UNIVERSITY OF CALIFORNIA SAN DIEGO

Characterization of Cytotoxic T-Lymphocyte Mediated Immunotherapy-Tolerant Cancer Persister Cells

A Thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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The Thesis of Filipe Araujo Hoffmann is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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ABSTRACT OF THE THESIS

Characterization of Cytotoxic T-Lymphocyte Mediated Immunotherapy-Tolerant Cancer Persister Cells

by

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Master of Science in Biology

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Professor Matthew Hangauer, Chair
Professor Yunde Zhao, Co-Chair

Following initial therapy responses, tumors often relapse leading to patient mortality. How cancer cells change from a therapy sensitive to a therapy resistant state is poorly understood, particularly in the context of cytotoxic CD8 T cells mediated immunotherapy. Multiple studies have identified mechanisms by which residual tumor cells avoid CD8 T cell activation, but thus far there have been no studies focused on whether residual tumor cells survive continuous exposure to and attack by activated CD8 T cells. We hypothesized that in addition to commonly proposed

evasive mechanisms, cancer cells can enter an immunotherapy-tolerant persister cell state to survive activated CD8 T cell attack. Here, we report the observation of a subpopulation of quiescent immunotherapy persister cells which survive through a reversible, non-genetic mechanism. Upon extended cytotoxic T cell pressure, a subset of immunotherapy persister cells reenter the cell cycle and regrow into overtly resistant colonies which may represent the initial events of acquired resistance and tumor recurrence. These findings suggest that cancer cells may survive initial T cell cytotoxicity exposure through a quiescent persister state for several weeks prior to relapse. Interestingly, we found that immunotherapy persister cells survive despite continual T cell activation and experience sublethal activation of apoptotic signaling. Together, these studies reveal a novel population of tumor cells which survive extended CD8 T cell attack and may seed tumor recurrence during acquired resistance to immunotherapy.

INTRODUCTION

As the second major leading cause of death in the United States, cancer is unarguably a major public health concern.[1] While the term "cancer" describes a broad group of diseases sharing a set of defining hallmarks, such as uncontrolled cell proliferation, the biological pathways that drive carcinogenesis vary from patient to patient.[2,3,4] In order to develop anti-cancer therapeutics, scientists generated drugs that inhibit essential pathways unique to a given patient's cancer progression.[5,6] Unfortunately, these therapeutics often target processes essential for normal cell function generating strong side effects such as nausea, fatigue, hair loss, anemia, and others.[7]

Different from more commonly used cancer therapeutics, our adaptive immune system contains anti-tumor properties and offer a greater degree of tumor specificity, especially CD8+ killer T cells.[8,9,10] Each mature CD8 T cell expresses a unique T cell receptor (TCR) carrying high specificity to a given antigen when presented by major histocompatibility complex 1 (MHC-I or HLA).[11, 12, 13] In the context of tumor immunosurveillance, CD8 killer T cells specifically target tumors presenting neo-antigens or tumor associated antigens in the context of MHC-I.[11, 12,13,14] Following MHC-I to TCR interaction, CD8 T cells secrete cytotoxic granules as well as cytokines on their respective target, which promotes cancer cell death. [11]

As an attempt to improve this tumor to T cell interaction, researchers generated a new class of anti-cancer drugs and therapies referred to as immunotherapeutics.[15] These approaches include utilizing chimeric antigen receptor (CAR) T cells possessing greater potential to recognize a wider variety of cancer epitopes than standard CD8 T cells.[16,17] Furthermore, adoptive T cell transfer has also been associated with improved cancer patient outcomes.[18] This approach is conducted by extracting CD8 T cells that specifically target a patient's tumor and

expanding these cells ex vivo.[18] These expanded T cells are then returned into the patient in order to target and destroy cancer cells.[18]

However, many reports have indicated that cancer cells may survive T cell cytotoxicity by avoiding T cell to tumor interactions.[13,18,19,20] One commonly discussed mechanism occurs through the upregulation of programmed death ligand 1 (PDL1).[13,21] When PDL1 interacts with programmed death 1 (PD1) expressed in the surface of T cells, the T cells become anergic and unable to target tumors.[13,21,22] To counter this issue, researchers developed checkpoint blockade immunotherapy, which utilizes monoclonal antibodies (mAb) that prevent PD1 to PDL1 interactions.[13] Unfortunately, checkpoint blockade therapy fails to provide robust durable responses for the majority of cancer patients.[24,25] Furthermore, little is known about how after initiating checkpoint blockade therapy, cancer cells survive early CD8 T cell exposure and eventually acquire durable resistance allowing these cells to relapse.[24]

In regards to acquired checkpoint blockade therapy resistance, a recent study has shown that residual *murine* organotypic tumor cells survive anti-PDL1 checkpoint blockade immunotherapy pressure through an "immunotherapy persister" state.[26] This state was hypothesized to seed tumor regrowth, but it remains unclear if analogous persister cells exist in human cancers or whether persister survival depends on T cell evasion.[26] Therefore, here we aim to better characterize the immunotherapy persister state hallmarks and expand these findings to human cancer cell lines. We propose that following cognate killer CD8 T cell exposure, cancer cells may enter a quiescent pro-survival immunotherapy persister state to tolerate initial CD8 T cell cytotoxicity. Subsequently, these immunotherapy persisters may reenter the cell cycle and regrow as overly resistant immunotherapy-tolerant expanded persisters (ITEP) despite continuous T cell pressure.

In order to test our hypothesis, we developed an *in vitro* co-culture model in which human A375 melanoma cells are co-cultured with mature NY-ESO-1 (ESO-1) targeting TCR CD8 T cells.[27] We utilized the A375 cancer cell line because they are adherent, which allows for washing non-adherent T cells and dead tumor cells from plates; as necessary. Consequently, we were capable of refreshing T cells every 3 days to avoid exhaustion.[28,27] This allowed us to specifically study surviving residual immunotherapy persister cancer cells with the following aims:

1. Identify an acquired immunotherapy persister state in the A375 melanoma cells.
2. Characterize the immunotherapy persister hallmarks in relation to chemotherapy persisters.
3. Identify immunotherapy persister survival mechanisms and vulnerabilities.

Chapter 1: Melanoma A375 and NY-ESO-1 Co-Culture Model

Following an initial positive response to chemotherapeutics, cancer cells can relapse leading to patient mortality. To uncover primary tumor chemotherapy resistance, scientists identified several cancer survival mechanisms, such as drug pumps, activation of complementary pathways, and EGFR mutations.[31,33,34] However, little is known about how acquired cancer resistance mechanisms emerge in response to drug therapy pressure.

To explain how cancer cells change from therapy sensitive to therapy resistant, scientists previously suggested that after the majority drug naive parental cancer cells die under initial chemotherapeutic pressure, residual quiescent cancer cells survive through a persister cell state.[34] These persister cells acquire a chromatin mediated state that promotes tumor survival following initial drug exposure.[34] Persister cells then acquire additional survival mechanisms and reenter the cell cycle as overly resistant drug-tolerant expanded persisters (DTEP) despite continuous drug pressure.[34,35] Interestingly, if early persisters are removed from drug treatment, they are re-sensitized to therapy when later re-exposed to chemotherapeutics.[34] This reversibility suggests that the persister state is maintained through an unstable epigenetic modification that is lost upon removal of drug pressure.[34]

We hypothesized that in the context of CD8 T cell mediated immunotherapy, cancer cells can acquire a similar pro-survival quiescent immunotherapy persister state. This would allow cancer cells to survive early T cell cytotoxicity for extended periods of time prior to relapsing as overly resistant and proliferative immunotherapy-tolerant expanded persisters (ITEP). Furthermore, the immunotherapy persister hypothesis is further supported by *murine* cancer studies indicating that tumors possibly acquire an immunotherapy persister phenotype, though direct survival of CD8 T cell attack has not been reported.[26]

To test the possibility of immunotherapy persister cells in human tumors, we generated a model in which the human BRAF V600E A375 melanoma cell line is co-cultured with a cognate mature CD8 T cell for several weeks. Due to T cell receptor (TCR) high specificity to a given antigen and MHC-I, we generated TCR CD8 T cells specific for the NY-ESO-1 (ESO-1) peptide presented by A375 cells in the context of HLA-A*02 (MHC-I).[12,27] We subsequently *in vitro* co-cultured those ESO-1 targeting T cells with A375 melanoma for several weeks while refreshing T cells every 3 days to avoid exhaustion.

If melanoma cells acquire an immunotherapy persister state following CD8 T cell co-culture, we expect to observe that following initial T cell exposure, the majority of A375 cells die. However, a residual population would enter a pro-survival, reversible and quiescent state that seeds subsequent tumor regrowth.

MATERIALS AND METHODS:

Cell Lines. BRAF V600E A375 melanoma cell line was purchased from ATCC. Retrovirus producing PG-13 cell line was kindly provided by the Restifo lab (NCI). Peripheral Blood Mononuclear Cells (PBMC) extracted from healthy patients following donor's approval were purchased from StemCell Technology.

ESO-1 TCR Expression Retrovirus Generation. Retrovirus producing PG-13 cells were thawed at least five days in advance prior to virus collection. PG-13 cells were cultured at 37°C in RPMI media supplemented with 10% FBS and 1% antibacterial and anti-fungal. Following three days of culture, virus containing media was collected and frozen at -80°C for storage.

CD8 T cell Isolation. CD8+ T cells were magnetically isolated from freshly thawed PBMCs through EasySepTM Human CD8 T cell isolation kit (Stem Cell Technology, #17953) following the manufacturer's protocol.

CD8 T cell Expansion. Isolated CD8+ T cells were cultured in ImmunoCultTM -XF T cell expansion medium supplemented with 1% antibacterial-antimycotic and 7.5ng/mL IL-2. Moreover, CD8+ T cells were stimulated with 25uL of ImmunoCultTM Human CD28/CD3/CD2 T cell activation mixture (StemCell Technology, #10970) per 1mL of media at day of isolation. ImmunoCult media supplemented with 7.5ng/mL IL-2 was refreshed every 2 days while maintaining the T cell density at 500,000 to 1,000,000 cells per mL. After 10 days of expansion T cells were re-stimulated with 1.25uL of CD28/CD3/CD2 activation mixture per mL of media. After 14 days of expansion, T cells were aliquoted at concentration of $1*10^7$ T cells per mL of cryostor® cell cryopreservation media and frozen at -80°C for preservation. Frozen cells were subsequently transferred to -120°C for long term storage.

CD8 T cell Transduction with Retrovirus Expressing ESO-1 TCR. In order to transduce CD8+ T cells with ESO-1 targeting TCR, we followed a published protocol from Restifo Lab (Patel et. al., 2017).

Immunopersister Co-culture Model. 20,000 A375 cells were seeded in 12 well plates and allowed to adhere and proliferate for 24 hours. 4,000 ESO-1 TCR T cells thawed one day in advance were then added to each well in RPMI media supplemented with 10% FBS, 1% antibacterial and antifungal and 7.5 ng/mL IL-2. T cells were refreshed every three days to avoid exhaustion. Quiescent residual A375 cells that survived at least 12 days in co-culture were considered immunopersisters while proliferative A375 cells co-cultured for at least 45 days were considered ITEPs.

Reversibility Assay. Immunopersisters derived through a 12-day co-culture model were allowed to grow in the absence of T cells at RPMI media supplemented with 10% FBS and 1% antibacterial and antifungal for 1 week. Regrown cells were subsequently re-exposed to T cell pressure for 12-days based on previously described co-culture model.

Microscopy. All wells were thoroughly washed three times with RPMI media prior to imaging. All pictures were then taken on EVOS M5000 microscope at 10x magnification.

Cell Viability Assay. 4,000 parental A375 cells were seeded in 12 well plates. 40,000 T cells were added to each well with the exception of untreated controls. Following 3 days of co-culture, plates were washed 3 times with RPMI media and cell viability was measured through Cell Titer Glo (CTG) (Promega) following the manufacturer's protocol.

RESULTS:

In order to investigate if melanoma cells can acquire an immunotherapy persister state, we generated an *in vitro* co-culture model in which ESO-1 targeting TCR CD8 T cells target the A375 melanoma line (Figure 1a). To generate ESO-1 TCR T cells, we infected mature polyclonal CD8+ T cells with retrovirus expressing ESO-1 targeting TCR. This led to the generation of ESO-1 TCR T cells with a high expression of ESO-1 targeting TCR (Figure 1b). These ESO-1 TCR T cells were subsequently sorted through flow cytometry and utilized in co-cultures.

After confirming that ESO-1 TCR T cells had a significantly enhanced capability of A375 killing compared to wild type mature polyclonal CD8+ T cells, we aimed to investigate whether A375s could acquire an immunotherapy persister state to survive the T cell attack (figure 1c). We found that after a period in which the majority of parental melanoma cells died in

co-culture, a residual melanoma population survived through a quiescent tolerant state (top two pictures of figure 1e). Moreover, after several weeks of constant T cell pressure, these quiescent melanoma cells reentered the cell cycle and proliferated as immunotherapy-tolerant expanded persisters (ITEP) (top third picture figure 1e). However, if early immunotherapy persisters were regrown in the absence of T cell pressure, these regrown persisters lose their T cell tolerant phenotype, which was marked by exacerbated death following re-exposure to ESO-1 TCR T cells (Figure 1e, bottom).

In summary, immunotherapy persister populations carry an overall quiescent, pro-survival and reversible state. These findings highly overlap with previous observations on chemotherapy persisters studies (Figure 1d). Therefore, we followed up our studies by trying to better characterize the immunotherapy persister state while attempting to identify its similarities with chemotherapy persisters.

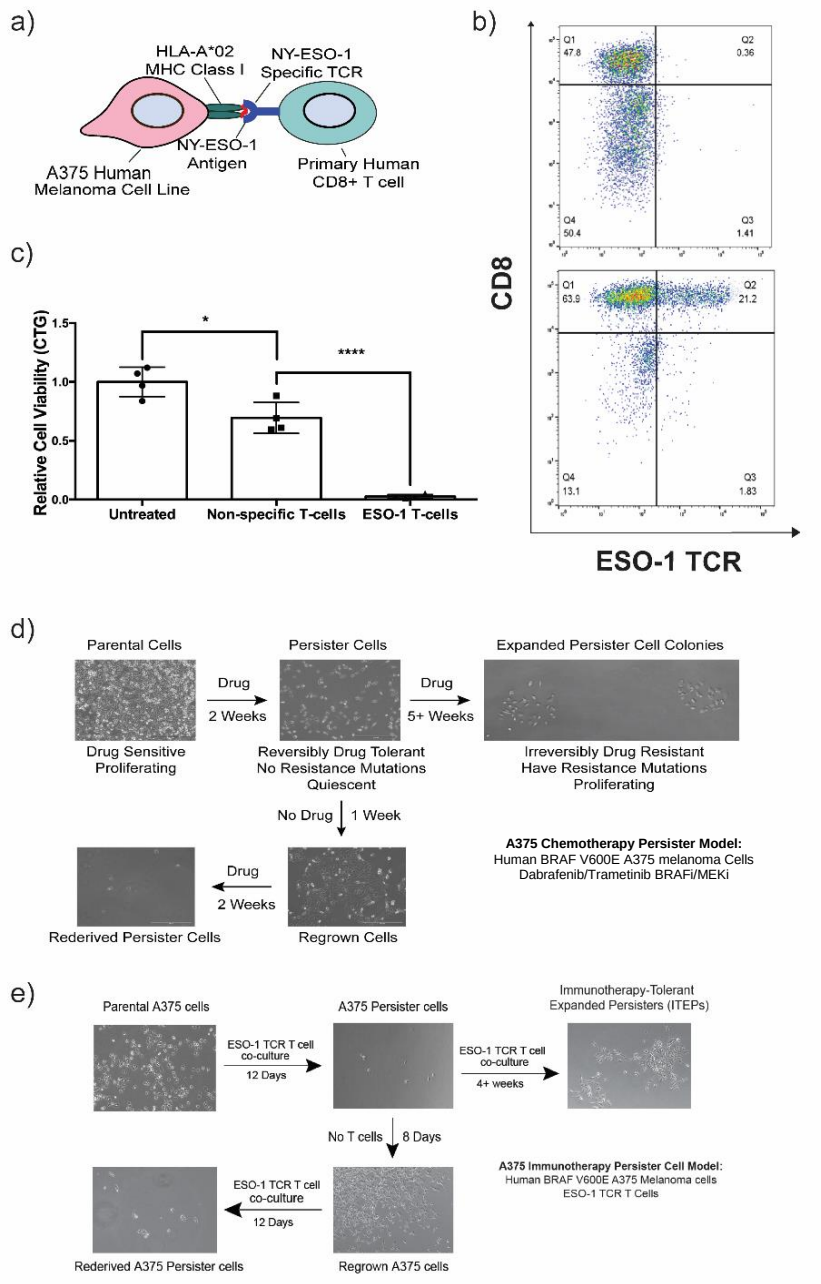

Figure 1. Human BRAF V600E A375 Melanoma Cells Survive Targeted Therapy and CD8 T cell Cytotoxicity by Reversibly Entering a Quiescent Pro-survival Persister State which Seeds Tumor Cell Regrowth. a) NY-ESO1-specific TCR T cell and melanoma A375 coculture model. b) Representative flow cytometry plot of primary human CD8 T cells stained for CD8 and NY-ESO-1 specific TCR expression. Top plot contains wild type human CD8 T cells and bottom plot contains CD8 T cells that were transduced with retrovirus expressing the NY-ESO-1 TCR. Prior to use for coculture experiment, ESO-1 TCR positive CD8 T cells were purified via FACS. c) A375 cells co-cultured for 3 days with polyclonal CD8 T-cells or NY-ESO-1 specific TCR T cells. Cell viability was measured with CellTiter Glo (CTG), ****$p < 0.0001$ ($n = 4$). d) Representative images of chemotherapy persister cells and DTEPs. e) Representative images of immunotherapy persister cells and ITEPs. d-e) These images demonstrate both the reversible therapy tolerant of persister cells and the long-term formation of regrowing tumor cells (DTEPs or ITEPs, respectively).

Chapter 2: Immunotherapy Persisters Lack Immunosuppressive or Immune Evasive Phenotypes

In the context of cancer immunosurveillance, CD8 T cells play a primary role in the eradication of tumors.[10,11,12] Unfortunately, a variety of primary immunotherapy resistance mechanisms were reported to prevent tumor to T cell interactions.[13,19] This may occur by direct loss of tumor associated antigen expression or loss of MHC-1 molecules essential for tumor recognition by T cells, which leads to T cell evasion.[36,37]

Alternatively, tumors can express cell surface proteins that directly impair T cell function.[13] One of these ligands is the program death ligand 1 (PDL1).[13,21] When PDL1 binds to programed death 1 (PD1) expressed in the surface of CD8 T cells, the T cells become anergic, allowing tumors to avoid cytotoxicity.[13,21,22] To counter this issue, checkpoint blockade immunotherapy was developed.[13,15] This approach uses monoclonal antibodies (mAb) that bind to and prevent the interaction of PDL1 and PD1.[13] In response, CD8 T cells remain active and target cancer cells.[13]

Additionally, loss of IFN-gamma receptor has been associated with cancer immune evasion and tumor survival.[23] IFN-gamma is a type II interferon secreted by CD8 T cells, and it possesses several anti-tumorigenic properties.[38,39,41] Its signaling acts through the JAK/STAT signaling pathway, which leads to the phosphorylation of the transcription factor STAT-1.[40] Phosphorylated STAT-1 (pSTAT-1) then causes broad changes in gene expression that improve tumor to T cell interactions.[38,39,40,43] For instance, it enhances antigen presentation on the surface of cancer cells both by upregulating MHC-I and enhancing antigen processing.[38,43] Moreover, high concentration of IFN-gamma alone can directly result in cancer cell death.[41]

Since cancer cells primarily evade T cells or suppress T cell function as a survival mechanism to CD8 T cell cytotoxicity, we initially hypothesized that immunotherapy persisters acquire a T cell evasive or immune suppressive phenotype to survive early CD8 T cell exposure. One potential mechanism is through loss of antigen presentation. For example, A375 immunotherapy persisters may lose ESO-1 antigen or HLA-A*02 expression. Alternatively, immunotherapy persisters may acquire an immune suppressive phenotype by upregulating PDL1 which would prevent CD8 T cells upregulation of T cell activation markers and prevent IFN-gamma secretion. In this chapter, we describe experiments to test each of these possibilities.

Western Blot. Immunotherapy persisters were derived through 12-day co-culture with 50,000 ESO-1 TCR T cells in 10 cm plates. A375 immunotherapy persisters and parental cells were washed with PBS and lysed using RIPA buffer (Thermo Scientific) supplemented with Phosphatase inhibitor (Thermo Scientific) and protease inhibitor (Thermo Scientific). Lysates were sonicated 3 times for 5 seconds with 1-minute intervals between each round of sonication. Lysate were then centrifuged at 15,000g at 4 °C for 15 min. Supernatant was removed from debris and protein concentration was quantified through Pierce BCA Protein Assay Kit. Quantified protein was mixed with sample buffer (Thermo Scientific) and denatured at 70°C for 10 min. Samples were separated by SDS–PAGE (NuPage 4–12% Bis-Tris Gel, Life Technologies), run with Chameleon 700 Pre-stained Protein Ladder, and transferred to a nitrocellulose membrane using an iBlot system (Life Technologies). Membranes were blocked with 10% BSA for 1 h at room temperature, and then incubated with primary antibody at 4 °C overnight. LICOR secondary antibodies were then incubated with the membrane for 1 h at room temperature, and the membrane was imaged using the LICOR Odyssey Imaging System. B-Tubulin levels were measured as a

loading control. Antibodies commercial sources: pSTAT-1 (CST, #9167); STAT-1 (CST, #92176); NY-ESO-1 (Sigma, N2038); B-Tubulin (Invitrogen, MA5-16308).

Flow Cytometry. 60-100,000 cells were collected and spined down at 300xg for 5 minutes to pellet and resuspended in 5 mL of cold PBS. Cells were then resuspended in 1mL of cold PBS supplemented with 1uL of Ghost Dye (CST, #59863) and incubated for 30 minutes on ice. Followingly, cells were washed two times with cold staining buffer (PBS + 1%FBS) and resuspended in 100uL of staining buffer supplemented with fluorochrome conjugated primary antibodies. Stained cells were incubated for 30 minutes at 4°C in the dark. Each antibody concentration followed the manufacturer's proposed protocol. T cells were then washed twice with a staining buffer and fixed in 300uL of 2%PFA. Fixed cells were then analyzed through flow cytometry. Antibodies commercial sources: CD69 (Invitrogen, #48-0699-42); CD25 (Invitrogen, #12-0257-42); CD8 (StemCell Technology, # 60022AZ.1); PDL1 (Invitrogen, #12-5983-42); HLA-A*02 (Invitrogen, #17-9876-42).

Flow Cytometry Analysis. Flow cytometry data analysis was conducted through FlowJo software. Only single cells gated for low Ghost Dye and high CD8 (T cells only) fluorescence were considered for further analysis.

IFN-gamma ELISA. Approximately 100,000 Immunopersister cells were derived through 12-day co-culture with 20,000 ESO-1 TCR T cells in 12 well plates. T cells were refreshed and co-cultured with immunopersisters for additional 3 days. Media was subsequently collected and centrifuged for 5 minutes at 14,000g. Supernatant extracted and stored at -20°C until usage. Moreover, 100,000 parental cells were either co-cultured with ESO-1 TCR T cells or cultured in the absence of T cells. Media was collected as previously stated. Media IFN-gamma concentration was measured through ELISA kit (StemCell Technologies, #02002) following manufacturer’s protocol.

Anti-PDL1 and Anti-DP1 Co-culture. 20,000 A375 cells were seeded in 12 well plates. During the following day, 4,000 ESO-1 TCR T cells were added to each well in RPMI media supplemented with 10% FBS, 1% antibacterial and antifungal and 7.5ng/mL IL-2. Anti-PDL1 condition was supplemented with 20ug/mL of Durvalumab (Selleckchem, #A2013), anti-PD1 condition was supplemented with 20ug/mL of Pembrolizumab (Selleckchem, #A2005), and no antibody control was co-cultured following standard co-culture protocol. Following 12 days of co-culture with T cells and antibodies refreshed every three days, cell viability was measured through Cell Titer Glo (CTG) (Promega).

RESULTS:

To test whether immunotherapy persisters acquire an evasive phenotype to survive CD8 T cell attack, we initially tested if A375 persisters could activate ESO-1 TCR T cells. To our surprise, after being exposed to persisters, ESO-1 TCR T cells significantly upregulated the T cell activation markers CD25 and CD69 instead of showing signs of exhaustion (figure 2a, b, c). Due to the high correlation between T cell activation with its potential to destroy target cells, these findings suggested that immunotherapy persisters were in fact targeted by T cells instead of evading cytotoxicity.

To further investigate whether T cells target immunotherapy persisters, we tested if ESO-1 TCR T cells were able to secret IFN-gamma when co-cultured with perister cells. We observed that T cells treated with immunotherapy persisters secreted a significantly higher amount of IFN-gamma in media when compared to co-culture with parental cells (Figure 3a). Again, these findings strongly suggested that immunotherapy persisters did not evade CD8 T cell cytotoxicity, but rather survived T cell attack.

With a clear indication that immunotherapy persisters did not evade T cells, we checked whether these cells survived T cell cytotoxicity through an IFN-gamma insensitive phenotype. However, immunotherapy persisters responded to IFN-gamma signaling as they significantly upregulated STAT1 and pSTAT1 when compared to parentals (figure 3b). These findings were further confirmed as immunotherapy persisters upregulated HLA-A*02 expression in response to T cell co-culture (figure 3c, d). In addition, A375 persisters did not lose the ESO-1 antigen expression as an evasive mechanism (figure 3b). Together, these data strongly suggests that immunotherapy persister cells do not evade ESO-1 TCR T cells.

To test the possibility of immunotherapy persisters surviving through an immunosuppressive phenotype, we investigated whether these cells upregulate PDL1. However, co-culture with ESO-1 TCR T cells did not select for an A375 persister population that upregulates PDL1 (Figure 4a, b, c). To further disconfirm that PDL1 expression promotes immunotherapy persister survival, we generated immunotherapy persister cells in media supplemented with Durvalumab (anti-PDL1) and Pembrolizumab (anti-PD1). As expected, these drugs did not enhance the killing of persisters (figure 4d). Therefore, the loss of IFN-gamma respos

In conclusion, we determined that different from commonly proposed models, immunotherapy persisters do not acquire an immune evasive or immunosuppressive phenotype. Therefore, we next focused on identifying tumor cell intrinsic survival mechanisms that may allow immunotherapy persisters to tolerate CD8 T cell cytotoxicity.

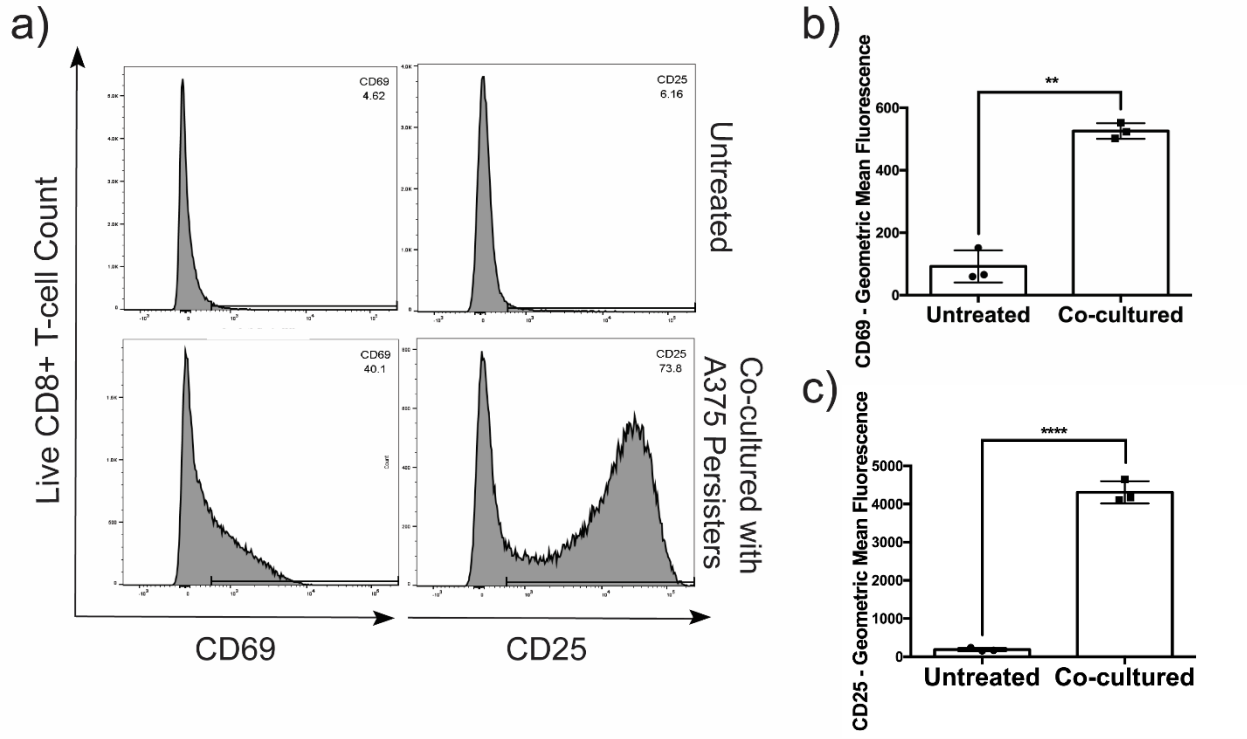

Figure 2. A375 Melanoma Immunotherapy Persister Cells Activate Cognate CD8 T cells. a) Flow cytometry histogram of ESO-1 TCR CD8 T cells that were either co-cultured for 3 days with A375 persisters (bottom plots) or untreated (top plots). These cells were co-stained for CD69 and CD25 expression. b) Quantification of CD69 geometric mean fluorescence intensity of untreated or 3-day co-cultured ESO-1 TCR CD8 T cells, $^{**}p < 0.01$ (n = 3). c) Quantification of CD25 geometric mean fluorescence intensity of untreated or 3-day co-cultured ESO-1 TCR CD8 T cells, $^{****}p < 0.0001$ (n = 3). Figure was generated with support from Brandon Mauch.

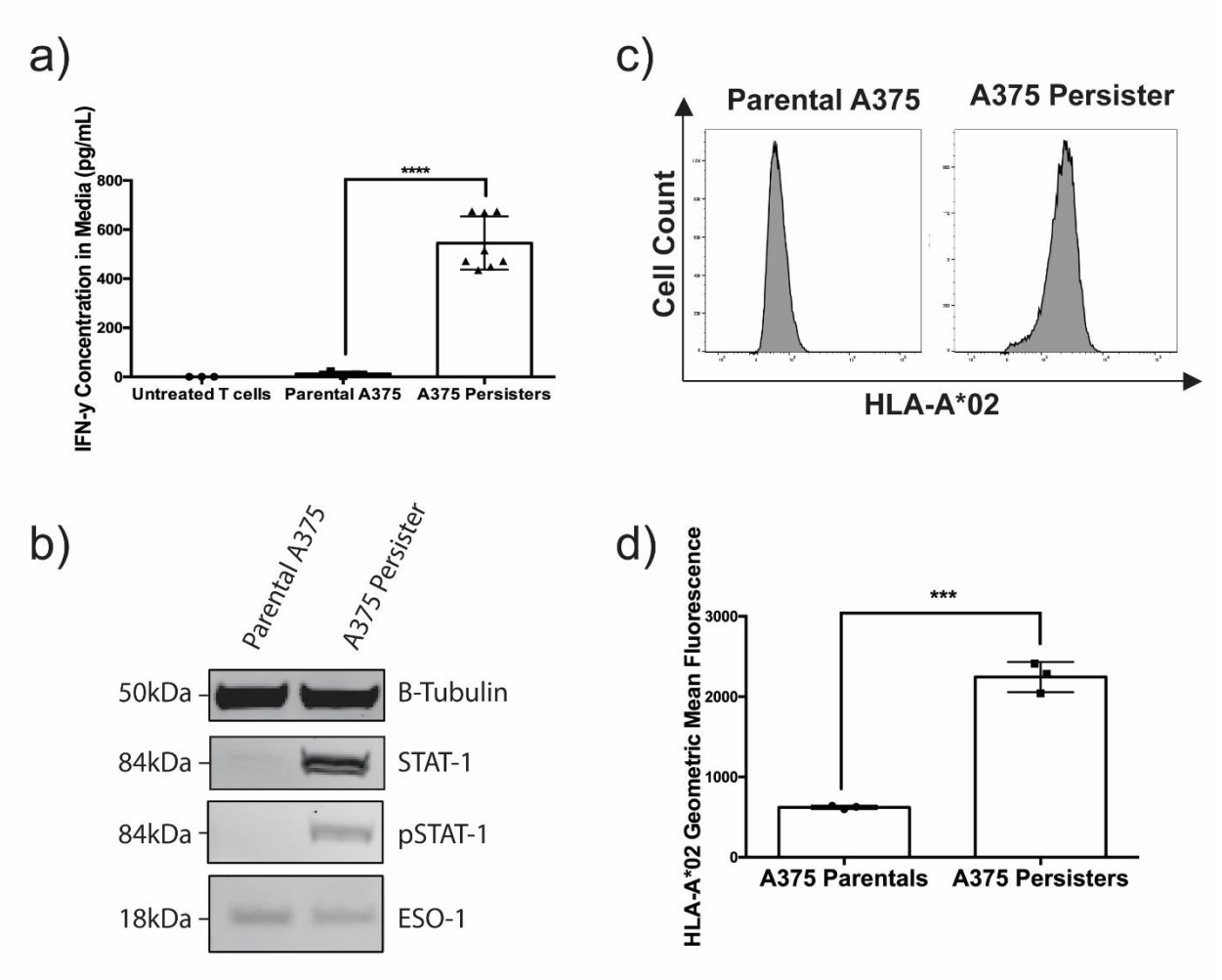

Figure 3. A375 Melanoma Immunotherapy Persister Cells Maintain ESO-1 Antigen Presentation. a) IFN-gamma concentration in media collected from ESO-1 TCR+ CD8 T cells alone (Untreated T cells) (n = 3) and ESO-1 TCR+ CD8 T cells co-cultured with parental A375 cells (n = 3) or A375 persister cells (n = 8), ****$p < 0.0001$. b) Western blot analysis of A375 parental cells and A375 immunotherapy persister cells. Samples were stained for -tubulin, STAT-1, pSTAT1 and ESO-1. c) Flow cytometry histogram of A375 parental and persister cells stained for HLA-A*02 expression. d) HLA-A*02 geometric mean fluorescence intensity of A375 parental cells and persister cells, ***$p < 0.001$ (n=3). Figure was generated with support from Brandon Mauch.

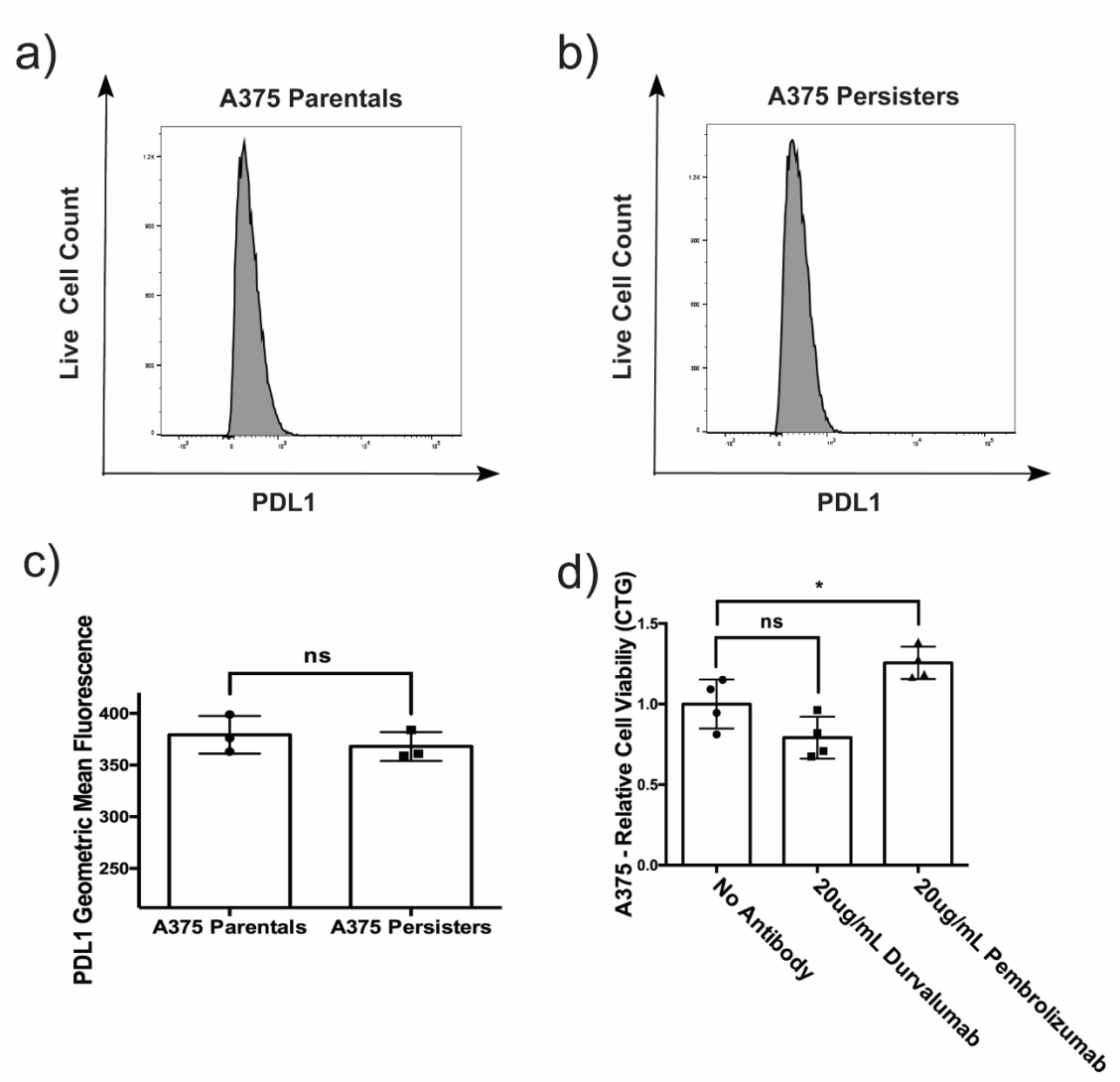

Figure 4. A375 Melanoma Immunotherapy Persister Cells do not Upregulate or Depend on PDL1. a) Representative flow cytometry histogram of A375 parental cells stained for PDL1 expression. b) Representative flow cytometry histogram of A375 persister cells stained for PDL1 expression. c) PDL1 geometric mean fluorescence intensity of A375 parental cells and persister cells, ns $p > 0.05$ ($n = 3$). d) A375 cells were co-cultured for 12 days with ESO1 TCR T cells in the presence of 20 ug/mL of Durvalumab (anti-PDL1) or 20 ug/mL of Pembrolizumab (anti-PD1), with antibodies and T cells replenished every 3 days. Cell viability was measured with Cell Titer Glo (CTG). ns $p > 0.05$, *$p < 0.05$ ($n = 4$). Figure was generated with support from Brandon Mauch.

Chapter 3: RNA Expression Hallmarks of Immunotherapy Persister State

Prior studies have identified that multiple unique populations of cancer chemotherapy persister cells can arise within a single treated plate.[42] More interestingly, each of these populations can utilize unique survival mechanisms that are distinct from their neighboring cells.[42] Based on these factors, it is likely that individual immunotherapy persister cells within a single co-culture plate may also carry unique survival mechanisms, which would be overseen in bulk cell analysis assays. Therefore, to better understand the heterogeneity of immunotherapy persister cell populations, we conducted a single cell RNA sequencing analysis in A375 parental, immunotherapy persister and ITEP cells. Moreover, scRNAseq analysis also allows for identification of potential immunotherapy persister survival mechanisms.

MATERIALS & METHODS:

Single Cell RNA Sequencing. 500,000 A375 cells seeded in 10cm plates were co-cultured with 100,000 ESO-1 TCR T cells. Immunotherapy persisters were co-cultured for 12 days, ITEPs were co-cultured for 45 days and parentals were cultured in the absence of T cells. At the end of treatment, cells were lifted with trypsin and loaded onto a 10X Chromium instrument (10X Genomics) following the established protocol. Libraries were generated using the 10X Chromium Single Cell 3' v3 kit as recommended. Quality control of the libraries was sequenced using NovaSeq S4.

Read Alignment and Data Processing. Fastq files were aligned to the human "refdata-cellranger-GRCh38-3.0.0" genome with Cell Ranger version 3.1.0 with the "cellranger count" command to generate single cell feature counts for each library. The "filtered_feature_bc_matrix" generated for each population was used to create a "Seurat object" in the Seurat R package version 4.0.3. Cells containing greater than 1,000 and less than 7,500

features, and with less than 20% mitochondrial reads were included in downstream analyses. A cell cycle score was calculated for each cell using the default Seurat method, and this score was used to regress the cell cycle during normalization and scaling with the SCTransform command.

Determining Variable Features and Mapping. The commands “RunPCA,” “RunUMAP,” “FindNeighbors,” and “FindClusters” were performed with default settings, with 30 dimensions used for “RunUMAP” and “FindNeighbors." In the “FindClusters” command, the resolution for A375 WT, A375 DFFB KO, and PC9 were set to 1.0, 0.4, and 0.2, respectfully, based on visualization of graphed clusters. The Seurat command “FindMarkerGenes” command was used with default parameters to identify differentially expressed genes between specified populations or clusters of cells.

Gene-set Enrichment Analysis (GSEA). GSEA was conducted with the ClusterProfiler R package (version 3.18.0) which calculated a normalized enrichment score (NES) using default parameters for each gene set. For the overlapping persister genes between A375 and PC9, genes differentially expressed in the same direction in both cell lines were analyzed with the GSEA/MSigDB website (http://www.gsea-msigdb.org/gsea) and a p-value and false discovery rate (FDR) was calculated with a hypergeometric test. The AUCell package version 1.12.0 was used to calculate gene set scores per cell, and the indicated thresholds were then visualized on a UMAP with Seurat.

Individual Gene Expression Analysis. Original count matrices were merged and filtered as previously described using Seurat. Expression values were then log-normalized and fold-change was calculated for a comparison between populations.

RESULTS:

Due to our prior determination that immunotherapy persister cells do not acquire an evasive or immunosuppressive phenotype, we hypothesized that persister cells instead utilize intrinsic mechanisms of survival. To broadly search for their hallmarks and survival mechanisms, we conducted a scRNAseq on A375 parentals, immunotherapy persisters and ITEPs. We found that each of these melanoma populations contained distinct RNA expression patterns and clustered independently (Figure 5a).

We then compared the RNA expression of A375 persisters and parental cells in order to better understand the hallmarks driving the immunotherapy persister phenotype. Not surprisingly, IFN-gamma pathways were greatly upregulated in immunotherapy persisters, which confers with our previous findings of pSTAT-1 and HLA-A*02 up-regulation (figure 5b, 4b, c, d). Moreover, proliferation markers such as MYC, mitotic spindle and E2F targets were significantly lower in immunotherapy persister cells, consistent with our prior observations of immunotherapy persister cells quiescence (figure 1e, 5b).

Specifically in immunotherapy persister and ITEP cells, we observed increased epithelial-mesenchymal transition (EMT) genes. EMT signaling has been previously associated with cancer survival mechanisms in chemotherapy, which provides a possible survival approach for immunotherapy persisters (figure 5b).[44] We also found that apoptotic markers were significantly upregulated in immunotherapy persisters. This finding is inconsistent with the possibility that immunotherapy persister cells acquire an immune evasive phenotype because the presence of apoptotic signaling suggests that immunotherapy persister survival mechanisms that are downstream T cell targeting of tumors. With a clearer characterization of the immunotherapy perister cell state at the RNA level, we opted to continue our studies by investigating whether the

RNA expression of apoptotic hallmarks was translated to the protein level. By doing so, we may better characterize the immunotherapy persister state and understand their survival mechanism.

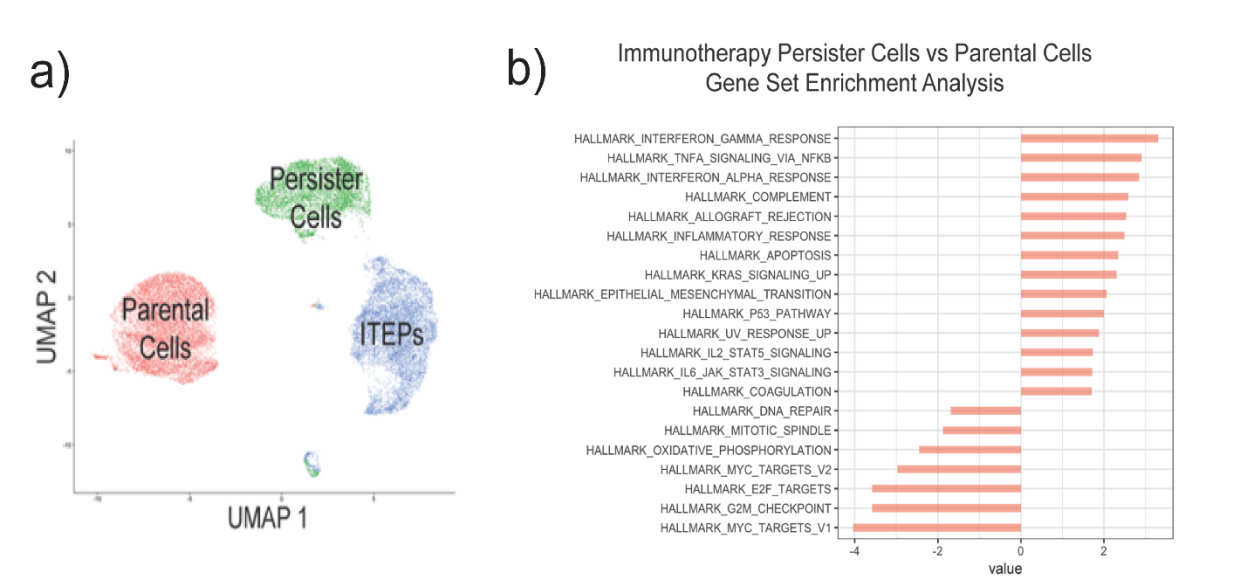

Figure 5. scRNAseq Analysis of Immunotherapy Persister Cells and ITEPs. a) Single cell RNA expression UMAP graph of A375 parental cells, persister cells and ITEPs. b) Gene set enrichment analysis of A375 persister cells compared to A375 parental cells. Figure was generated with support from August Williams.

Chapter 4: Immunotherapy and Chemotherapy Persisters Survive Despite Strong Apoptotic Signaling

Following activation, CD8 T cells secrete granules that promote apoptosis in target cells.[45,46,47] In this context, major lytic protein packages of perforin mediate perforation of the target cell's plasma membrane. This allows for cytotoxic granules - termed Granzyme B - to enter the cancer cell cytosol.[48,] Granzyme B then initiates a caspase cascade event in which several pro-apoptotic enzymes are cleaved and activated, ultimately leading to cell death.[45]

Among the direct targets of granzyme B, the pro-apoptotic executioner caspase 3 plays a major role in pushing apoptotic signaling past its lethal threshold.[50,51] Once cleaved, caspase 3 furthers apoptosis by indirectly activating caspase 3 activated DNases (CAD).[50,51] In response, CAD degrades the DNA as one of the final events leading to programmed cell death.[52]

Moreover, Granzyme B enhances activity caspase 3 by stimulating apoptotic signals upstream from caspase cleavage.[53] Among those, Granzyme B directly activates the pro-apoptotic enzyme BID (22kDa) by cleaving BID into its truncated form tBID (15kDa).[54,55] Subsequently, tBID activates BAX and BAK, which both lead to mitochondrial outer membrane perforation (MOMP).[54,56] When MOMPs occur, components of the mitochondrial electron transport chain such as Cytochrome C leak into the cytosol.[56] Cytochrome C then enhances the apoptotic signaling and increase downstream cleavage of caspase 3.[56]

Based on scRNAseq analysis, immunotherapy persister cells express apoptotic signaling. Therefore, we hypothesized that persister cells may survive CD8 T cell cytotoxicity through a sublethal response to pro-apoptotic markers. This could be mediated by downregulation of pro-apoptotic enzymes in surviving persister cells which may confer tolerance to T cell attack.

MATERIALS & METHODS:

Western Blot. Immunotherapy persisters were derived through 12-day co-culture with 50,000 ESO-1 TCR T cells in 10 cm plates. Chemotherapy persisters were generated through 15-day culture in 250nM Dabrafenib and 25nM Trametinib in RPMI media supplemented 10% FBS and 1% antibacterial and antifungal. Remaining western blot analysis follows protocol described in chapter 2. Antibodies commercial sources: B-Tubulin (Invitrogen, MA5-16308); Cleaved Caspase 3 (CST, #9664); Granzyme B (CST, #4275), BID (CST, #2002), BAX (CST, #5023), PUMA (CST, #12450), NOXA (CST, #14766), BAD (CST, #5023), BIM (CST, #2933), BAK (CST, #12105), MCL-1 (CST, #94296), BCL-XL (CST, #2764), BCL-2 (CST, #15071), BCL-w (CST, #2724), BFL-1 (CST, #14093).

Flow Cytometry. 60-100,000 cells were lifted with trypsin and collected in 15mL test tubes. Cells were washed 3 times by spinning them down at 300xg for 5 minutes to pellet and resuspended in 5 mL of cold PBS. After final wash, cells were resuspended in 1mL of cold PBS supplemented with 1uL of Ghost Dye 510 (CST, #59863) and incubated in the dark for 30 minutes at 4°C. After 1x wash with staining buffer, cells were resuspended in 100uL of 4% PFA for 10 minutes at room temperature. Samples were then resuspended in permeabilizing buffer (0.3% Triton-X) for 10 minutes at room temperature. Samples were washed two times with cold staining buffer (PBS + 1%FBS) and resuspended in 100uL of staining buffer supplemented with fluorochrome conjugated primary antibodies following manufacturer's indications. Stained cells were incubated for 30 minutes at 4°C in the dark. Additional two washings with staining buffer were conducted to remove antibodies. Cells were lastly fixed in 300uL of 2%PFA and analyzed through flow cytometry. Antibodies commercial sources: granzyme B (Invitrogen, #560213); cleaved caspase 3 (CST, #560213).

RESULTS:

Based on the scRNAseq analysis showing that immunotherapy persisters contain an apoptotic signature, we considered the possibility that persister cells have an altered pro-apoptotic protein expression. We were particularly interested in determining whether the main CD8 T cell effector protein granzyme B was capable of penetrating the cytosol of immunotherapy persister cells. To investigate this possibility, we conducted a western blot analysis on A375 parental and immunotherapy persister cells in order to test for the presence of intracellular granzyme B. Interestingly, we discovered that granzyme B was present within the bulk immunotherapy persister population but not in parental cells (figure 6a). Through the utilization of flow cytometry, we also observed that the majority of persister cells contained granzyme B (figure 6b).

Followingly, we explored whether other main apoptotic hallmarks were present in persister cells. Therefore, we tested for the expression of cleaved caspase 3 in immunotherapy persister cells through western blot and flow cytometry. Similar to our previous findings on granzyme B, the majority of persister cells harbored elevated expression of cleaved caspase 3 (Figure 6c, d).

Based on these observations, we hypothesized that living persister cells may face a sublethal apoptotic signal through the down or up regulation of certain apoptotic enzymes. Consequently, we analyzed the expression of apoptotic proteins in A375 parental, immunotherapy persisters and chemotherapy persisters. We found that when compared to parentals, persisters upregulated most pro-apoptotic enzymes while downregulated anti-apoptotic enzymes (figure 7a, b).

Overall, chemotherapy and immunotherapy persisters have similarly strong apoptotic signaling expression. The only exceptions are the pro-apoptotic enzymes BID and NOXA, which are specifically downregulated in immunotherapy and chemotherapy persisters, respectively (figure 7a, c). Based on these findings, we can conclude that persister cells contain specific changes in the apoptotic machinery. Yet, it remains unclear as to whether these changes underlie the persister cell survival.

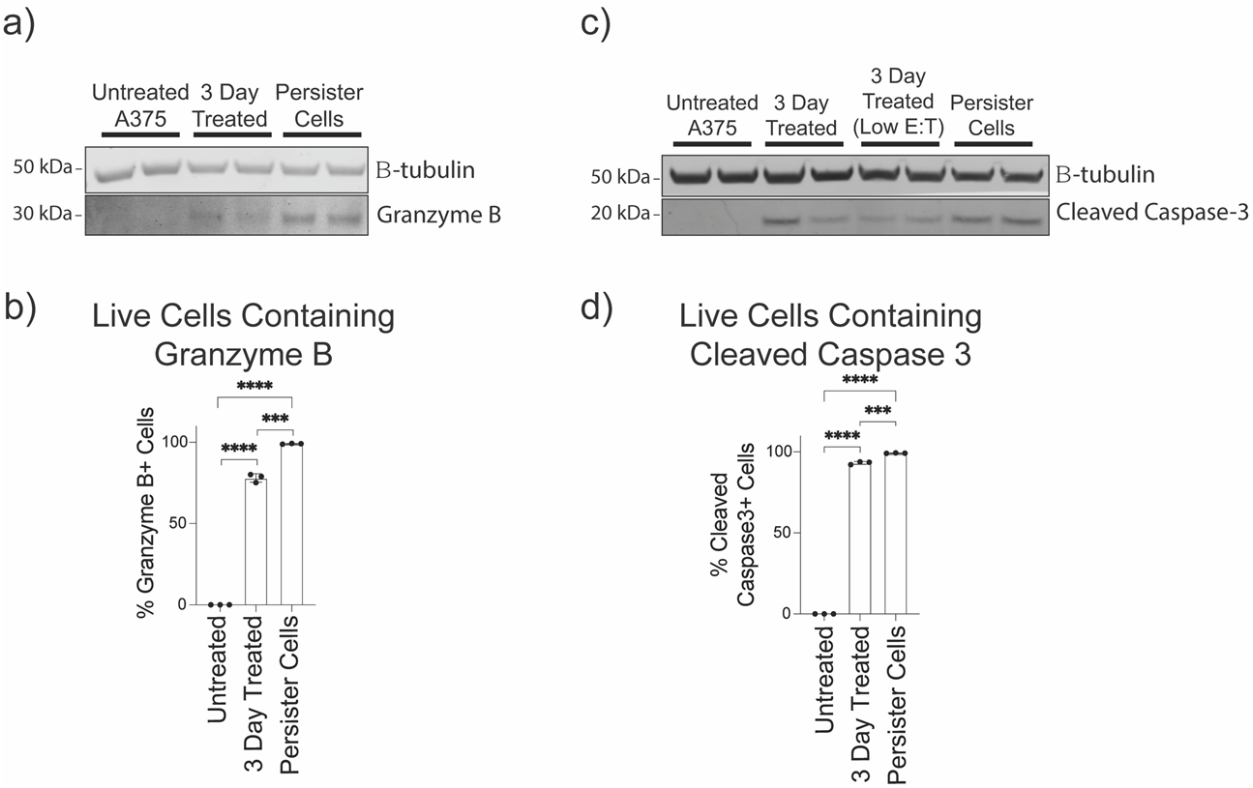

Figure 6. Immunotherapy Persister Cells Present Apoptotic Hallmarks. All panels illustrate the protein expression analysis of untreated A375 parental cells, 3 days co-cultured parental A375 cells and 12 days co-cultured persister A375 cells. a) Western blot analysis of Granzyme B expression. b) Flow cytometry analysis of Granzyme B expression, ***$p < 0.001$, ****$p < 0.0001$ (n=3). c) Western blot analysis of cleaved Caspase 3 expression. d) Flow cytometry analysis of Caspase 3 expression, ***$p < 0.001$, ****$p < 0.0001$ (n=3). Figure was generated with support from Michael Wang.

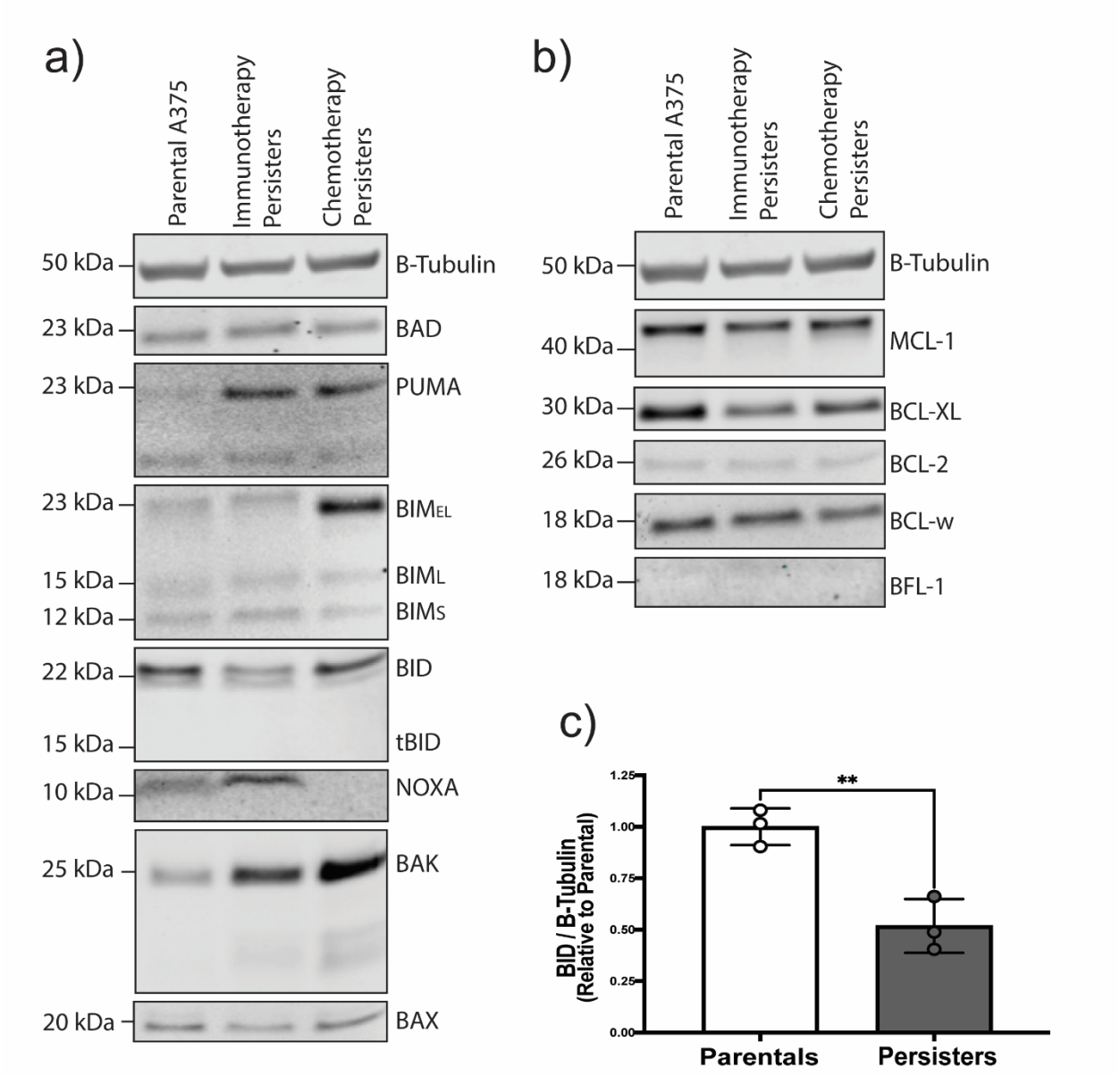

Figure 7. Pro- and Anti-Apoptotic Gene Expression in A375 Chemotherapy and Immunotherapy Persister cells. a-b) Western blot of A375 untreated parental, chemotherapy, and immunotherapy persister cells. c) Western blot quantification of BID relative to -tubulin expression, **p < 0.01 (n=3). Figure was generated with support from Michael Wang.

Chapter 5: Immunotherapy and Chemotherapy Persister Cells Express Distinct Epigenetic Modifications

Different from genetic mutations in which the DNA sequence is permanently modified, epigenetics refers to DNA modifications that do not alter the DNA sequence.[57] Epigenetic changes describe the alteration of gene expression by either condensing or opening coding regions of the genome.[57] To accomplish this, positively charged proteins, termed histone, bind to DNA in order to condense and ultimately repress certain genes.[58] Moreover, histone acetylases and methylases can alter the histone ability to bind DNA and therefore regulate gene expression.[59]

Modifications in cell epigenetics allow for cells to respond to situational environmental changes.[60,61] For instance, chemotherapy persister cells express an epigenetic state distinct from drug naïve parental cancer cells.[34] This distinction is believed to promote chemotherapy persister survival following initial drug exposure.[34] Furthermore, these epigenetic changes are reversed in absence of drug pressure, which explains the reversibility of the chemotherapy persister state.[34] Here, we hypothesized that a chromatin mediated change also contributes to immunotherapy persister formation, possibly explaining why these cells have a reversibly T cell tolerant phenotype.

MATHERIALS & METHODS:

Western Blot. Immunotherapy persisters were derived through 12-day co-culture with 50,000 ESO-1 TCR T cells in 10 cm plates. Chemotherapy persisters were generated through 15-day culture in 250nM Dabrafenib and 25nM Trametinib in RPMI media supplemented 10% FBS and 1% antibacterial and antifungal. Cells were washed with PBS and lysed using RIPA buffer (Thermo Scientific) supplemented with Phosphatase inhibitor (Thermo Scientific) and protease inhibitor (Thermo Scientific). Lysates were sonicated 3 times for 5 seconds with 50% amplitude.

Samples were allowed to rest on ice for 1-minute intervals between each round of sonication. Lysate were then centrifuged at 15,000g at 4 °C for 15 min. Supernatant collected and protein concentration was quantified through Pierce BCA Protein Assay Kit. Quantified protein was mixed with sample buffer (Thermo Scientific) and denatured at 70°C for 10 min. Samples were separated by SDS–PAGE (NuPage 4–12% Bis-Tris Gel, Life Technologies), run with Chameleon 700 Pre-stained Protein Ladder, and transferred to a nitrocellulose membrane using an iBlot system (Life Technologies). Membranes were blocked with 10% BSA for 1 h at room temperature, and then incubated with primary antibody at 4 °C overnight. LICOR secondary antibodies were then incubated with the membrane for 1 h at room temperature, and the membrane was imaged using the LICOR Odyssey Imaging System. Total H3 levels were measured as a loading control. Antibodies commercial sources: H3K14ac (CST, #7627); H3K9me3 (CST, #13969); H3K27me3 (CST, #9733); H3K4me3 (CST, #9751); total H3 (CST, #3638).

Cell Viability Assay. Relative cell viability was measured through Cell Titer Glo (CTG) (Promega) following the manufacturer's protocol.

RESULTS:

To test the possibility of a chromatin-mediated immunotherapy persister state, we investigated the levels of histone modifications in A375 parentals, chemotherapy persisters and immunotherapy persisters. Although most epigenetic markers were similarly expressed in both A375 parental and immunotherapy persisters, H3K4me3 was upregulated on the latter (figure 8a). Interestingly, immunotherapy persisters were particularly sensitive to Histone Deacetylase (HDAC) inhibitors in comparison to parentals and chemotherapy persisters (figure 8b). Together, these data suggest that immunotherapy persister cells harbor epigenetic changes that promote survival under T cell pressure, but additional studies are needed to prove causality.

We also observed that both immunotherapy and chemotherapy persisters possess distinct modified histone levels compared to one another (figure 8a). For instance, chemotherapy persisters lowly expressed H3K14ac and H3K4me3, while highly expressing H3K9me3 and H3K27me3 in comparison to both immunotherapy peristers and parentals (figure 8a). These findings imply that although both immunotherapy and chemotherapy persisters contain altered chromatin remodeling compared to parental cells, they do not share similar epigenetic states.

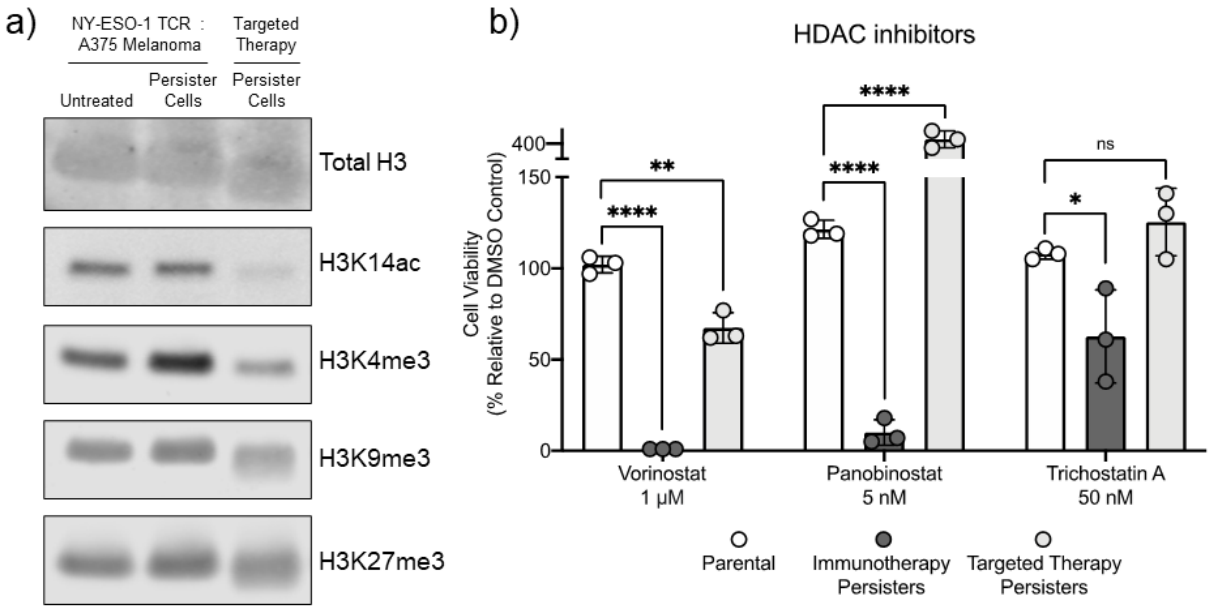

Figure 8. Comparison of A375 Chemotherapy and Immunotherapy Persister Cell Epigenetic States. a) Epigenetic marker western blot analysis of A375 immunotherapy persister cells and chemotherapy persister (targeted therapy) cells. b) A375 cells treated with HDACi for 3 days (parental), co-treated with HDACi and NY-ESO-1 TCR T cells for 15 days (Immunotherapy Persisters), or co-treated with HDACi and 250 nM Dabrafenib and 25 nM Trametinib for 15 days (Targeted Therapy Persisters). Cell viability was measured using CellTiter-Glo, ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ ($n = 3$). Figure was generated with support from Michael Wang.

DISCUSSION

Here, we have demonstrated that a residual cancer cell population acquires a reversible, quiescent, and pro-survival immunotherapy persister state to survive cytotoxic CD8 T cell attack. Interestingly, these immunotherapy persister cells survive for several weeks under continuous cognate T cell pressure and eventually re-enter the cell cycle as overtly resistant ITEPs. These findings are similar to previous observations from chemotherapy persister cell studies.[34]

Immunotherapy persister cells remarkably do not avoid T cell activation through commonly described mechanisms.[13,19,20] Instead, they maintain antigen presentation, activate CD8 T cells, and receive lytic granule component granzyme B. Furthermore, we observed persister cell response to IFN-gamma signaling, which confers with recent findings on residual *murine* tumor cells that survive checkpoint blockade therapy.[26]

Since immunotherapy persister cells express several apoptotic hallmarks and survive, we proposed that immunotherapy peristers might survive due to down regulation of certain pro-apoptotic enzymes. Specifically, it is possible that the down regulation of BID, which we found to be lowly expressed in immunotherapy persister cells, provides a survival advantage. This hypothesis is supported by prior reports showing that BID plays a crucial role in granzyme B mediated apoptosis.[55] Therefore, the loss of BID may prevent immunotherapy persisters from reaching a lethal threshold of caspase activity, which allows these cells to survive T cell exposure. Yet, additional studies are required to confirm this hypothesis.

Furthermore, because immunotherapy persisters do not maintain their T cell tolerance in the absence of continuous T cell pressure, their phenotype is not driven by permanent genetic changes. Immunotherapy persister cells also have distinct histone modification levels compared to parental cells and are sensitive to HDAC inhibitors, potentially reflecting an altered epigenetic

state. Together, these data illustrates that the immunotherapy persister state may be driven by epigenetic modifications, similar to chemotherapy persister cells.[34] However, it is unclear whether ITEP formation is caused by epigenetic changes as opposed to genetic mutations and further work is needed to characterize the ITEP cell state.

It is also important to notice that instead of being an acquired state, immunotherapy persister cells might be innately present in rare preexisting naïve A375 melanoma cells. These preexisting T cell-tolerant persisters could be selected under T cell pressure due to specific markers such as lower MHC I expression. This may contribute to cancer survival following initial CD8 T cell attack. However, we were unable to identify subpopulations of immunotherapy persister cells within parental cancer cells through scRNAseq analysis since A375 parental and persister cells clustered independently. In future experiments, we will search whether any of these preexisting mechanisms are present in rare parental cells.

In summary, our study revealed that residual immunotherapy persister cells can survive CD8 T cell attack without avoiding T cell activation. This has important implications for understanding how residual tumors survive during T cell mediated immunotherapies. The acquisition of an immunotherapy persister state in human cancer patients may serve as a temporary mechanism for tumors to survive early exposure to CD8 T cells during CD8 T cell-mediated immunotherapy. These residual cancer cells may then regrow with a long-lasting T cell resistant mechanism leading to tumor relapse in patients. While it remains unclear as to whether an immunotherapy persister state arises in human cancer patients, an immunotherapy persister state appears to play a role in primary tumor organoids and in genetically engineered mouse models.[55]

In the future, it will be important to determine whether immunotherapy persister cells are a key surviving cell population within human patients during cancer immunotherapy acquired resistance.[20,25] Therefore, further research aiming to identify immunotherapy persister hallmarks in multiple cell lines and mouse models should be conducted. This may elucidate the mechanism of persister cell survival and promote the identification of immunotherapy persister drug targets. If successful, these studies could be exploited in order to help prevent cancer relapse following cancer immunotherapy.

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