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Successful ex vivo expansion of tumor infiltrating lymphocytes with systemic chemotherapy prior to surgical resection

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Short Report

Keywords: Tumor-Infiltrating Lymphocyte, Adoptive Cell Therapy, Chemotherapy, Solid Tumor, Cancer Immunotherapy

Posted Date: May 18th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2939447/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Cancer Immunology, Immunotherapy on July 19th, 2023. See the published version at https://doi.org/10.1007/s00262-023-03500-9.

Abstract

Tumor Infiltrating Lymphocytes (TIL) have demonstrated efficacious clinical outcomes for many patients with various types of solid cancers, including melanoma, gastrointestinal cancer, lung cancer, and head & neck cancer. Currently, majority of clinical trials require that patients did not receive systemic therapy right before tumor tissue resection to avoid the interference of chemotherapy in the ex vivo TIL expansion. The primary disadvantage of this strategy is limiting the accessibility of TIL therapy for many eligible cancer patients. Over the past decade, substantial progress has been made for ex vivo expansion technologies in T cells. In this study, we investigated the possibility of enrolling patients who underwent chemotherapy prior to surgical resection. We collected seventeen tumor tissues from treatment naive cases, and five from cases that underwent chemotherapies. Cancer indications enrolled in this study were colorectal, lung, and brain cancers from both primary and metastasis cancers. TILs from these tumors were expanded ex vivo to 2.1E8 on average, with an overall success rate of 90.9%. Subsequently, TIL phenotypes and cytokine production were analyzed using flow cytometry and ELISA, respectively. We demonstrated functional TIL expansion from tumor tissues despite chemotherapy prior to surgical resection. We observed no significant phenotypic or functional differences nor changes between groups with and without chemotherapy. TIL expansion rate and characteristics were similar regardless of chemotherapy prior to resection, thereby providing a possibility to recruit patients with the most recent chemotherapy history in TIL therapy trials.

Introduction

Adoptive Cell Therapy (ACT) has emerged as one of the most promising cancer immunotherapies. One such ACT, Tumor Infiltrating Lymphocytes (TIL) therapy has been widely studied and has exhibited favorable clinical outcomes for solid tumors [4–6]. For example, a recent study by Chesney *et al.* achieved an objective response rate (ORR) of 36% in 66 advanced melanoma patients, with tumor progression after Immune Checkpoint Inhibitors (ICI) and targeted therapies [4]. A two arm, randomized phase 3 trial study by Rohaan et al. showed a median overall survival (OS) of 25.8 months in melanoma patients receiving TIL, in contrast to an OS of 18.9 months in the 2nd group of patients treated with ipilimumab (anti-CTLA-4), demonstrating a strikingly better efficacy with TIL than with ICI [5–6].

One of hurdles to recruit patients in immunotherapy clinical trials has been a history of chemotherapy prior to enrollment. Given that chemotherapy is one of standards of care in cancer, exclusion of patients who have received chemotherapy from a TIL therapy trial would greatly impact patients due to lack of treatment options. A study by Aydin *et al*. shows that TIL can be successfully expanded from a rare indication, penile cancer regardless of neoadjuvant therapy status or HPV status of the patient [7]. However, successful generation of TILs from tumor tissues following systemic therapies in other types of solid cancer has remained elusive. We hypothesized that chemotherapy would not significantly impact ex vivo TIL generation. Thus, we procured tumor tissues from patients with or without chemotherapy and examined TILs from these two groups. Here we report the effective generation and expansion of functional TILs from tumor tissues that were resected after systemic therapies for multiple indications including metastatic cancers, with a success rate of 83% (5 out of 6 cases) with chemotherapy and 94% (15 out of 16 cases) without chemotherapy. Success was defined as fold expansion ≥ 2 during rapid expansion protocol (REP). These findings provide a possibility to extend inclusion criteria to enroll cancer patients with the most recent chemotherapy history for TIL therapy.

Materials and Methods

Patient samples

Tissue samples were provided by the Cooperative Human Tissue Network (CHTN (RRID: SCR_004446), https://www.chtn.org/) funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects. The tissues were obtained as either anonymized, de-identified, or a limited data set. The de-identified pathological reports were provided for each sample. Sixteen treatment naïve cases and five cases with treatment were included in this study. The patient demographics and baseline characteristics are listed in Table 1.

Table 1
Patient demographics and baseline characteristics

ID	Age (years)	Sex *	Indication	Pathology Stage Classification	Chemo-therapy prior to surgery (days)	Cell count	Cell count/mg tissue	Fold expansion
1	56	M	Mucinous adenocarcinoma of the cecum/ileocecal valve	pT3 pN0 pM1c	16	4.87E7	1.40E + 06	16
2	37	M	Adenocarcinoma with medullar and mucinous features of the colon	pT4b pN2b	33	8.3E7	1.38E + 06	2
3	44	F	Metastatic colon adenocarcinoma to liver	Unknown	488	7.19E5	6.60E + 03	5
4	68	F	Adenocarcinoma, Metastatic colon cancer to liver	pT2 pN0 pM1a	60	7.70E7	2.78E + 04	33
5	50	F	Adenocarcinoma of ascending colon with liver metastasis	Unknown	14	1.67E8	2.94E + 05	56
6	60	F	Adenocarcinoma in the left frontal lobe of the brain metastasis from lung	cT2b cN2 pM1c	None	2.00E7	3.28E + 02	244
7	48	F	Metastatic adenocarcinoma of the abdominal wall	Unknown	None	1.00E7	3.53E + 04	72
8	60	M	Adenocarcinoma of sigmoid colon	pT3 pN1b	None	2.39E7	5.60E + 05	4
9	77	M	Metastatic small cell carcinoma of the right cerebellum	Unknown	None	11.88E6	3.53E + 04	6.6
10	71	F	Adenocarcinoma of the colon	Unknown	None	10.23E7	8.94E + 05	34.1
11	78	M	Carcinoma of the colon	Unknown	None	37.7E6	6.94E + 05	6.7

*F: Female, M: Male

#Removed from downstream analysis due to lack of in vitro TIL expansion

ID	Age (years)	Sex *	Indication	Pathology Stage Classification	Chemo-therapy prior to surgery (days)	Cell count	Cell count/mg tissue	Fold expansion
12	71	F	Invasive papillary adenocarcinoma of the lung (Upper lobe)	pT2a pN0	None	6.34E7	4.15E + 05	6
13	62	F	Invasive acinar adenocarcinoma of the lung (Upper lobe)	pT2a pN0	None	1.55E8	2.73E + 05	16
14	85	F	Metastatic colon cancer to liver	Unknown (Primary colon cancer: pT3 pN0)	None	1.06E9	8.36E + 05	106
15	72	F	Mucinous adenocarcinoma of the cecum	pT4a pN1a	None	1.02E8	2.04E + 05	34
16	69	F	Colonic adenocarcinoma of the splenic flexure	pT4b pN0	None	1.42E8	9.49E + 04	47
17	88	M	Adenocarcinoma of sigmoid colon	pT3 pN0	None	1.94E7	5.10E + 04	6
18	64	F	Adenosquamous carcinoma of the right lung	pT1c pN2	None	2.12E9	3.19E + 06	212
19	70	M	Neuroendocrine carcinoma of the Ileum, metastatic to liver	pT2 pN1 pM1a	None	3.00E6	3.73E + 04	5
20	72	F	Adenocarcinoma rectosigmoid	pT3 pN0 (BMT A7)	None	1.00E7	3.75E + 04	3
21	60	F	Adenocarcinoma of the descending colon	pT3 pN2b	None	3.66E5	1.05E + 05	1
#22	53	F	Metastatic colorectal cancer to liver	Unknown	568	Failed to expand during initiation phase	N/A 4.04E + 02	N/A
*F: Female, M: Male								
#Removed from downstream analysis due to lack of in vitro TIL expansion								

Generation of TILs from a tumor tissue

A resected tumor tissue was stored in HypoThermosol FRS (Stemcell Technologies) with 0.1% Gentamicin Sulfate (Gibco) and 1% Amphotericin B (R&D Systems) and kept at 4°C until processed. TILs were expanded as previously described [8]. Briefly, for the initiation phase, tumors were dissected into < 2 mm^3 fragments and cultured with AIM-V media (Gibco) containing 10 µg/mL Gentamicin Sulfate, 25 mM HEPES (Lonza), 2 mM L-Glutamine (Gibco), 10% Human AB serum (Access cell culture) supplemented with 6,000 IU/mL of rhIL-2 (R&D Systems). Half media changes were performed every 3 to 4 days with fresh culture media with IL-2 (6,000 IU/mL) and cultured in 37°C and 5% CO2 until Day 14. On Day 14, TILs were harvested and counted and then proceeded to the REP. TILs were co-cultured with feeder cells which were irradiated peripheral blood mononuclear cells (PBMCs) from healthy donors at a ratio TIL:PBMC of 1:100 in AIM-V media with 5% Human AB serum, 2 mM L-Glutamine, 25 mM HEPES, 30 ng/mL of anti-CD3 (OKT3 clone, ThermoFisher Scientific) and 3,000 IU/mL rhIL-2. Half media changes were performed every 3 to 4 days with fresh media with IL-2 (3,000 IU/mL) for 14 days. On Day 28, cells were harvested, counted, and downstream assays were conducted, or cryopreserved in LN2 till used.

Characterization of Generated TILs using Flow Cytometry

Phenotypic analysis of TILs: single cell suspensions were stained with a master mix of antibodies (Abs) for surface stains after blocking with 10 µL of FcR blocking reagent (Miltenyi Biotec) for 10 min at room temperature (RT). The following Abs were used, anti-CD3-FITC (Biolegend, Cat# 300415), anti-CD4-PerCP (Biolegend, Cat# 344608), anti-CD8-V500 (BD Biosciences, Cat# 560774), anti-PD-1-BV421 (BD Biosciences, Cat#564323), anti-4-1BB-PE (Biolegend, Cat# 309804), anti-TIGIT-PE-Cy7 (Biolegend, Cat# 372714), anti-CD39-APC (BD Biosciences, Cat# 560239), anti-CD69-APC.Cy7 (BD Biosciences, Cat# 560737). Live/Dead Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific, Cat# L34959) was used to exclude dead cells from the analysis. Samples were fixed with 2% paraformaldehyde.

Functional analysis of TILs: single cell suspensions were incubated with Protein Transport Inhibitor Cocktail (eBioscience, Cat# 00-4980-03), anti-CD107a-PE-Cy7 (BD Biosciences, Cat# 561348) with/without Cell Stimulation Cocktail (eBioscience, Cat# 00-4970-03) for 4 hours at 37°C. After 4 hours incubation, cells were washed, and stained with a master mix of Abs for surface stains after blocking with 10 µL of FcR blocking reagent (Miltenyi Biotec) for 10 min at RT. The following Abs were used: anti-CD3-FITC (Biolegend), anti-CD4-PerCP (Biolegend), anti-CD8-V500 (BD Biosciences), anti-CCR7-APC (Biolegend, Cat# 353214), anti-CD45RA-APC-H7 (Biolegend, Cat# 304128). After incubation, cells were permeabilized with BD Cytofix/Cytoperm (BD Biosciences, Cat# 554722) for 20 min on ice, washed twice with BD Perm/Wash buffer (BD Biosciences, Cat# 554723), then intracellular cytokines were stained with anti-IFN-γ-BV421 (BD, Cat# 562988) and TNF-α-PE (BioLegend, Cat# 502909). Live/Dead Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific, Cat# L34959) was used to exclude dead cells from the analysis.

All the samples were acquired using LSRFortessa (BD) and analyzed with FlowJo software (TreeStar).

ELISA

Cryopreserved cells were thawed using assay media [RPMI with 10% FBS (R&D Systems) and 2mM L-Glutamine]. The viable 8E4 cells were transferred to a well in 200 µL with/without stimulation in a 96-well plate. CD3/CD28

Dynabeads (Gibco, Cat#11131D) were added at a ratio 2:1 Beads: Cells. After 24 hours of incubation, the plate was centrifuged, and supernatants were collected and used immediately for the ELISA assay or frozen at -80^{o}C until used. IFN-γ levels were measured using Human IFN-gamma Quantikine ELISA kit (R&D Systems, Cat# SIF50C) according to the manufacturer's instructions.

Statistical analysis

Principal component analysis (PCA) was performed using a truncated singular value decomposition method [9] implemented in the R package of irlba (version 2.3.5). Pearson correlation coefficients were calculated using the R function, cor, from the package of stats (version 4.0.3). The heatmaps with hierarchical trees are drawn using the R function, pheatmap in the package of pheatmap (version 1.0.12) using euclidean distance metric and complete linkage rule.

Statistical analyses were performed using Excel 16.69 (Microsoft). Paired *t*-tests were performed using Excel 16.69 (Microsoft). *P*-values less than 0.05 were considered to be statistically significant.

Results

Pre-surgery chemotherapy shows no impact on in vitro TIL generation and expansion

Recent work revealed successful TIL generation from patient samples with penile cancer [7], however, there has been no evidence of treatment effect on TIL generation in other indications. Therefore, it remains unclear whether TIL can be initiated from patient samples with other cancer types after chemotherapy. To verify this, we generated TILs from tumor tissues with 17 colorectal and 5 lungs, including 9 metastatic cancers (Table 1). The tissue weight varied from 50 mg to 2,560 mg. Therefore, we normalized the number of TIL per mg of tissue to be able to compare samples. First, we sought to determine the correlation between the number of TILs generated and the time period between chemotherapy and surgical resection. We observed that there was no significant correlation between TIL numbers and the length of time after chemotherapy (R^2= 0.2695, Supplemental Figure 1). We then examined the cell numbers and expansion rate with/without chemotherapy. The average number of TIL per mg of tumor tissue was 3.9E+5 cells for patients with chemotherapy prior to resection and 4.7E+5 for patients without chemotherapy before surgery (Figure 1A). The mean and SD of expansion rate of the TILs that were generated from tissues with and without chemotherapy were 23 ± 20 times and 27 ± 32 times, respectively (Figure 1B). These generated TILs were proceeded to REP, then subsequently used for further analyses.

TILs display similar phenotypic profiles regardless of pre-surgery chemotherapy

Next, we performed flow cytometric analysis to further characterize TILs derived from chemotherapy treated and untreated samples. Expanded TILs exhibited predominantly CD3+ T cells, average 94.6% with chemotherapy and 85.6% without chemotherapy (Figure 2A). In the CD3+ T cell populations, CD4+ T cells were dominant with an average of 68.2% and 65.3%, treatment+ and treatment naive, respectively (Figure 2B) while CD8+ T cells were 27.4% and 25.6% with and without chemotherapy respectively (Figure 2C). A study has shown that TIL with stem-like phenotype (CD8+ CD39- CD69-) were associated with cancer regression and that CD39+ CD69+ TIL have poor persistence [10]. We examined our TILs and found that there were no significant differences observed in CD39/CD69 double staining between the chemotherapy and no chemotherapy groups (18.2% vs 18.6% for the CD39- CD69-, 32.9% vs 25.6% for the CD39+ CD69+ with and without chemotherapy respectively, Figure 2D). We

then examined the T cell activation marker 4-1BB which was equally expressed on TILs with or without chemotherapy, both in CD4+ (15.4% vs 27.4%) and CD8+ (17.7% vs 21.6%) populations (Figure 2E). Programmed cell death receptor 1 (PD-1) is highly expressed on tumor specific T cells and plays a vital role in immune responses [11, 12]. We found that the expression of our TILs was comparable in CD4+ (14.4% with chemotherapy vs. 23% without chemotherapy, Figure 2F) and CD8+ populations (5.8% with chemotherapy and 11.3% without chemotherapy, Figure 2F). Similarly, T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) which is upregulated by immune cells and expressed by activated CD8+ T and CD4+ T cells [13], is a promising new target for immunotherapy. Our results indicated that it was expressed in 63.3% and 65.1% of CD4+ TILs with and without chemotherapy respectively. TIGIT expression in CD8+ T cell population demonstrated a similarly unremarkable profile of 57.9% with chemotherapy and 61.6% without chemotherapy (Figure 2G). Interestingly, we found that the vast majority of TILs were T effector memory cells (Tem: CD45RA-CCR7-, Figure 2K), and other phenotypes were T central memory (Tcm: CD45RA-CCR7+, Figure 2J), Naïve-like-phenotype T cells (CD45RA+CCR7+, Figure 2I), and Terminally Differentiated Effector Memory (TEMRA: CD45RA+CCR7-) T cells (Figure 2H).

Finally, we confirmed TILs with and without chemotherapy were undisguisable when combining the effects of all surface marker expressions. PCA was performed to search for a multi-variable pattern based on the expression of 10 surface markers. Figure 3 shows that TILs with and without chemotherapy are not separable by top 2 principal components (PCs) that explain 64.1% of total variances. Pearson correlation coefficients between each pair of surface marker expressions are shown in Supplemental Figure 2A. The proportions of total variance are explained by top 10 PCs are shown in Supplemental Figgure 2B. In addition, we also examined the correlations between top 3 PCs with each surface marker in Supplemental Figure 2C. PC1 is negative correlated with CD4+PD-1+ (Pearson correlation = -0.92) and CD4+4-1BB+ (-0.94) and positively correlated with CD8+TIGIT+ (0.83) and CD8+CD69+(0.77).

TILs exhibit comparable cytokine producing capacity irrespective of chemotherapy status

We confirmed that chemotherapy did not change TIL phenotypes and then sought to examine their functionality. The cells were harvested and stimulated with anti-CD3/anti-CD28 coated beads for 4 hours in the presence of protein transport Inhibitor and anti-CD107a antibody. Anti-CD107a was used as a marker for degranulation. As expected, significant amounts of IFN-g,TNF-a., and CD107a were detected. There was no statistically significant difference between treated and nontreated group in both CD4+ and CD8+ populations (Figure 4A and 4B, respectively). To confirm this, the production of IFN-g was quantified using ELISA (n=4 for the group “with chemotherapy” and n=6 for the group “without chemotherapy”). The mean and SD of secreated IFN-g from with and without chemotherapy were 6,989 ± 4,017 pg/mL and 12,379 ± 10,328 pg/mL, respectively (Figure 4B). Hence, we confirmed that systemic treatment did not change TIL functionality.

Discussion

In this study, we demonstrated successful TIL generation and expansion from tissues of patients with varies cancer types who received chemotherapy as little as 14 days before surgical resection. These TILs possessed phenotypes and functionalities similar to treatment naïve TILs. Exceptionally, we included metastatic cancers as well as primary cancers from multiple indications and found that functional TILs could be expanded from

multiple indications in both primary and metastatic sites. This would be beneficial to patients with metastasized sites as well as patients who received chemotherapy prior to surgical resection.

One of the crucial factors for the success of TIL therapy is TIL phenotype, especially the CD8+: CD4 + ratio and their ability to recognize and kill tumor cells. CD8 + T cells are known as cytotoxic T cells and play an important role in tumor killing; however, CD4 + TIL have a supportive role that is essential for antitumor properties [13–14]. The balance between CD8 + and CD4 + TILs could be a key factor for their persistence and their cytotoxic effects in tumor microenviroment. It remains unclear in the prevailing literature as to which TILs are the most important against solid tumors. Krishna *et al.* showed that TILs with stem-like phenotype (CD39 CD69-CD8+) were associated with cancer regression and CD39 + CD69 + TIL had poor persistence [10]; den Bulk and others indicated the importance of CD39 + CD103 + as neoantigen-specific cytotoxic T cells [15–16]. This will need to be futher investigated with clinical trial samples and correlation with their clinical outcome.

To further develop TIL therapy, it could be effectively paired with agonistic antibodies like Urelumab, which targets CD137 (4-1BB) [17] to enhance expansion rate and elevate CD8 + TIL in the product. Additionally, Pearce *et al.* demonstrated that the combination of PD-1 and TIGIT checkpoint blockade can significantly overcome the immunosuppressive effect of PD-1 and TIGIT engagement on T cell activity in pancreatic ductal adenocarcinoma [18].

Our study suggests that TIL can be generated and expanded from cancer patients who have received systemic therapies prior to surgical resection. These findings provide a possibility to extend inclusion criteria to enroll cancer patients with the most recent chemotherapy treatment for TIL therapy.

Declarations

Acknowledgments

We acknowledge CHTN for tissue procurement. We thank all patients, staff and investigators at the CHTN network. We thank Drs. Fuli Yu and Imtiaz Yakub for technical assistance and Mrs. Jing Zhong for administrative coordination.

Author Contributions

JB, AR performed experiments, analyzed data, and wrote the manuscript. WX and HY contributed the bioinformatics analyses and revised the article. JM and AL performed experiments and analyzed data. SM developed the concept, analyzed data, managed the project, coordinated author activities, revised the article, and provided final approval of the version to be submitted.

Disclosure

Nothing to disclose.

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Figures

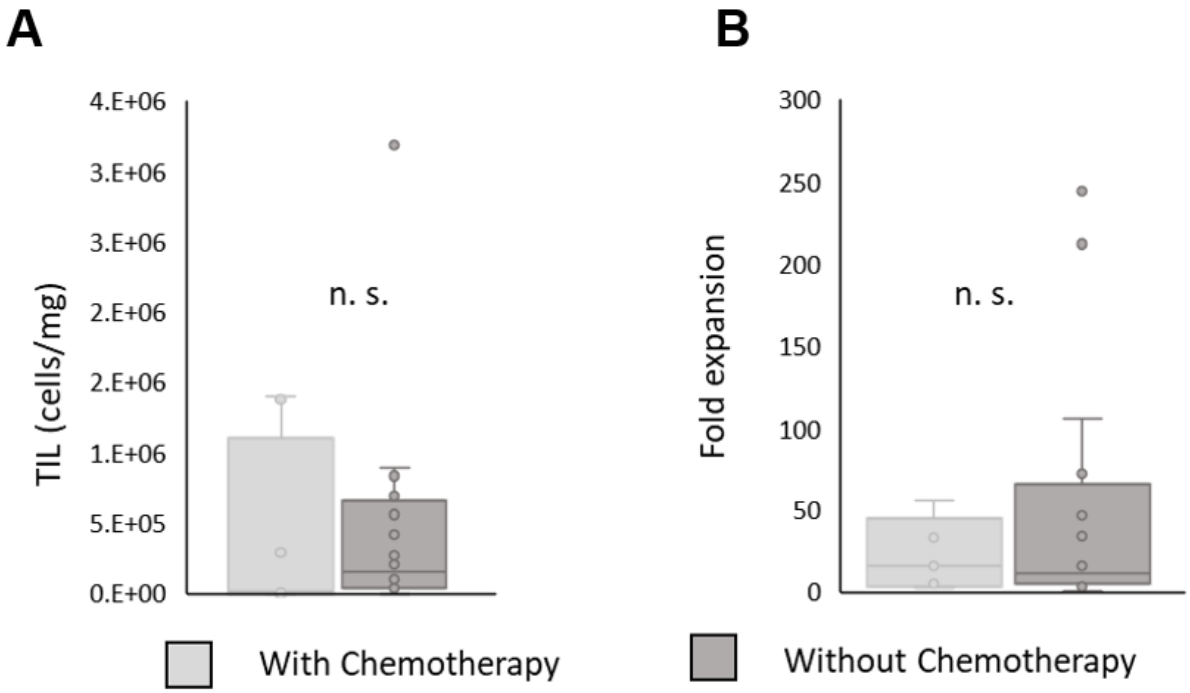

Figure 1

Effects of systemic therapy on TIL generation and expansion in vitro

(A) TIL number per mg of tumor tissue with/without chemotherapy was monitored. (B) Fold change of TILs with or without preliminary chemotherapy was calculated. Data points indicate individual patients (n=21). n. s.: not significant.

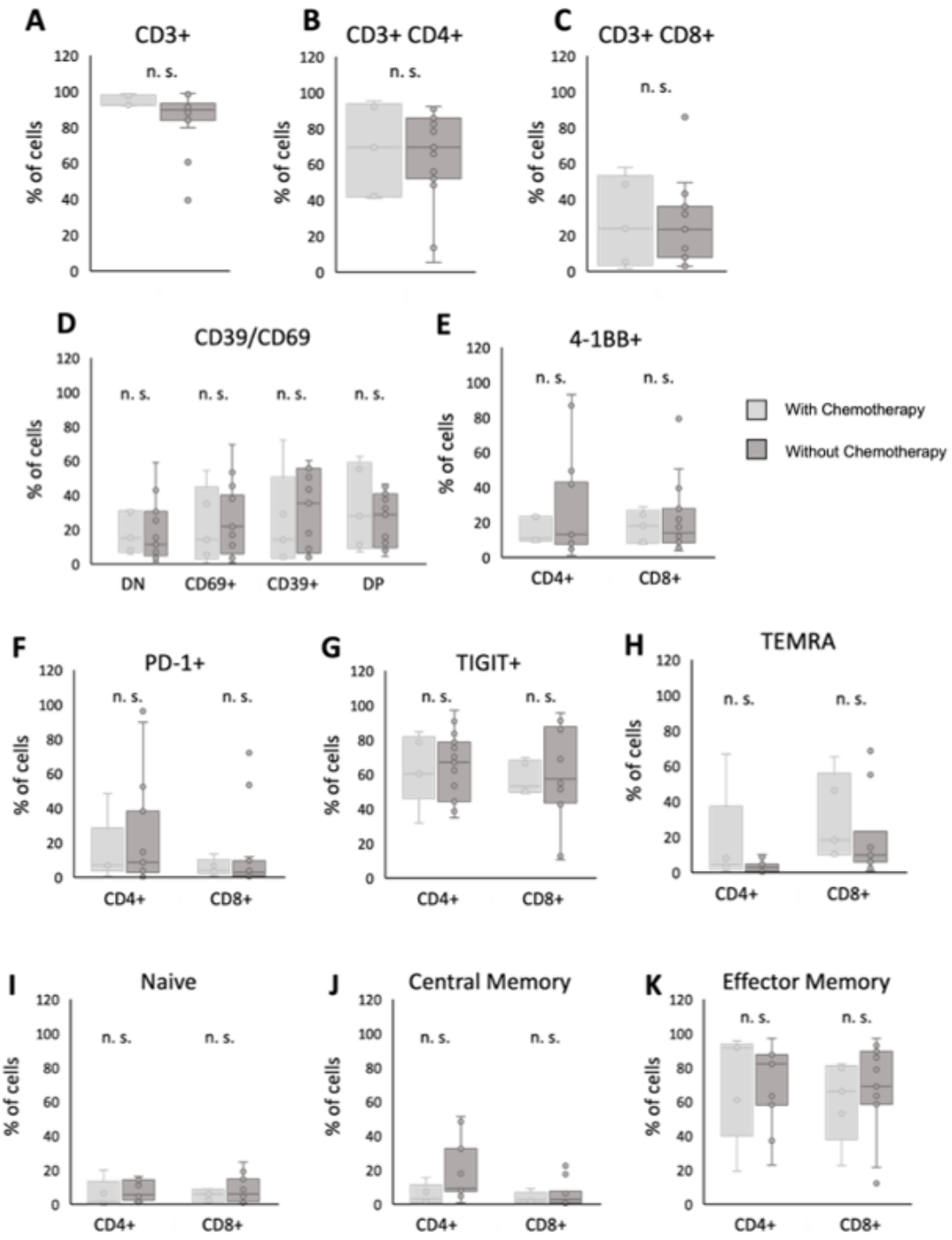

Figure 2

Characteristics of TILs generated with or without chemotherapy

(A) Generated TILs were dominantly CD3+ T cells, and CD4+ T and CD8+ T cells ratio were shown (B and C, respectively), (D) double-positive (DP) and double-negative (DN) CD39/CD69, (E) expression of 4-1BB, (F) PD-1, and (G) TIGIT (n=21). Cells were stained with CD45RA and CCR7 to determine the percentage of memory phenotypes, (H) TEMRA (CD45RA+ CCR7-), (I) Naïve-like-phenotype T cells (CD45RA+, CCR7+), (J) Central Memory (Tcm, CD45RA-, CCR7+) and (K) Effector Memory (Tem, CD45RA-, CCR7-), in CD4 and CD8 populations (n=16). n. s.: not significant.

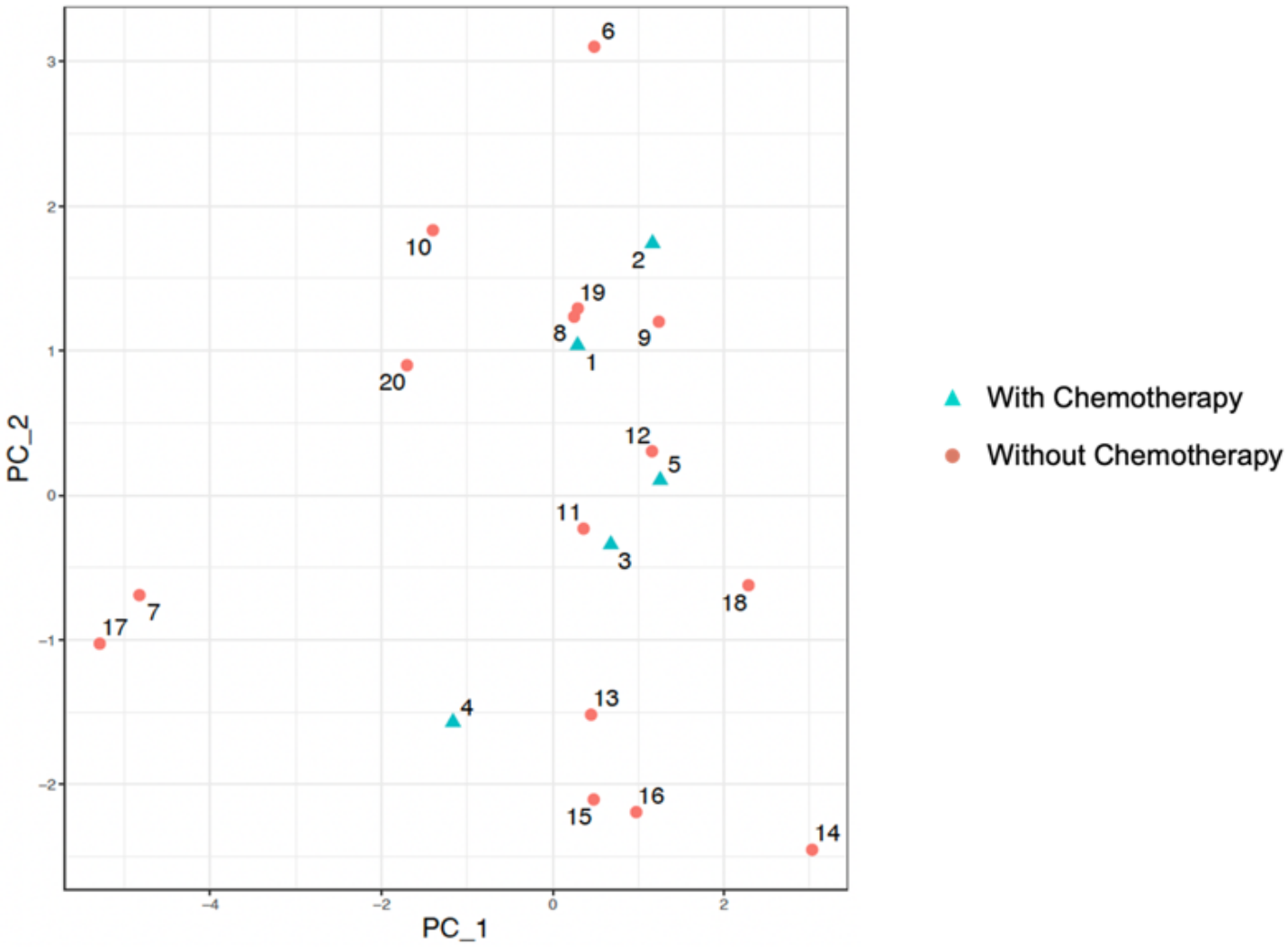

Figure 3

PCA of all surface marker expressions.

TILs without chemotherapy are not deviated from those with chemotherapy in a multi-marker-expression analysis. PC1 and PC2 explains 44.0% and 20.1%, respectively, of total variance within expressions of 10 surface markers and do not separate TILs with or without chemotherapy.

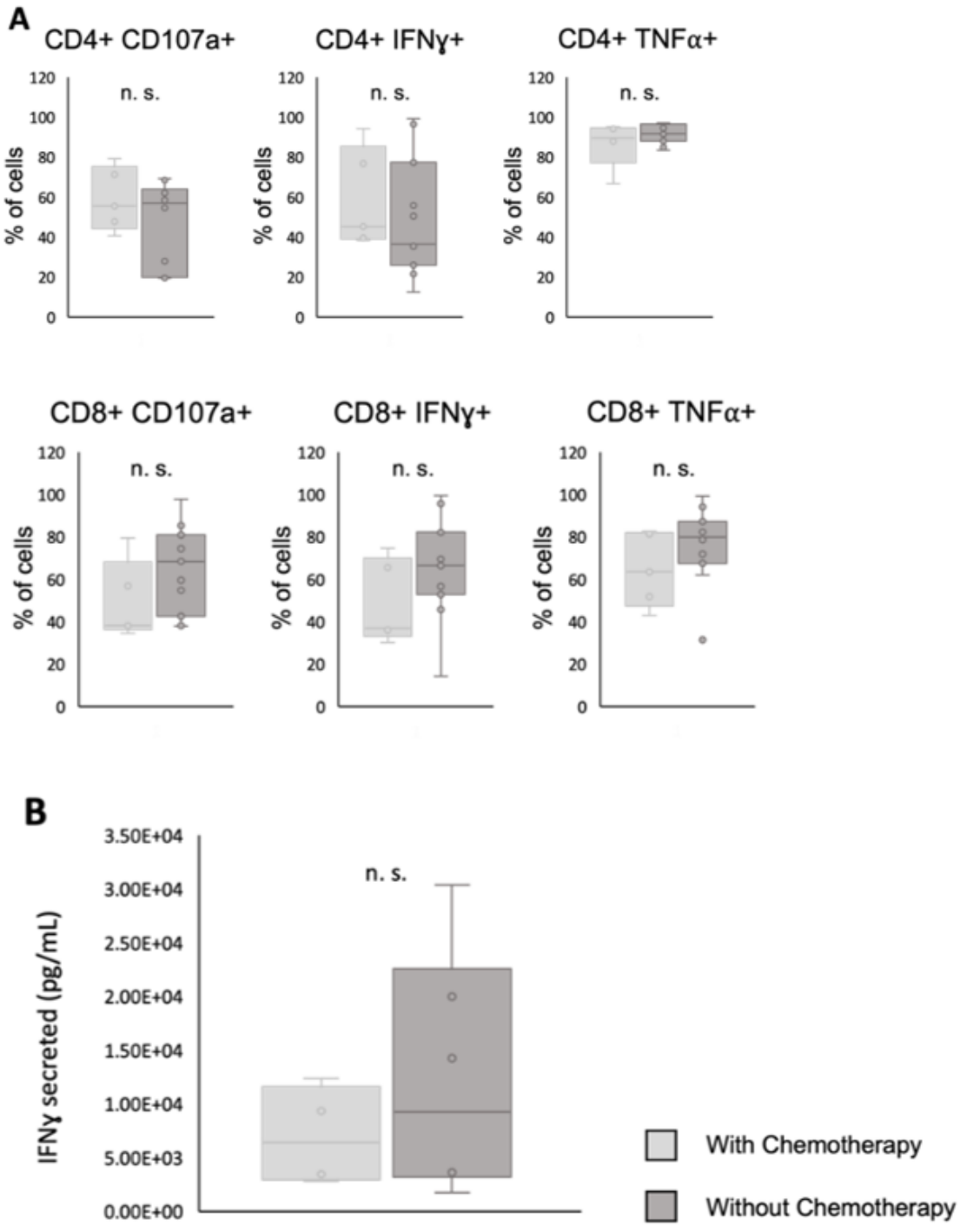

Figure 4

Analysis of TIL capability to produce cytokines

(A) Percentage of the secretion of CD107a, IFN-g and TNF-a in the CD4+ T and CD8+ T cell populations. Values from the unstimulated cells were subtracted from the stimulated as background for this analysis (n=16). (B) Secreted IFN-g was quantified by ELISA from the TILs with or without chemotherapy prior to surgery (n=10). n. s.: not significant.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- S1.png
- S2.png