45 and human CD3 antibodies. Percent TILs (human CD3+ murine CD45- cells) were quantified via flow cytometry at each time point, with cytoTIL15 demonstrating a peak in expansion at Day 14, followed by continued persistence through day 53 post-transfer (3 donors; n=5 mice/group).

cytoTIL15 maintain memory T-cell subpopulations after 14 days *in vivo*



cytoTIL15 persist long-term in splenic and bone marrow compartments *in vivo*

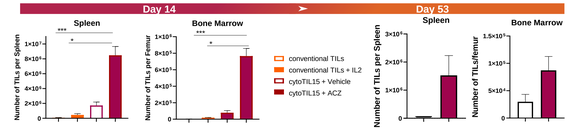


Figure 6. Persisting cytoTIL15 are enriched in spleens and bone marrow and exhibit a favorable memory-like phenotype. *Top:* At 14 days following adoptive cell transfer, conventional TILs and cytoTIL15 were assessed from processed peripheral blood, spleens, and bone marrow of the above 4-5 mice/group by flow cytometry to characterize T cell memory subsets. *Bottom:* Cohorts of 4-5 mice were sacrificed at 14 and 53-days post-transfer, and spleen and bone marrow harvested, processed into single cell suspension, and stained with murine CD45 and human CD3 antibodies. Absolute number of TILs (percent human CD3+ murine CD45- cells x total cell count per tissue) were quantified via flow cytometry. cytoTIL15 demonstrated a significantly higher persistence in splenic and bone marrow samples both at Day 14 and Day 53 post-transfer. (Conventional TILs are indicated by the orange icons and bars, and cytoTIL15 are indicated by the maroon icons and bars; data from TILs generated from Donor 3; n=4-5 mice/group).

cytoTIL15 demonstrate enhanced cytotoxicity against allogeneic tumors *in vitro*

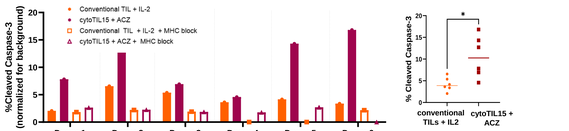
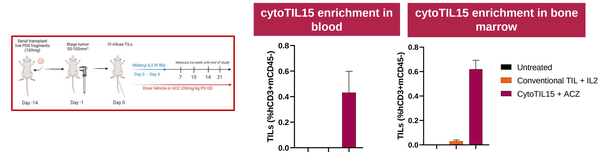


Figure 7. cytoTIL15 elicit MHC-I-dependent cytotoxicity *in vitro*. *Left:* Un-engineered conventional TILs and cytoTILs were cryopreserved at the end of REP. Cryopreserved TILs were thawed and rested in cytokine-free conditions overnight, after which cells were co-cultured with Cell Trace Violet-labeled, HLA-matched melanoma cells at a 1:1 and 5:1 effector-to-target (TIL:target) ratios. To assess for MHC-1 dependent cytotoxicity, melanoma cells were pre-treated with 80ug/mL HLA ABC MHC blocking antibody for 2 hours prior to the assay. After 3 hours of co-culture, cells were harvested and evaluated for expression of intracellular cleaved-caspase 3 (a marker for irreversible commitment to cell death) on melanoma cells by flow cytometry. Quantified cleaved caspase 3 was normalized to that of target cells alone (spontaneous or background release) * denotes p<0.05; n=6 donors.

cytoTIL15 control tumor growth in allogeneic PDX models



cytoTIL15 demonstrates superior anti-tumor efficacy compared to conventional TILs + IL2

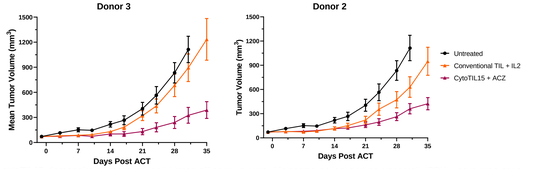


Figure 8. cytoTIL15 demonstrate improved anti-tumor efficacy *in vivo*. Melanoma patient-derived xenograft (PDX) tissue was serially passaged as 100mg fragments implanted subcutaneously onto the flanks of female NSG mice. Animals were randomized 13-days following PDX-implant, and ACT with conventional TILs or cytoTIL15 was performed 14-days following PDX-implant. Conventional TIL and cytoTIL15 were generated from two different HLA-matched, allogeneic donors. cytoTIL15 (with 200mg/kg ACZ PO QD) demonstrated enhanced anti-tumor efficacy in a xenograft melanoma model as compared to conventional TILs (receiving high dose IL2 twice daily for the first 4 days post-adoptive cell transfer; n=8/group). Cohorts of 4-5 mice were sacrificed at 35 days post-transfer and spleen, cardiac blood, and bone marrow harvested, processed into single cell suspension, and stained with a panel of antibodies including murine CD45, and human CD3 antibodies. The frequency of TILs (percent human CD3+ murine CD45- cells, out of singlets-live-lymphocytes in mouse blood) were quantified via flow cytometry.

Conclusions

The superior persistence and potency of cytoTIL15 in the complete absence of IL2 highlights this novel TIL product with a potential for enhanced safety and efficacy in clinical trials for indications such as metastatic melanomas and other solid tumors.

- cytoTIL15 are **polyfunctional** and preserve T cell memory subpopulations as compared to IL2-derived TILs, all while maintaining TCR Vβ diversity.
- cytoTIL15 enhance long-term persistence in vitro and in vivo, **independent of IL2,** and persist in immunological niches.
- cytoTIL15 demonstrate potent **anti-tumor cytotoxicity** against both autologous and allogeneic tumor targets *in vitro*.
- ACT with cytoTIL15 **controls tumor growth** in the allogeneic setting, and persistent TILs have a favorable anti-tumor phenotype.

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For questions, please reach out to Rachel Burga (rburga@obsidiantx.com). Schematic generated from biorender.io.

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