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Research Article

Expansion of Tumor-Infiltrating Lymphocytes (TILs) with Substantial Stemness Properties from Vulvar Cancer

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Abstract

Purpose: The promising clinical trial efficacy of Tumor-Infiltrating Lymphocyte (TIL) treatment in certain types of solid tumors has been proved repetitively. According to the available data of a phase 2 pivotal trial, definite curative effect on cervical cancer is observed with an objective response rate up to 44.4%. Pronounced biological and immunological similarities are reported between vulvar cancer and cervical cancer. However, the therapeutic potential of TIL in vulvar cancer remains to be further explored.

Methods: In this study, we described one convincing and reliable manufacturing procedure which could induce clinical-scale TILs expansion within the therapeutic window from both vulvar cancer and cervical cancer cases with a high success rate of production and preparation. The immune-phenotype and cytotoxicity of expanded TILs were characterized via flow cytometry and functional assay. Paired with matched autologous Peripheral Blood Mononuclear Cells (PBMCs) control, the TCR clonality of the final TIL product was analyzed via next generation sequencing(NGS) platform.

Results: Result of immuno-phenotype analysis showed that TILs from vulvar cancer are prone to maintain a higher percentage of progenitor-like phenotype and behave stronger tumor-killing capacity compared to counterpart TILs derived from cervical cancer. TCR clonality analysis indicated that all TIL samples encompass more enriched TCR clones than PBMCs, which may be due to tumor-reactive expansion and tend to be individually tumor antigen specific.

Conclusion: Our study provides a feasible method of tumor specific TIL expansion for clinical administration and a potential innovative therapeutic strategy for vulvar cancer treatment.

Keywords: Cervical cancer; Immunotherapy; Stemness; Tumor infiltrating lymphocytes; Vulvar cancer

Introduction

Vulvar cancer is a malignant tumor which histologically originates from the vulva and representing around 5% of all gynecological malignancies. The overall incidence and mortality of vulvar cancer are rising in the past few decades [1]. Nearly 90% of vulvar cancer cases are classified as Squamous Cell Carcinoma (SCC) in most conditions, followed by melanoma. So far, limited treatment options are available for vulvar cancer. Surgery still served as the major choice of vulvar cancer, with radio- and/or chemotherapy as secondary or complementary choices in certain cases. In the cases of accepting classical therapies, the five-year survival of patients with localized regional cancer lesion or patients with emerging distant metastasis are 53% and 19%, respectively [2]. Therefore, it is urgent to develop new therapeutic strategies. Cancer immunotherapy, represented as PD-1/PD-L1 blockade or adoptive CAR-T transfer, has revolutionized the history of clinical cancer treatment and significantly prolonged the survival of patients, especially for patients with advanced or metastatic tumors [3,4]. However, quite few randomized clinical trials of immunotherapy had been performed on vulvar cancer and limited published data is available so far [5]. Immunohistochemistry analysis revealed that the existence of considerable infiltrated immune cells in Tumor Micro-Environment (TME) which could be the hint of potential immunotherapy [6,7]. Notably, around 78% of HPV-induced SCC has shown high intraepithelial T cell infiltration and 40-60% of HPV negative SCC are indicated the immune 'hot' or 'inflamed'. Moreover, it is reported that higher intraepithelial infiltration of CD3+ T cells are associated with improved overall and recurrence-free survival [7], which would be the solid evidence and basis for immunotherapy.

As the indispensable optional cell resource of adoptive cellular therapy, TILs would be isolated from tumor biopsy or surgery excision, then expanded to certain order of magnitude *in vitro* with cytokines such as interleukin-2 (IL-2) or validated cytokine cocktails to meet clinical requirement before reinfused back intravenously to the patient [8]. Compared with CAR-T cell therapy, which still set hematological malignancies as its main concerning of application so far, TIL therapy holds relatively explicit advantages in solid tumors treatment. TILs derived from the tumor site would logically encompass considerable tumor specific TCRs which could recognize heterogeneous tumor associated antigens and/or tumor specific neoantigens, while CAR-T cells are only characterized to focus on pre-set target epitopes. Having been stimulated by tumor antigens in TME, it is reasonable that TILs would eventually express certain chemokine ligands/receptors such as CXCR3 or CXCL13 which would enable or endow the TILs with superior homing capacity of tumor site after adoptive transfer [9,10]. In addition, off-target toxicity is seldomly reported in TIL therapy.

Dr. Steven Rosenberg, and his colleagues at the National Institutes of Health (NIH) US. started to explore the clinical application of TILs in several patients with metastatic melanoma in 1988, furthermore, the reported objective response rate (ORR) of these early cases achieved up to 60% [11]. While for a long time, the successful experience of TILs treatment was only confined to melanoma. Currently, extensive administration of TILs in clinical is being explored for more cancer types, such as non-small cell lung cancer, ovarian cancer, colorectal cancer, bile duct cancer, breast cancer and cervical cancer et.al. and promising results were observed in distinct clinical trials [12-14]. In a recent phase 2 trial of autologous TIL therapy, the reported the ORR achives 44.4% in 27 enrolled patients with cervical cancer who had received multiple lines of conventional treatment before or together with TILs infusion [15]. The relatively high T cell infiltration in situ indicates the high therapeutic potential of TIL for patients with vulvar cancer, but the feasibility of TIL isolation and expansion from vulvar cancer lesion hasn't been investigated yet. Therefore, we isolated and expanded TILs from vulvar cancer (VC TILs) in comparison with those from cervical cancer (CC TILs) in the present study. Compared with similar international products, our process selected better conditions for the preservation and expansion of stem cells, and have a higher proportion of stem cells in the final products (such as TCF1+ proportion, Tcm+Naive proportion, etc.). The TCR diversity and clonal types of TIL samples were further

analyzed in paralleling with PBMC (peripheral blood mononuclear cell) from the same donor. By evaluating and comparing multiple indicating markers of TILs from two tumor types, we demonstrated that our manufacturing process could successfully expand VC TILs with favorable effector functions, and both VC and CC TILs products show dominant enriched TCR clones than autologous PBMCs.

Materials and Methods

Patient Subjects

Participants included were female between 18 and 70 years old have at least one tumor lesion with a diameter of more than 1.5 cm, and could be treated with reasonable tumor reduction surgery in accordance with NCCN clinical guidelines. Patients who received radiotherapy within 28 days before surgery were excluded. Fifteen cervical cancer and four vulvar cancer samples were covered in this study. Among them, three samples (2CC and 1VC) obtained first-grade drainage lymph node metastases (confirmed as tumor metastases by PET-CT and intraoperative pathology), and the remaining samples were sampled from the primary tumor. PBMC was obtained from peripheral blood collected during surgery. Primary tumor and superficial lymph node metastases were obtained by surgical operation. Patient consent was written and the consent was for research. This is a case control study, patients selected by the informed consent.

TIL Preparation

The tissue was placed in tissue preservation solution for 2~8 degrees after tissue isolation, and processed within a certain period of time. Primary tumors or lymph node metastases were mechanical dissociation into 1-20mm3 fragments and placed in a Grex or gas permeable bag. In preREP phase, the tissues were expanded with timely replenished or replaced medium for about two weeks. Culture medium (CTSTM OpTmizerTM T Cell Expansion SFM, Thermo) contained 3000IU/mL rhIL-2 (T&L Biotechnology). In the following REP phase, TILs were stimulated with anti-human CD3 antibody (OKT3, T&L Biotechnology) in the presence of irradiated feeder cells and then expanded for about another two weeks.

Flow Cytometry

Expanded TILs were stained with 1:100 fluorescent conjugated antibodies specific for human CD3 (BD, 564713), CD4 (BD, 557852), CD8 (BD, 564526), CD56 (BD, 555516), CD28 (BD, 555730), CD103 (Biolegend, 350216), CD39 (BD, 563679), TCF1 (Biolegend, 655208), PD-1 (BD, 564323), TIM-3 (BD, 565558), LAG-3 (BD, 565720), CD45RA (BD, 562885), CCR7 (BD, 565867). All cells were stained with a Live/Dead viability dye (eBioscience, 65-0865-14). For intracellular staining of TCF1, cells were fixed with Transcription Factor Buffer Set (BD, 562574), and then TCF1 was stained in accordance with the manufacturer's protocol (Biolegend). Data were acquired on an MACSQuant Analyzer 10 flow cytometer (Miltenyi) and analyzed using FlowJo software (Treestar).

Functional Assay

For CD107a expression analysis, TILs were seeded in a 96-well plate at the density of 1e5/well, and three duplicated wells were set for each group. Reagent TransAct (Miltenyi) was added to stimulate TILs overnight. On the next day, CD107a expression was analyzed by flow cytometry. For cytokine production and cytotoxicity analysis, HeLa cells labeled with CFSE were seeded in a 96-well plate at the density of 5e4/well and 1.5e5/well TILs were added on top with the purpose of co-incubation. The supernatant was collected after18hrs' co-culture and cytokine production was analyzed by CBA (BD). DAPI was added into the co-culture system after supernatant collection in order to quantify TILs killing function via detecting tumor death by flow cytometry.

RNA Isolation and TCR Sequencing

PBMCs were separated by density gradient centrifugation. Total RNA was extracted using QIAamp® RNA Blood Mini Kits (QIAGEN, USA) according to the manufacturer's protocol. TCR libraries were constructed with the SMARTer Human TCR a/b Profiling Kit (TAKARA, USA) following the manufacturer's protocol. First-strand cDNA synthesis from RNA was primed by the TCR dT Primer and used the SMART-Seq v4 Oligonucleotide for template switching at the 5' end of the transcript. Following the reverse transcription, two rounds of PCR were performed in succession to amplify cDNA sequences corresponding to variable regions of TCR-α and TCR-β transcripts and add the sequencing adapter and sample index. The PCR product was then purified with magnetic beads. The libraries were pooled and sequenced on an Illumina MiSeq sequencer (2 x 300 bp read length). The TCR gene segments were determined by MiXCR [16] on the reference sequences from the Immunogenetics (IMGT) database (V 3.1.34, http://www.imgt.org).

Statistical Analysis

Statistical analyses were performed using Prism 7.0 (GraphPad Software) and R 4.1.0. Bar or dot plots show means ± SEM. Non-paired t test (two-tailed) was used for the comparison between CC and VC datasets. P values are marked in the corresponding figures and two side p values < 0.05 were considered as statistically significant. Vegan package in R were utilized to calculate Shannon-Weaver index of TCR CDR3 sequence.

Results

Patient Samples

Primary tumor samples and Lymph Node (LN) metastases

obtained from surgical specimens were collected from 10 patients with cervical cancer and 4 patients with vulvar cancer, covering FIGO stage from Ⅰ to Ⅳ. All samples were successfully prepared. The feasibility of TIL expansion would be evaluated within these two types of tumors. Sample information including demographic data is summarized in Table 1.

Sample#	Cancer Types	Batch	Tumor Source	Size	Stage
1	Cervical Caner	200203	Primary	0.5g	ⅡA1
2	Cervical Caner	200207	Primary	0.5g	ⅠB2
3	Cervical Caner	200311	Primary	1.0g	ⅢC2
4	Cervical Caner	200315	Primary	1.1g	ⅠB3
5	Cervical Caner	200401	Primary	0.5g	ⅠB1
6	Cervical Caner	200402	Primary	0.4g	ⅢC1
7	Cervical Caner	200409	Primary	1.5g	ⅠB3
8	Cervical Caner	200418	Primary	0.3g	T1b1N0M0
9	Cervical Caner	200620	Primary	1g	ⅡB
10	Cervical Caner	200621	Primary	1g	ⅠB2
11	Cervical Caner	200813	Primary	1g	ⅠB1
12	Cervical Caner	200921	Primary+ LN	1.5g	ⅢC
13	Cervical Caner	201006	Primary	0.9g	ⅢC1p
14	Cervical Caner	201101	Primary+ LN	2.9g	ⅢC2
15	Cervical Caner	201106	Primary	0.5g	ⅠB1
16	Vulvar Cancer	200205	Primary	0.7g	ⅠB
17	Vulvar Cancer	200722	LN	0.5g	Ⅳ
18	Vulvar Cancer	200801	Primary	2.5g	ⅢA(i)
19	Vulvar Cancer	200803	Primary	0.8g	ⅠB

Table 1: Sample information.

Expansion of TIL from vulvar and cervical tumor lesion

VC TILs could be successfully expanded with the same procedure as that for CC TILs. Briefly, after the surgical tumor specimen acquisition and transportation, TIL cells were isolated from the tumor fragments, cultured in a previous rapid expansion phase (preREP), and then amplified in a rapid expansion phase (REP). Expanded TIL cells were then cryopreserved and transported to the clinical center (Figure 1A). The average cell number of the four batches of VC TILs in preREP round are no less than 2×107 (Figure 1B), and the average expansion folds in REP round are no less than 1000 folds (Figure 1C), which are higher than that in CC TILs expansion. In general, VC TILs can be successfully cultured and amplified *in vitro*.

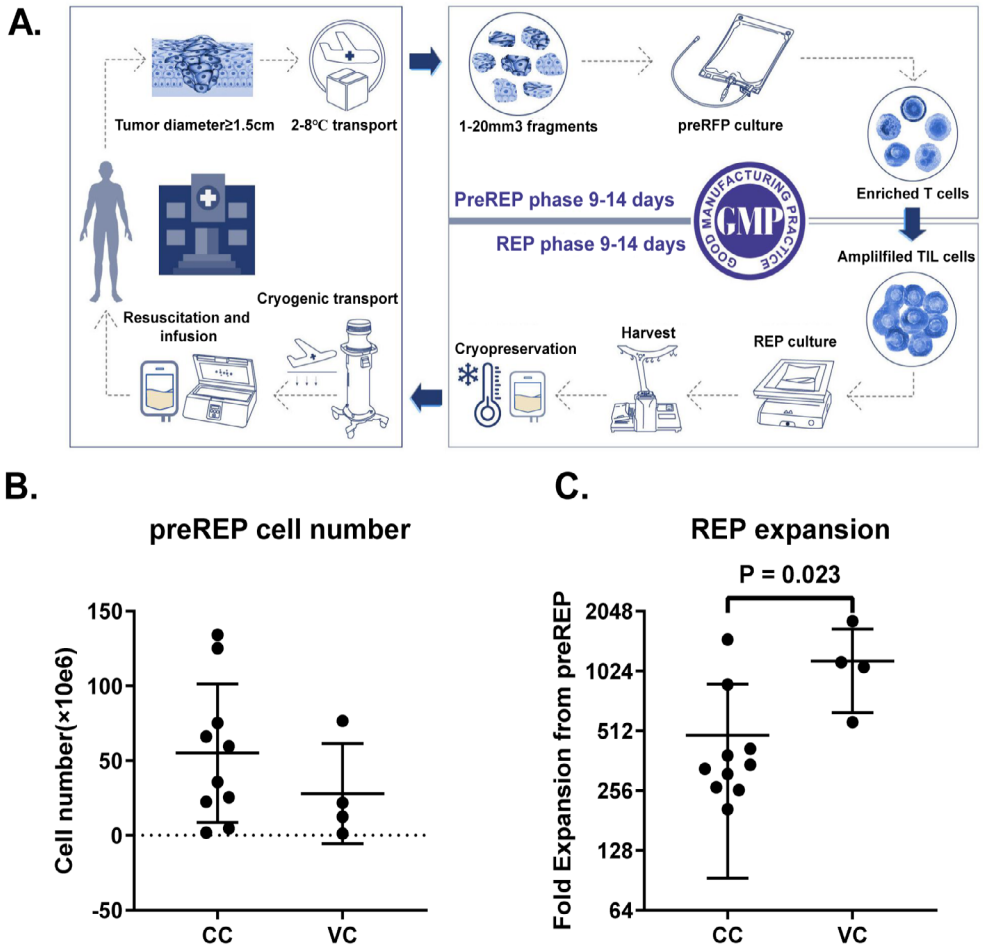


Figure 1: TIL can be successfully expanded from cervical and vulvar cancer. (A). A schematic representation of the TIL preparation process. **(B).** The total number of TILs expanded from cervical or vulvar tumor fragments were measured at the end of preREP culture. Each point represents the total TIL number generated from the tumor specimen of an individual patient. **(C).** The fold expansion of each TIL batch from preREP to the end of REP.

VC TILs share similarities in immune-phonotype with CC TILs

To characterize the subgroup and phenotype of expanded VC TILs, we analyzed the immune subsets as well as indicator markers involved in T cell activation, differentiation and function. At the end of preREP, the majority of TILs products in most cases are CD3+CD56- T cells (mean>50%) with a small fraction of CD3-CD56+ NK cells (around 10%) (Figure 2A). The percentage of CD8+ and CD4+ T cells showed high variance among different individuals in both cancer types (Figure 2B). Both VC and CC derived TILs showed high expression of CD28, a marker associated with T cell activation and tumor elimination [17], suggesting a functional phenotype in preREP product (Figure 2C,2D). At the end of REP, T cells subsets were further expanded(most>90%) and the NK population shrunk into minimal size (Figure 3A). In the preREP stage, the proportion of T cells in VC samples was lower than that in CC samples, but reversed to the REP stage, indicating that the TIL of VC samples was well expanded (Figure 2A and 3A). CD8+ or CD4+ subsets within T cell population could still be stably observed in certain cases in both types of cancer in the meanwhile of CD4/CD8 polarization (Figure 3B). Similar to TILs in the preREP stage, the CD28+ T cells remained at a high level in REP product, while CD28+CD8+ T cells in VC TILs showed an even higher percentage compared to CC TILs (Figure 3C). We further analyzed the expression of inhibitory receptors as TIM-3 and LAG-3, which are involved in T cell exhaustion [18]. Both CC and VC TILs highly expressed TIM-3, while VC TILs showed comparable lower expression of LAG-3 on both CD8+ and CD4+ subsets (Figure 3D). Percentage of Tregs in both VC and CC TILs are

under 5% (Figure 3E). Taken together, both VC and CC TILs are T cells predominant at the end of REP, meanwhile VC TILs may have a more active CD8+ T cell counterparts as indicated by higher CD28 and lower LAG-3 expression (Figure 3F).

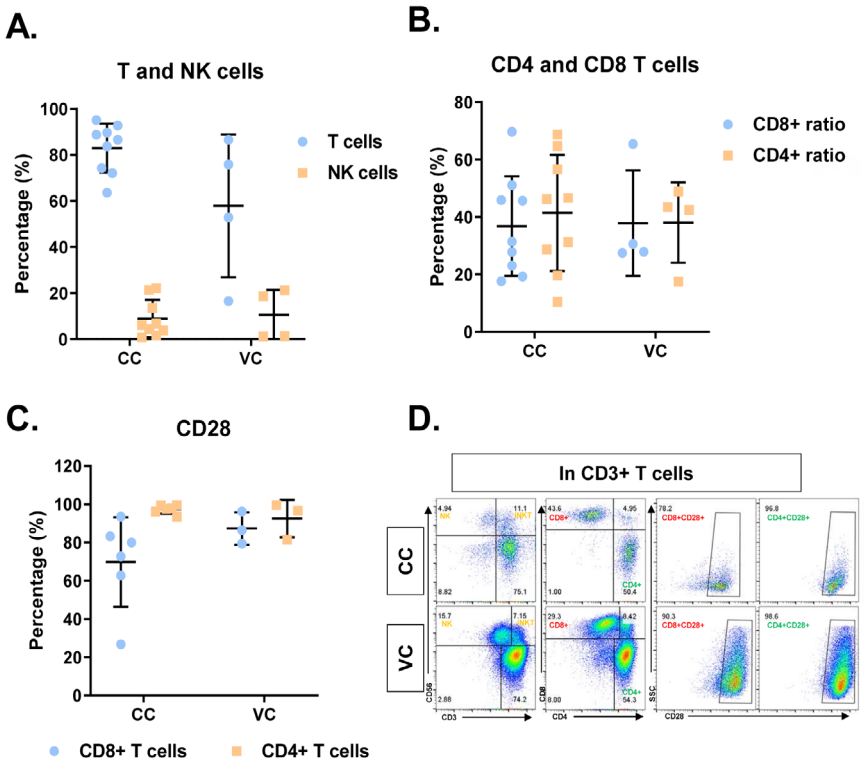


Figure 2: Profiling of CC and VC TILs after preREP. (A-B). At the end of preREP culture, TILs were collected and the percentage of $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD3^-CD56^+$ NK cells were detected by flow cytometry. **(C).** CD28 expression on $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry. **(D).** Representative flow chart for each marker expressed.

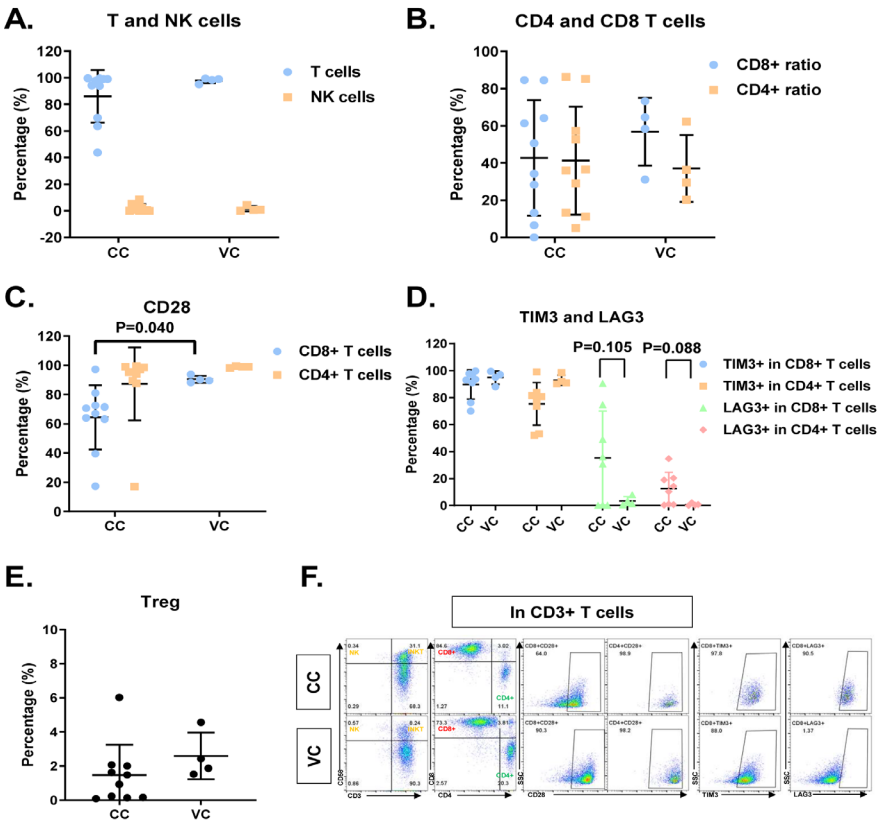


Figure 3: Profiling of CC and VC TILs at the end of REP. (A-B). At the end of REP culture, TILs were collected and the percentage of $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD3^-CD56^+$ NK cells was measured by flow cytometry. **(C).** CD28 expression on $CD4^+$ and $CD8^+$ T cells was detected by flow cytometry. **(D).** TIM3 and LAG3 expression on $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry. **(E).** Treg ratio in TILs was analyzed with CD25 and Foxp3 staining by flow cytometry. **(F).** Representative flow chart for each marker expressed.

VC TILs harbor higher stem-like property than CC TILs

A recent study found that tumor-infiltrating CD8+ T cells could be divided into two functionally distinct subgroups: a terminally differentiated group marked by high expression of immune-checkpoint molecules such as PD-1(programmed death receptor 1, also known as CD279) and TIM-3, and a stem-like group with low checkpoints but high TCF1 and CD28 expression [19]. PD-1 is a co-inhibitory molecule which may restrain T cell function and anti-tumor response, and considered as a marker of T cell exhaustion [20]. TCF1 is a key transcription factor in stem cell-like T cells, and plays a critical role in sustaining immune response against tumor and chronic virus infection [21,22]. To better characterize the functional profile of TILs undergoing the REP process, we focused on the PD-1 and TCF1 expression on VC and CC TILs. The percentage of PD-1 positive CD4+ and CD8+ T cells in CC TILs is only around 10%, which is even lower in VC TILs (Figure 4A). Almost 100% of CD8+ VC TILs are TCF1 positive, which is significantly higher than that in CC TILs (Figure 4B), indicating the fact that VC TILs may possess higher stemness and even further may linked with preferable potential of amplication and survival in vivo than CC TILs. We further examined the memory phenotype of VC and CC TILs based on CD45RA and CCR7 expression and found higher percentage of naïve and central memory T cells (Tcm) (Figure 4C) and a lower percentage of effector memory and effector memory RA T cells (Tem and TemRA) (Figure 4D) in VC TILs than that in CC TILs, especially in CD4+ T cells who are also dominant in stemness marker expression within VC TILs.

In addition, we run CD103 and CD39 staining on TILs products as CD103+CD39+ tumor-infiltrating CD8+ T cells may represent tumor-reactive cells in solid tumors and are associated with better overall survival [23]. We observed more than 20% CD103 and CD39 double positive CD8+ T cells within 6/10 of CC TIL samples and 2/4 of VC TIL samples, which are straightforwardly lower in CD4+ T cells (Figure 4E).

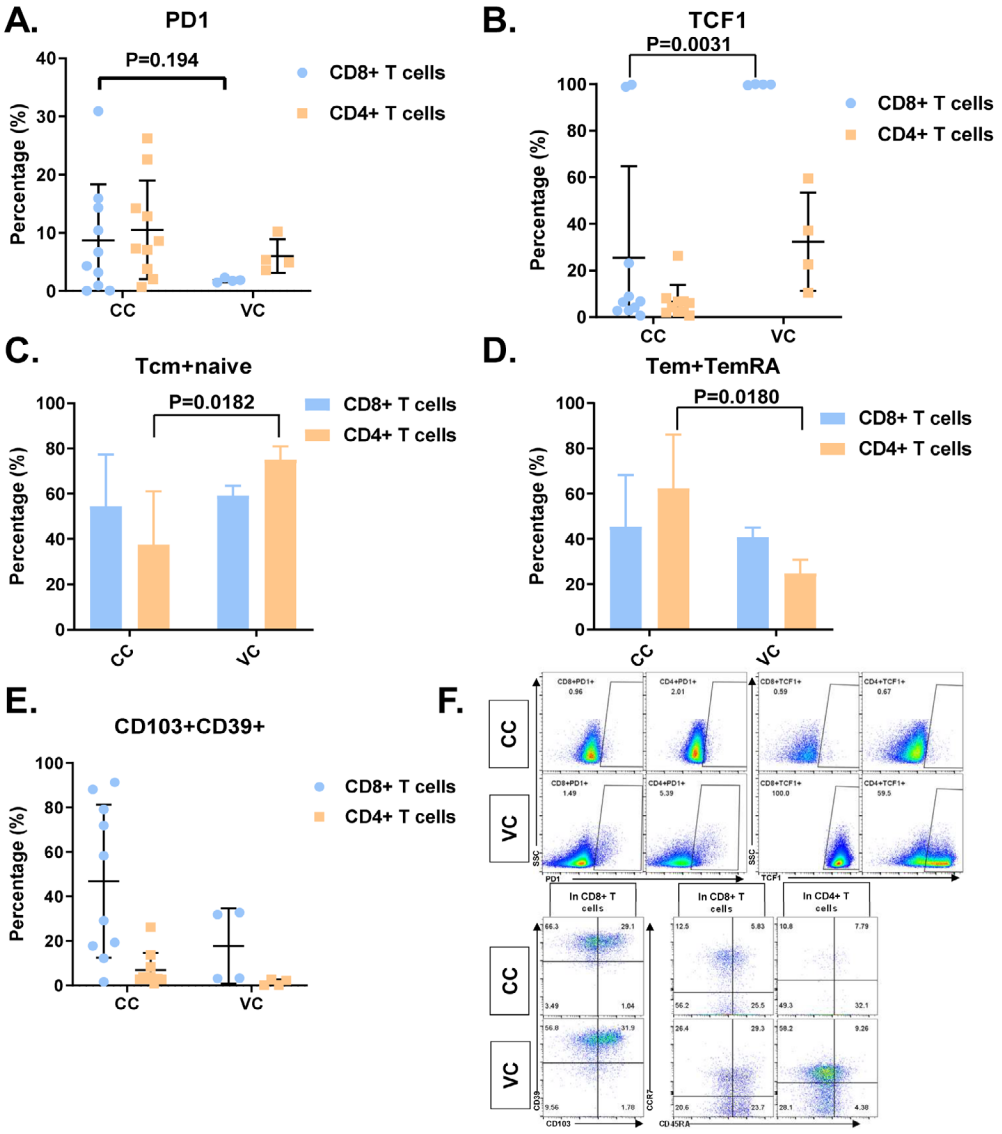


Figure 4: Stemness and memory phenotype analysis of CC and VC TILs. TILs were analyzed for the expression of indicated markers using flow cytometry after 9-14 days of rapid expansion. **(A-B).** The percentage of PD1 or TCF1 expression in TILs of each batch is shown. **(C-D).** The percentage of different T cell memory phenotypes is shown. Naïve, central memory, effector memory and effector memory RA T cells are defined by $CD45RA^{+}CCR7^{+}$, $CD45RA^{-}CCR7^{+}$, $CD45RA^{-}CCR7^{-}$ and $CD45RA^{+}CCR7^{-}$, respectively. **(E).** The percentage of CD103 and CD39 expression in TILs of each batch is shown. **(F).** Representative flow chart for each marker expressed.

uCDR3 Magnitude and Diversity Characterization

The number of unique CDR3 (uCDR3) in both VC and CC samples were determined via CDR3 nucleic acid sequencing. The number of uCDR3 in the TIL samples summarized across donors (mean = 14,122.38, median = 8,087) was significantly lower than that in the PBMC (mean = 63679.62, median = 56350.5) (Figure 5A, P = 0.00133). In addition, the number of the uCDR3 in each TIL sample was comparably lower than that in PBMC from the corresponding donor (Figure 5B), both in CC and VC. The accumulate relative abundance of Top10 ranked uCDR3 in every TIL sample (mean = 50.49, median = 49.83) is higher than that in the PBMC sample (mean = 16.32, median = 13.72) from the same donor. Notably, the accumulate relative abundance of Top10 uCDR3 is more than 50% in 4

out of 8 TIL samples. Moreover, we analyzed the Shannon Entropy in each sample, which is a quantitative measure that simultaneously takes both richness and evenness into account. A higher Shannon entropy indicates that the TCR sample has more types of uCDR3 or a more even distribution of CDR3 reads. The Shannon Entropy of TIL (mean = 4.93, median = 4.96) was significantly lower than that of PBMC (mean = 9.01, median = 9.31) (Figure 5D, P = 0.0004). The Shannon Entropy of each TIL sample was lower than that in its paired PBMC sample (Figure 5E). All experimental observations above suggest that the TIL samples have more enriched T cell clones than the PBMC.

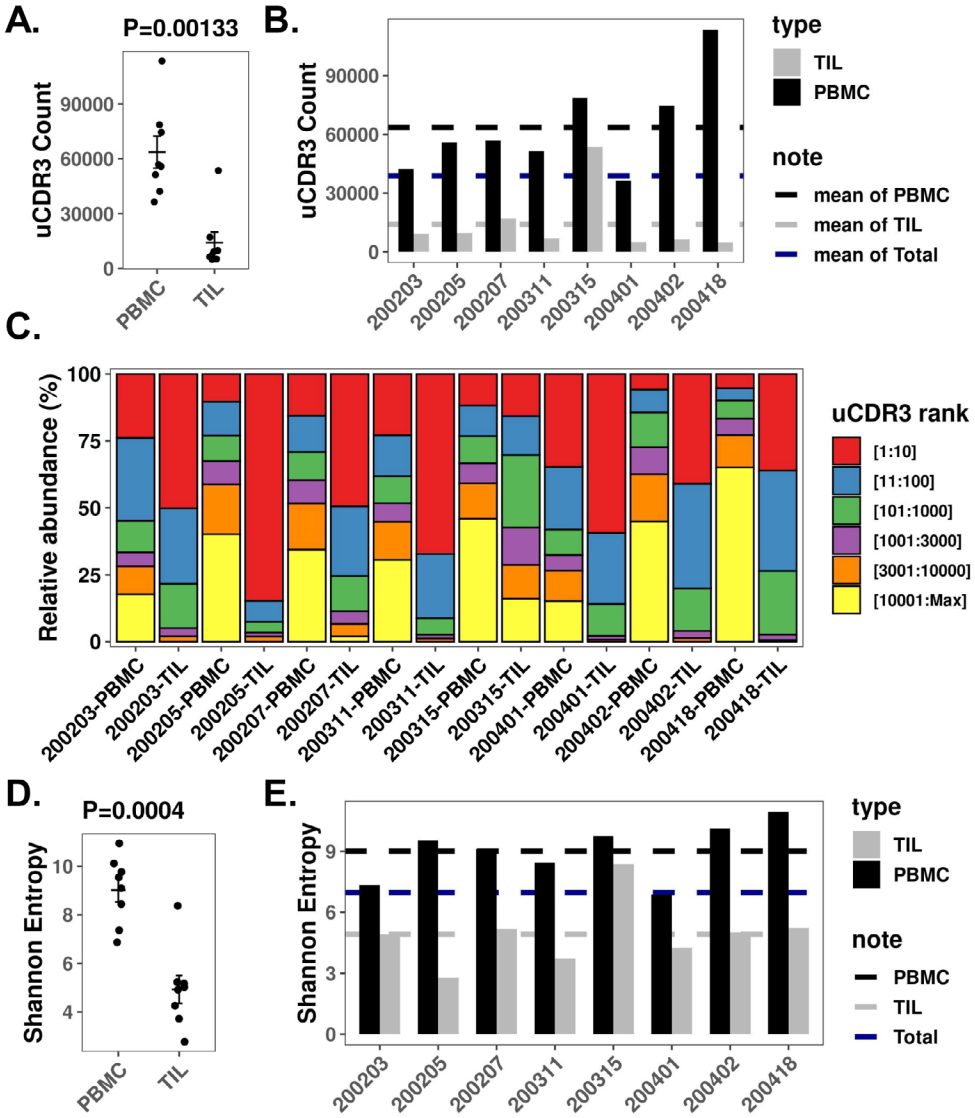
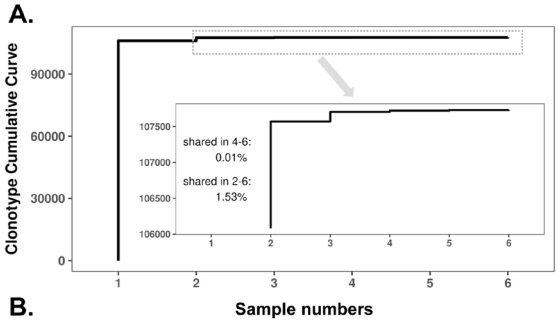


Figure 5: uCDR3 diversity characterization between PBMC (n = 8) and TIL (n = 8). (A). Unique CDR3 comparison between PBMC and TIL. Paired t test P-value was 0.00133. **(B).** Unique CDR3 counts in each sample. Mean uCDR3 counts of PBMC, TIL and total samples were shown in colored dash lines as indicated. **(C).** Percentage stacked bar-plot by sample. Colored bars indicate uCDR3 ranked by uCDR3 clone abundance. For example, red bar shows the total percentage of the Top10 ranked uCDR3 in each sample. **(D).** The Shannon Entropy comparison between PBMC and TIL. Paired t test P-value was 0.0004. **(E).** The Shannon Entropy in each sample was shown. Mean Shannon Entropy of PBMC, TIL, and total samples were shown in colored dash lines as indicated.

TCR Clonotype Analysis in TIL Product

A total of 107,732 clonotypes were identified in all TIL samples. Detection times of each clonotypes in TIL samples were summarized in Figure 6A. Most clonotypes (98.467%) were detected in only one TIL sample. Only 1.53% clonotypes were found in more than one sample and 0.01% were found in 4 or more samples, suggesting that the repertoire of TIL TCR were patient-specific (Figure 6A). Reads of most shared clonotypes represented low fraction, with 77.8% of clonotypes accounting for less than 0.001% reads fraction and 18.2% clonotypes accounting for 0.001%-0.01% (Figure 6B).



Clonotype Reads Fraction	Clonotype Number	Percent (%)
0% to 0.001%	85251	38.896
0.001% to 0.01%	19986	9.119
0.01% to 0.1%	3644	1.663
0.1% to 1%	602	0.275
1% to 10%	95	0.043
10% to 20%	7	0.003
> 20%	3	0.001

Figure 6: Counts and frequency distribution of clonotypes between PBMC (n = 8) and TIL (n = 8). (A). Detection times of each clonotype in TIL samples. X axis indicates the number of samples having the same clonotype. Y axis indicates the cumulative number of clonotypes. Clonotypes were determined by the combination of CDR3 sequence, V gene and J gene. The cumulative number of shared clonotypes were highlighted in the zoomed subplot. Clonotypes detected in more than one TIL samples were considered as shared clonotypes. 1.53% clonotypes were detected in more than one TIL samples, and 0.01% clonotypes were detected in 4-6 TIL samples. **(B).** Reads fraction of shared clonotypes is shown. The reads fraction ranges at which these shared clonotypes were represented in their respective TIL samples were summarized in the left column. The clonotype number in each category and their corresponding percentage in total clonotype numbers were shown in the middle and right column, respectively.

Function Analysis of CC and VC TILs

To investigate whether the expanded TILs are responsive to extracellular restimulation, VC and CC TILs are restimulated overnight with T cell activator. Upregulation of CD107a, a marker of degranulation which would indicate T cell cytotoxicity, could be observed on a portion of VC and CC TILs (Figure 7A). We further analyzed the cytokine production of TILs in response to HeLa cells, a cervical cancer cell line, and found that both CC and VC TILs produced high levels of IFN-γ and TNF-α, and VC TILs tended to release more among all samples (Figure 7B and C). Finally, the cytotoxicity potential of TILs was examined by HeLa cell lysis in a co-culture system. VC TILs tended to show higher HeLa cell killing of compared to CC TILs, which suggesting the stronger tumor killing potential of VC TILs (Figure 7D).

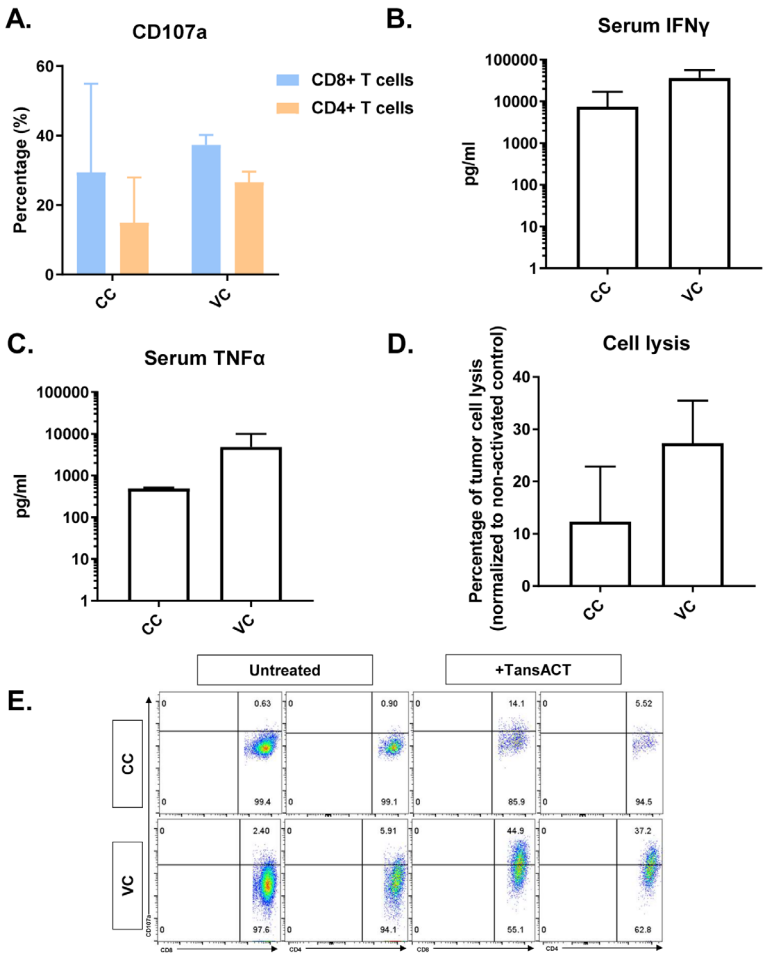


Figure 7: Functional characterization of CC and VC TILs. TILs were assessed for cytokine secretion and tumor killing capacity after rapid expansion. **(A).** TILs were re-stimulated by antibodies and then the CD107a expression was analyzed by flow cytometry. **(B-C).** IFN-γ and TNF-α were detected by CBA in the supernatants of TIL after co-culture with HeLa cells line overnight with an effector to target ratio of 3:1. Mean of IFN-γ and TNF-α level (pg/ml) is shown above in each bar. **(D).** Tumor killing capacity of TILs was shown by the percentage of CFSE labeled Hela cell lysis in the co-culture system. **(E).** Representative flow chart for CD107a expression on TILs after co-culture.

Discussion

Orchestrating and harnessing the host immunity via manifold cancer immunotherapy strategies would enable the occurrence of tumor elimination, which may induce promising clinical benefits in patients with advanced cancers. However, in most tumor cases, especially solid tumor, unsatisfied irresponsive immunotherapy and tumor escape is frequently reported with unmet clinical requirements [24]. TIL is a type of adoptive cellular therapy with distinctive advantages in solid tumors treatment, including but not limited to its diverse TCR clones capable of heterogeneous tumor antigens recognizing, its superior homing ability to the tumor site and low off-target toxicity. Relatively, the antigen targets of CAR-T are limited, and it is hard to inter the tumor naturally. So far, TIL therapy has shown promising clinical results in several types of solid tumors, such as metastatic melanoma and cervical cancer. However, there are few reports on TIL of vulvar cancer. Vulvar cancer is a lethal gynecological malignancy with increasing incidence in the last few decades, while effective therapeutic strategies are still urgently needed, especially for the refractory vulvar cancer. As the published beneficial predictor of Overall Survival (OS) and Recurrence-Free Survival (RFS) in multiple cancer types, CD3+T cell or CD3+CD8+ T cell more precisely infiltrating in tumor milieu according to the published data of immunohistochemical(IHC) staining

or bulk/single cell sequencing, would provide solid theoretical and practical foundation for TIL clinical administration in vulvar cancer patients. Therefore, we explored the feasibility of TILs isolating and expanding *in vitro* for vulvar cancer treatment.

A manufacturing process with optimized culture conditions based on previous TIL studies were employed in TILs expansion, and TILs product were successfully expanded for potential therapeutic value with desired prosperities. In this study, we showed that VC TILs could be arised from both tumor tissue and lymph node metastases. Even in samples with a lower percentage of T cells at the preREP stage, VC TILs are eventually expanded in subsequent expansion round with around 95% CD3+ T cells in the final product. Tumor specific TILs within TME are highlighted by the comprehensive expression of exhaustion related markers as PD-1 and impaired tumor killing function, while high CD28 and low PD-1 expression on VC and CC TILs after REP in our study indicate the potential antitumor reactivity after the rejuvenating expansion. Besides, the percentage of Tregs in the final product is comparably low (below 5% percent).

The uCDR3 count and diversity analysis revealed the expansion and enrichment of certain TCR clonotypes in the TIL product. The relative abundance of Top10 and Top100 ranked uCDR3 in TILs is much higher than that in autologous PBMC. Most clonotypes were detected only in one sample, and most shared clonotypes were clones with low frequency, which suggested that most of the TCR repertoires in TIL product are patient-specific. As reported by Bobisse et al., these patient-specific TCRs could be identified even in some tumor cases harboring relatively low mutational loads such as ovarian cancer. The patient-specific TCRs may be induced and generated by differential expression of tumor antigens as the expression of patient-specific neoantigens for instance [25] or patient-specific responding process against autologous tumor [26]. Functional analysis showed that our TIL product, especially VC TILs, harvest with upregulated CD107a expression and IFN-γ and TNF-α production upon tumor or antibody stimulation, meanwhile, cytotoxicity against HeLa cells were also observed. The phenotype and functionality of the TIL products expanded according to our Standard Operation Procedure (SOP) are concordant to those reported melanoma TILs [27], indicating the effectiveness of our TILs expansion procedure. A major challenge to evaluate the tumor specificity of TILs in our assay is the availability of autologous tumor samples. Currently, we are working on the methods of Patient-Derived Cancer Cells (PDCs) and Patient-Derived Organoids (PDO) establishment from vulvar cancer samples *in vitro*, which could be recruited in the tumor specific responses measurement in the future.

Compared with cervical cancer, the cell count of TILs from vulvar cancer samples after preREP round is close, but the average amplification rate during the REP stage is twice of that in cervical cancer, which demonstrated the potent amplification capacity of VC TILs in culture. VC and CC TILs have similar T and NK cell composition, but the activation marker CD28 and stem cell marker TCF1 expression is higher on VC TILs. Moreover, VC TILs showed better cytokine production and tumor-killing capability compared with CC TILs, which indicated better effector state of VC TILs. Further assays included in this study, such as T cell receptor repertoire profiling and TCR specificity against tumor associated antigens or neoantigens analysis would facilitate a better understanding of VC TILs. Taken together, we had demonstrated in this report that TILs could be successfully expanded from tumor tissue and tumor related lymph nodes from vulvar cancer lesions and authorized with stem-like functional effector prosperities due to the expression of activation surface markers and transcriptional factors. Therefore, as a potential promising novel therapeutic strategy for vulvar cancer, the clinical application of TIL therapy should be further investigated in vulvar cancer patients.

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References

1. Tan A, Bieber A K, Stein J A (2019) Diagnosis and management of vulvar cancer: A review. J Am Acad Dermatol 81: 1387-1396.
2. (2019) Survival rates for vulvar cancer. American cancer society.
3. Couzin-Frankel J (2013) Breakthrough of the year 2013. Cancer immunotherapy. Science 342: 1432-1433.
4. June CH, O'connor RS, Kawalekar OU (2018) CAR T cell immunotherapy for human cancer. Science 359: 1361-1365.
5. Nobbenhuis MA, Lalondrelle S, Larkin J (2014) Management of melanomas of the gynaecological tract. Curr Opin Oncol 26: 508-513.
6. Sznurkowski JJ, Zawrocki A, Emerich J (2011) Prognostic significance of CD4+ and CD8+ T cell infiltration within cancer cell nests in vulvar squamous cell carcinoma. Int J Gynecol Cancer 21: 717-721.
7. Kortekaas KE, Santegoets SJ, Abdulrahman Z (2019) High numbers of activated helper T cells are associated with better clinical outcome in early stage vulvar cancer, irrespective of HPV or p53 status. J Immunother Cancer 7: 236.
8. Geukes Foppen MH, Donia M, Svane IM (2015) Tumor-infiltrating lymphocytes for the treatment of metastatic cancer[J]. Mol Oncol 9: 1918-1935.
9. Bedognetti D, Spivey TL, Zhao Y (2013) CXCR3/CCR5 pathways in metastatic melanoma patients treated with adoptive therapy and interleukin-2. Br J Cancer 109: 2412-2423.

10. Mikucki ME, Fisher DT, Matsuzaki J (2015) Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. Nat Commun 6: 7458.

11. Rosenberg SA, Packard BS, Aebersold PM (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med 319: 1676-1680.

12. Creelan B, Wang C, Teer J (2020) Abstract CT056: Durable complete responses to adoptive cell transfer using tumor infiltrating lymphocytes (TIL) in non-small cell lung cancer (NSCLC): A phase I trial[J]. Cancer Research 80: CT056-CT056.

13. Tran E, Robbins PF, Lu YC (2016) T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. N Engl J Med 375: 2255-2262.

14. Zacharakis N, Chinnasamy H, Black M (2018) Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer[J]. Nat Med 24: 724-730.

15. Jazaeri AA, Zsiros E, Amaria RN (2019) Safety and efficacy of adoptive cell transfer using autologous tumor infiltrating lymphocytes (LN-145) for treatment of recurrent, metastatic, or persistent cervical carcinoma. Journal of Clinical Oncology 37: 2538-2538.

16. Bolotin DA, Poslavsky S, Mitrophanov I (2015) MiXCR: software for comprehensive adaptive immunity profiling. Nature Methods 12: 380-381.

17. Powell DJ, Dudley ME, Robbins PF (2005) Transition of late-stage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy. Blood 105: 241-250.

18. Datar I, Sanmamed MF, Wang J (2019) Expression Analysis and Significance of PD-1, LAG-3, and TIM-3 in Human Non-Small Cell Lung Cancer Using Spatially Resolved and Multiparametric Single-Cell Analysis. Clin Cancer Res 25: 4663-4673.

19. Jansen CS, Prokhnevska N, Master VA (2019) An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. Nature 576: 465-470.

20. Chen L, Han X (2015) Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. J Clin Invest 125: 3384-3391.

21. Siddiqui I, Schaeuble K, Chennupati V (2019) Intratumoral Tcf1(+)PD-1(+)CD8(+) T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. Immunity 50: 195-211.e10.

22. Utzschneider DT, Charmoy M, Chennupati V (2016) T Cell Factor 1-Expressing Memory-like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections. Immunity 45: 415-427.

23. Duhen T, Duhen R, Montler R (2018) Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat Commun 9: 2724.

24. Ribas A, Wolchok JD (2018) Cancer immunotherapy using checkpoint blockade. Science 359: 1350-1355.

25. Gros A, Parkhurst MR, Tran E (2016) Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. Nat Med 22: 433-438.

26. Crane CA, Han SJ, Ahn B (2013) Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein 19: 205-214.

27. Turcotte S, Gros A, Hogan K (2013) Phenotype and function of T cells infiltrating visceral metastases from gastrointestinal cancers and melanoma: implications for adoptive cell transfer therapy. J Immunol 191: 2217-2225.