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Dynamics of Tumor-infiltrating Lymphocytes During Neo-Adjuvant Chemotherapy in Ovarian Cancer

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The Graduate School, Yonsei University

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Dynamics of Tumor-infiltrating Lymphocytes During Neo-Adjuvant Chemotherapy in Ovarian Cancer

Directed by Professor Jung-Yun Lee

The Master's Thesis
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Master of Medical Science

Yup Kim

December 2021

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December 2021

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ACKNOWLEDGEMENTS

In suffering corona pandemic era, it is my deepest regret that I never attended classes off-line. 2 years maybe long or short depend on what one may see, but for me it was quite short.

I am truly thankful to Professor Jung-Yun Lee. Professor led me to the world of scholars. I also present my gratitude to professor Junsik Park, who kindly taught me how to survive in a wet lab. Professor Su-Hyung Park gave me pierce advices in writing this thesis. Although always very busy, professors spared their precious time and care for me.

I also want to thank my family. My parents in law, who supported and helped me all along writing this thesis, my parents, who gave birth to me and raised me, literally fed me were always behind my back. My beloved daughter, Eunha, was born in Feb 2021, in my graduation school days. She was a heaven-sent gift who always made me happy.

Lastly, I would like to thank my husband, Junhee Lee. He was always supportive and helped me along the whole course. Without him, I could not even dream of starting something new, such as master's degree.

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ABSTRACT

Dynamics of Tumor-infiltrating Lymphocytes During Neo-Adjuvant Chemotherapy in Ovarian Cancer

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(Directed by Professor Jung-Yun Lee)

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Ovarian cancer has the highest fatality-to-case ratio of all the gynecologic malignancies. There is no effective way of early detection and 75% are diagnosed in advanced cases. Prognosis is poor. In most advanced cases, neoadjuvant chemotherapy is done before optimal surgery. Owing to bone marrow toxicity, chemotherapy was expected be generally immunosuppressive. But studies found that chemotherapy change tumor microenvironment. Recently, the immunotherapy utilizing PD-L1 and PD-1 blockades have become the most promising treatment modality for numerous types of cancer. This study aims to identify whether and how TILs change after neoadjuvant chemotherapy and what consequence it might bring.

Among samples, we chose 15 pair of samples which were collected both before and after neoadjuvant chemotherapy. We also chose unpaired samples- 14 samples not treated with chemotherapy, and 11 samples after the neoadjuvant therapy. Each sample was divided to perform 1. staining without

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stimulation and 2. Staining after intracellular cytokine stimulation.

As a result, CD8+ TILs increased after NAC, whereas CD4+ TILS and Treg decreased after NAC. CD8/Treg ratio and conventional CD4+/Treg ratio increased after NAC. Suppressive effector Treg cells were decreased after NAC. Expression of 4-1BB were decreased, but other immune checkpoints were not changed in CD8 TILs and CD4 TILs after NAC. Proliferative Ki-67+ TILs were decreased after NAC. TCF1 was increased after NAC. TOX was decreased after NAC. Staining after intracellular cytokine stimulation, samples in pair did not show significant change associated with NAC.

T-cell exhaustion is characterized by the stepwise and progressive loss of T-cell functions and can culminate in the physical deletion of the responding cells. TCF-1 and TOX are important markers of T-cell exhaustion. Increasing TCF-1 and decreasing TOX shows TILs become less exhausted after neoadjuvant chemotherapy. Cytokines in T cells generally increased after NAC but was not statistically significant.

In conclusion, neoadjuvant chemotherapy makes T cell less exhausted and restores cytokine secreting capacity, leading to better tumor microenvironments

Key words: advanced ovarian cancer, neoadjuvant chemotherapy, immune population, immune checkpoint inhibitor

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Dynamics of Tumor-infiltrating Lymphocytes During Neo-Adjuvant Chemotherapy in Ovarian Cancer

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(Directed by Professor Jung-Yun Lee)

I. INTRODUCTION

1. Research background

A. Introduction to ovarian cancer

In Korea, ovarian cancer occurs approximately 2500 case each year an 5 year survival rate is around 60% in recent 10 years.(1) Epithelial cancers are the most common ovarian/fallopian malignancy, and at initial diagnosis, over two-thirds of patients have advanced disease. Ovarian cancer represents a major surgical challenge as the aim is to resect all visible disease. Optimal therapy includes maximal attempt at surgical cytoreduction to no gross disease, or at least optimal debulking (to <1 cm of residual disease), followed by platinum-based combination chemotherapy. It has the highest fatality-to-case ratio of all the gynecologic malignancies. There is no effective way of early detection and 75% are diagnosed in advanced cases, prognosis is poor. Advanced cases show 30% of 5-year survival rate.

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B. Introduction to neoadjuvant chemotherapy

Neoadjuvant chemotherapy is chemotherapy performed prior to cytoreductive surgery. Studies showed that patients with disease progressed more than stage III showed no difference in 5-year survival rate between standard care and 2-3 rounds of neoadjuvant chemotherapy prior to the optimal debulking surgery.(2) If performance status is poor, mortality is high in surgery, or optimal debulking is hard, Neoadjuvant chemotherapy is considered after biopsy. Neoadjuvant chemotherapy led to decrease in pleural effusion/ascites and performance status was better leading to decrease in postoperative morbidity.

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C. Previous study on neoadjuvant chemotherapy induced change

Chemotherapy was expected be immunosuppressive, owing to bone marrow toxicity, but recent studies found that chemotherapy change tumor microenvironment. Chemotherapy augments pre-existing TIL responses but fails to relieve major immune-suppressive mechanisms or confer significant prognostic benefit.(3) TILs and PD-L1 expression increase following NACT.(4) In ovarian cancer, chemotherapy modulates tumor microenvironment by T cells trafficking, neo-antigen presentations etc. resulting in better prognosis of immunotherapy. Neoadjuvant chemotherapy in ovarian cancer was focused on difference in survival compared to primary optimal debulking. Neoadjuvant chemotherapy of ovarian cancer reduces regulatory T cells and increase infiltrations and activation of CD8 + Tumor infiltrating lymphocytes (TILs). In EOC, TILs levels are prognostic at diagnosis and remain prognostic after NACT. TILs and PD-L1 expression increase following NACT.(4)

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D. Introduction to immune therapy

Basic science discoveries elucidating the molecular and cellular biology of the T cell have led to new strategies in this fight, including checkpoint blockade, adoptive cellular therapy, and cancer vaccinology. The T lymphocyte, especially its capacity for antigen-directed cytotoxicity, has become a central focus for engaging the immune system in the fight against cancer. (5) Immune checkpoint inhibitor, most notable anti-PD-1, changed the paradigm of cancer treatment. To date, the immunotherapy utilizing PD-L1 and PD-1 blockades have become the most promising treatment modality for numerous types of cancer, including melanoma, lung cancer, kidney cancer, bladder cancer, head and neck cancers, and Hodgkin's lymphoma. (6)

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E. Immune exhaustion and associated transcriptional factor

T cells are key mediators of antitumor function that specifically recognize and react to tumor-expressing antigens and have proven critical for cancer immunotherapy. T cell exhaustion is defined T cells entering a dysfunctional or exhausted state, which is characterized by sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells.(7) Exhausted T cells have a unique molecular signature that is markedly distinct from naive, effector or memory T cells. Reversing exhausted T cells and restoring anti-tumor potential represents an inspiring strategy to treat cancer. (8)

Exhausted CD8 T cells comprise heterogeneous cell populations, with TCF1 being an important determinant in this heterogeneity. Functionally, TCF1 terminally exhausted tumor-infiltrating CD8 lymphocytes (TILs) expressed more interferon-γ (IFN-γ) and granzyme B, TCF1+ stem-like TILs were more polyfunctional. TCF1 is critical for the development of stem-like progenitor cells. TCF1 appears to be a critical regulator during the progressive differentiation to exhausted CD8 T cells. (9). TCF1+ T cells represent a unique precursor population that is essential for long-term maintenance of T cell responses to viral infections and tumors and that is critical for the success of PD1 inhibitor therapies. (10)

Transcription factor thymocyte selection-associated high mobility group box (TOX) appears to be an essential regulator for inducing exhausted T cells in general. TOX, downstream of NFATC1, is emerging as a central regulator of T cell exhaustion. TOX appears to be specifically expressed in chronically activated T cells. (10) Robust expression of TOX results in commitment to Tex cells by translating persistent stimulation into a distinct Tex cell transcriptional and epigenetic developmental program. (11) TOX inhibition can potentially

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impede T cell exhaustion and improve ICI efficacy.(12)

In conclusion, ovarian cancer is immunogenic tumor, and immunotherapy can be one strategy, but highly sophisticated immunosuppressive network hinders from effective management. Over decades, ovarian cancer treatment scheme was not as rapid progressive as other cancers. Emergence of immunotherapy including immune checkpoint inhibitors is a big hope for the patients. In vitro study using cancer cell lines or mouse model showed neoadjuvant chemotherapy resulting in change of immune system, but it is still doubtful whether real ovarian cancer patient will show the same result. In this regard, study whether neoadjuvant chemotherapy influence on tumor microenvironment and in real in vivo setting is needed.

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Ⅱ. MATERIALS AND METHODS

1. Patients and lymphocyte isolation

Patients diagnosed with high grade serous ovarian cancer from April 2019 to June 2021 at Severance Hospital in Seoul, Republic of Korea, were enrolled in the study. Demographic is provided in Table 1. Tumor tissues and peripheral blood were collected on the day of resection. Tumor infiltrating lymphocytes were isolated from tumor tissue by centrifugation in Percoll (GE healthcare, Uppsala, Sweden) and cryopreserved. This study was approved by Institutional Review Board, and all enrolled patients agreed in participating the study with informed consent. This study was conducted in accordance with Declaration of Helsinki.

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Table 1. Clinical information of the advanced ovarian (high grade serous carcinoma) cancer patients.

Pt	Age	Sample	Origin	stage	BRCA	CRS1	CRS2	CRS3	Resid	Pre TM	Post TM	Study enrolled
1	44	Pair	ovary	IIIC	wild	2	2	2	<5mm	670	78.3	NCT03522246, KGOG 3042
2	79	Pair	tube	IVB	wild	3	2	2	R0	2055	68.4	KGOG 3042
3	72	Pair	ovary	IIIC	VOUS	2	2	N/A	<5mm	387	27.2	KGOG 3042
4	62	Pair	ovary	IVB	N/A	2	2	2	R0	1258	38.1	
5	63	Pair	ovary	IVB	N/A	2	3	3	R0	4109	24	MK7339_001
6	43	Pair	ovary	IVA	BRCA1	2	2	2	<5mm	10579	138	KGOG 3042
7	56	Pair	ovary	IIIC	wild	2	2	2	<5mm	1153	57.6	
8	56	Pair	ovary	IVB	wild	3	x	2	R0	1719	26.5	
9	73	Pair	ovary	IVA	BRCA1	2	x	2	R0	1807	103	KGOG 3042
10	67	Pair	ovary	IVB	BRCA2	2	2	2	<5mm	1733	146	
11	60	Pair	ovary	IVB	wild	2	2	2	0	2283.3	132.9	NCT03737643
12	73	Pair	ovary	IVB	wild	3	2	2	R0	3280	73	MK7339_001
13	66	Pair	ovary	IIIB	wild	N/A	N/A	N/A	R0	26.8	24.1	
14	60	Pair	ovary	IVB	wild	1	1	1	R0	622	58.6	
15	75	Pair	ovary	IVB	BRCA1	2	2	3	R0	1262	11.8	

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16	60	Pre	ovary	IIIA	BRCA1	N/A	N/A	N/A	R0	252	N/A	
17	55	Pre	ovary	IIIB	wild	N/A	N/A	N/A	R0	138	N/A	MK7339_001
18	67	Pre	ovary	IVB	wild	N/A	N/A	N/A	R0	210	N/A	MK7339_001
19	50	Pre	ovary	IIIB	BRCA2	N/A	N/A	N/A	R0	334	N/A	
20	56	Post	ovary	IVA	BRCA2	3	3	2	<5mm	868.3	23.1	NCT03737643
21	51	Post	ovary	IVA	wild	2	2	3	R0	11719.9	21.3	
22	48	Post	ovary	IVA	BRCA1	2	2	2	R0	12642.9	247	KGOG 3042
23	52	Post	ovary	IVB	wild	2	N/A	1	R0	3931.6	147.1	NCT03522246
24	44	Post	ovary	IIIC	VOUS	2	2	2	R0	752	6.1	
25	61	Post	ovary	IV	BRCA2	3	2	N/A	R0	1647	14.3	
26	55	Post	ovary	IIIB	BRCA1	2	2	2	R0	665	23.9	
27	48	Post	ovary	IVB	BRCA1	2	2	2	R0	2203	88.6	
28	57	Post	ovary	IVB	wild	N/A	N/A	N/A	D-lapa	895	57.3	MK7339-001
29	74	Post	tube	IVB	wild	2	2	2	R0	139	40.2	
30	60	Post	ovary	IVB	wild	2	2	2	<5mm	>300	148.5	
31	56	Pre	ovary	IVB	wild	3	2	2	R0	23844.6	23.6	KGOG3046
32	67	Pre	ovary	IIIC	wild	3	3	3	R0	1773.7	15.2	KGOG3046
33	62	Pre	ovary	IVB	N/A	3	3	3	R0	50.1	13.6	KGOG3046
34	59	Pre	ovary	IVB	BRCA1, BRCA2	3	3	2	R0	529	15	KGOG3046

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35	43	Pre	ovary	IVB	wild	2	2	2	R0	782	70	KGOG3046
36	68	Pre	ovary	IVA	wild	3	3	3	R0	573	28	KGOG3046
37	54	Pre	ovary	IVB	wild	3	3	2	N/A	1139	13.4	KGOG3046
38	56	Pre	ovary	IVB	wild	2	1	1	<5mm	3767	769	KGOG3046
39	60	Pre	ovary	IVB	wild	3	3	3	R0	13.3	9.6	KGOG3046
40	51	Pre	ovary	IVB	wild	1	1	1	<5mm	851	113	KGOG3046

Pt = patient

BRCA = BRCA (Breast Cancer Susceptibility Gene, 1 and 2) mutation

CRS1 = Chemotherapy response score 1: Omentum

CRS2 = Chemotherapy response score 2: Right ovary

CRS3 = Chemotherapy response score 3: Left ovary

Resid = Residual tumor

PreTM = Tumor marker (CA125) before NAC

PostTM = Tumor marker (CA125) after NAC

Study enrolled = Clinical study enrolled

Tube = Fallopian tube

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2. Sample preparation - Cell thawing

Cryotube was put in 37℃ hot water bath for 5 minutes. DNase I (50×) 40μl was put in cryotube and 1ml of PBS (total volume of 2ml in each cryotube) was added. After 5 minutes, we gently transfer the cells to 15ml conical tube. We washed cryotube with 2ml PBS to ensure no cell left in cryotube. We counted cells and split then into 2 groups. Group 1 was to perform FACS right after the thawing and group 2 for intracellular staining. For group 2, I added PBS to make total volume of 5-10ml and centrifuge 300g 10 minutes. I resuspended with RPMI 10% and rest cells in 36°C incubator.

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3. Flowcytometry and immunostaining

FACS analysis using anti-PD1, CD3, Ki-67, CD4, CD39, Granzyme B, TOX, FOXP3, TCF-1 and CD8 were done. Briefly, the prepared cells were washed with FACS buffer (PBS containing 2% FBS) and then surface-stained at 4°C in the dark for 30min with the following antibodies: BV 421-labeled anti-PD-1 (BioLegend, CA, USA), BV 510 labeled anti-CD3 (BD, USA), BV786 labeled anti CD4(BD), BB515-labeled anti-CD39 (Ber-ACT8; BioLegend), AF-700 labled anti-CD8 (BD). Dead cell staining was performed using APC-Cy7-labeled live/dead™ fixable near-IR stain. Intracellular staining followed fixation and permeabilization (fixation/permeabilization buffer kit; eBioscience, San Diego, CA, USA). Cells were intracellular-stained at 4°C in the dark for 30min with BV605-labeled anti-ki-67 (BioLegend), PercP-Cy5.5-labeled anti-granzymeB (BioLegend), PE-labeled anti-TOX (eBioscience), PE-Cy7-labeled anti-FOXP3 (eBioscience), APC-labeled anti-TCF-1 (DX2; BioLegend).

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Table 2. Resources used in the experiments

REAGENT or SOURCE	SOURCE	IDENTIFIER
Antibodies		
BV 421-labeled anti-PD-1	BioLegend	Cat#329920
BV 510 labeled anti-CD3	BD Bioscience	Cat#564713
BV786 labeled anti CD4	BD Bioscience	Cat#563877
BB515-labeled anti-CD39	BioLegend	Cat#565469
AF-700 labeled anti-CD8	BD Bioscience	Cat#557945
BV605-labeled anti-ki-67	BioLegend	Cat#350522
PercP-Cy5.5-labeled anti-granzymeB	BioLegend	Cat#372212
PE-Cy7 labeled anti-FOXP3	eBioscience	Cat#25-4776-42
APC-labeled anti-TCF-1	BioLegend	Cat#37636
BV421 labeled CCR8	BD Bioscience	Cat#566379
BV605 labeled CD45RA	BD Bioscience	Cat#566897
FITC-labeled anti-TNFα	BioLegend	Cat#502906??
PE-Cy7-labeled anti-IL17A	eBioscience	Cat#25-7179-42
PE-labeled anti-TOX	BioLegend	Cat#12-6502-82
APC-labeled anti-IFNɣ	BioLegend	Cat#502512
Chemicals and peptides		
Live/dead™ fixable near-IR stain.	ThermoFisher	L34976
Permeabilization buffer	Invitrogen	Cat#00-8333-56
DNase I (50×)	Morthington-Biochem	#LS002138
PBS	Cytiva	SH30028.02
RPMI 10%	Cytiva	SH30027.01
FACS buffer (PBS containing 2% FBS)		
Permwash	Invitrogen	Cat#2333698
αCD3	BioLegend	Cat#317326
αCD28	BioLegend	Cat#302934
Brefeldin	BioLegend	Cat#420601
Monensin A	BioLegend	Cat#420703
Software		

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FlowJo software (Tree Star, OR, USA).		N/A
Prism		N/A
Others		
FACSLyric	BD Bioscience	N/A
96well plate	Nonpyrogenic	

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4. Stimulation and intracellular staining

A. Well preparation for Intracellular cytokine staining

I prepared 2 wells for each sample. Dilute αCD3 1mg/mL to 400ng/mL, so that final working dose could be 100ng/mL and well contain 50μL. I diluted αCD28 1mg/mL to 4μg/mL, so that final working dose could be 1μg/mL and well contain 50μL. For control, I put RPMI 50μL, and put αCD3 50μL αCD28 50μL in the other well.

B. Incubation and harvesting

I counted cells and make density of each tube to 1×10^6 cells per 100μL. I put 50μL (0.5×10^6 cells) in control well and put 100μL (1×10^6 cells) in experimental well. After 1 hour of resting, put Brefeldin A (GolgiPlub, BD Bioscience) 1μL and Monensin (GolgiStop, BD Bioscience)1μL to each well. After 5 hours resting in 37°C in a 5% CO_2 incubator, cells were harvested.

C. FACS Staining

After 6 hours of resting, live/dead, surface, and intracellular staining were performed. Prepared single cells were washed with FACS buffer (PBS containing 2% FBS) and then surface-stained at 4°C in the dark for 30min with the following antibodies: BV421 labeled CCR8 (BD), BV510-labeled anti-CD3 (BD), BV605 labeled CD45RA(BD), BV786 labeled CD4(BD) AF700 labeled CD8. Dead cell staining was performed using APC-Cy7-labeled live/dead™ fixable near-IR stain. Intracellular staining followed fixation and permeabilization (Fixation/permeabilization buffer kit; eBioscience, San Diego, CA, USA). Cells were intracellular-stained at 4°C in the dark for 30min with 4°C in the dark for 30min with FITC-labeled anti-TNFα (BioLegend), PE-Cy7-labeled anti-IL17A (eBioscience), PE-labeled anti-TOX (UCHL1; BioLegend), APC-labeled anti-IFNγ(BioLegend).

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5. Statistical Analysis

Stained cells were assessed using a FACSLyric (BD Bioscience) and analyzed using FlowJo software (Tree Star, OR, USA). Statistical analyses were performed using Prism software 6 (GraphPad Software, San Diego, CA). All tests of significance were 2-tailed and $P \leq 0.05$ considered significant.

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Ⅲ. RESULTS

1. TIL population changed after NAC

Several studies confirmed that the presence of CD8+ T cells was associated with improved survival in ovarian cancer patients. Although CD4+ T cells are usually non-cytotoxic, they can help recruit and activate CD8+ T cells to contribute to anti-tumor response. Several reports indicate that the presence of CD4+ TILs also correlate with improved outcomes in ovarian cancer patients. (13)

First, I analyzed the population of TILs before and after neoadjuvant chemotherapy. CD8+/CD3+ TILs increased after NAC, whereas CD4+/CD3+ TILS and Treg/CD3+ TILs decreased after NAC. CD8/Treg ratio and conventional CD4+/Treg ratio increased after NAC.

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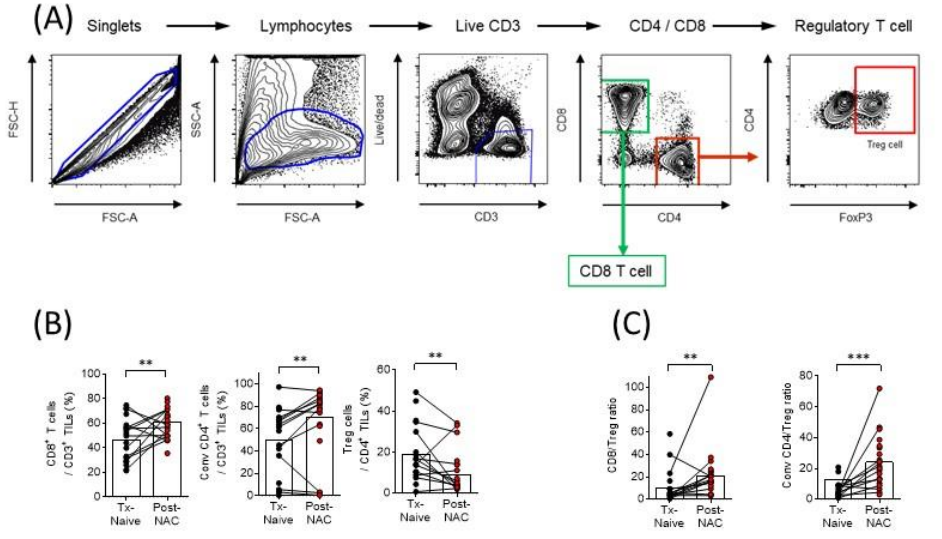

Figure 1. . Gating strategy for flow cytometric analysis and ratio change in TILs.

Gating strategy for flowcytometric analysis of TIL T cells (A). Sample from 30 patients (Pair12, pre7, post11) were collected and analyzed. (B and C). Statistical analysis was performed by the paired t-test or Wilcoxon sign-rank test. ** $P < 0.01$; *** $P < 0.001$.

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2. TIL memory subset changed after NAC

Analyzation by CCR7 and CD45RA was done to clarify change of memory subset differentiation. Differentiate status of CD8 TILs was not changed significantly. There was no significant finding, but trends showed CCR7+CD45RA+ T cells which are naïve T cells decreased and CCR7-CD45RA- T cells, effector memory T cells increased in CD4 and CD8 TILs.

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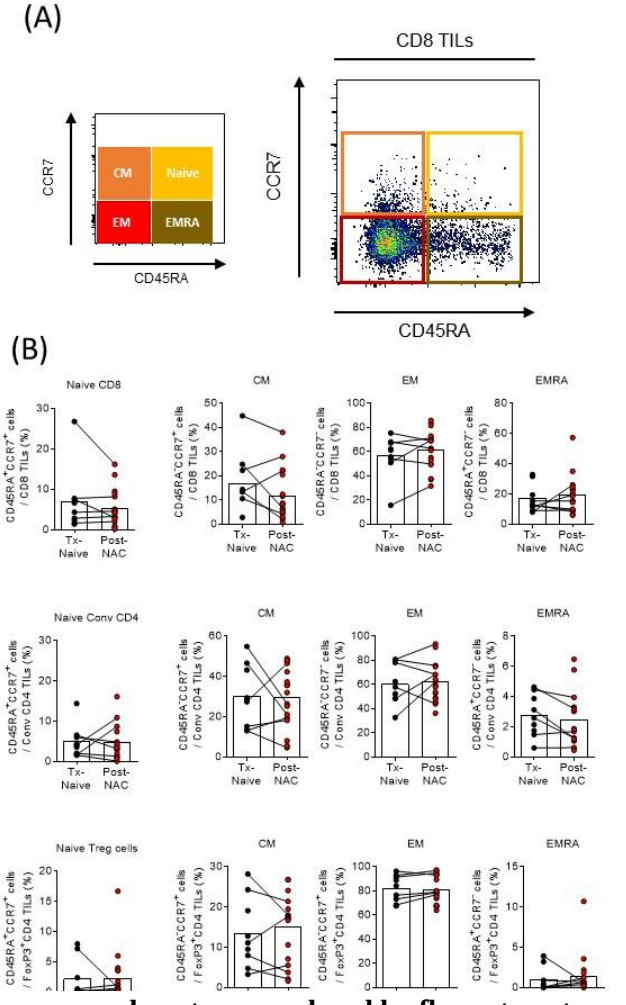

Figure 2. TIL memory phenotypes analyzed by flowcytometry.

TIL memory phenotypes were analyzed using markers CCR7 and CD45RA. Representative plots are presented in (A). CD8, conventional CD4, Treg memory phenotypes were analyzed. (B) Statistical analysis was performed by the paired t-test or Wilcoxon sign-rank test. ns, non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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3. TIL Treg fraction changed after NAC

To see functional change of regulatory T cell, Treg was analyzed by CD45RA and FOXP3. Fraction 1, CD45RA+FOXP3+ naïve Tregs decreased in TILs. FOXP3+ and CD45RA- Effector Tregs increased in TILs.

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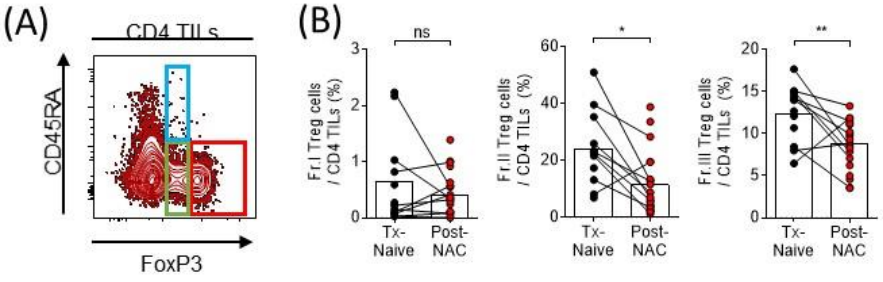

Figure 3 Regulatory T cell fractions analyzed by flowcytometry

Treg fraction was analyzed using markers FOXP3 and CD45RA. Representative plots are presented in (A). Fraction 1,2,3 change in TIL Treg is shown in (B). Statistical analysis was performed by the paired t-test or Wilcoxon sign-rank test. ns, non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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4. Immunophenotypes changed in CD4 and CD8 TILs

A. Proliferation marker 4-1BB decreased

Expression of multiple immune phenotypic markers were done by flowcytometry: immune checkpoint receptor PD-1, proliferation marker 4-1BB, exhaustion marker CD39.

PD-1, also known as CD279 is a 288-amino acid type I transmembrane protein, was demonstrated to be a negative regulator of immune responses. The protein is predominantly expressed on antigen-experienced memory T cells in peripheral tissues and less commonly on B cells, activated monocytes, dendritic cells, and natural killer (NK) cells.(14).

4-1BB (CD137, tumor necrosis factor receptor superfamily 9) is an inducible costimulatory receptor expressed on activated T and NK cells. (15).

CD39 is an enzyme which is responsible, together with CD73, for a cascade converting adenosine triphosphate into adenosine diphosphate and cyclic adenosine monophosphate, ultimately leading to the release of an immunosuppressive form of adenosine in the tumor microenvironment.(16)

Granzyme B is the most abundant serine protease which is stored in secretory granules of cytotoxic T lymphocytes CTLs and NK cells. The granzyme B-induced cell death has been traditionally viewed as a primary mechanism that is used by CTLs and natural killer (NK) cells to eliminate harmful target cells including allogeneic, virally infected and tumor cells.(17)

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CD69 is a membrane-bound, type II C-lectin receptor. It is a classical early marker of lymphocyte activation due to its rapid appearance on the surface of the plasma membrane after stimulation.(18)

In CD4 and CD8 TILs, Expression of 4-1BB were decreased. But other markers - PD-1, CD39, Granzyme B - were not changed in CD8 TILs and CD4 TILs after NAC.

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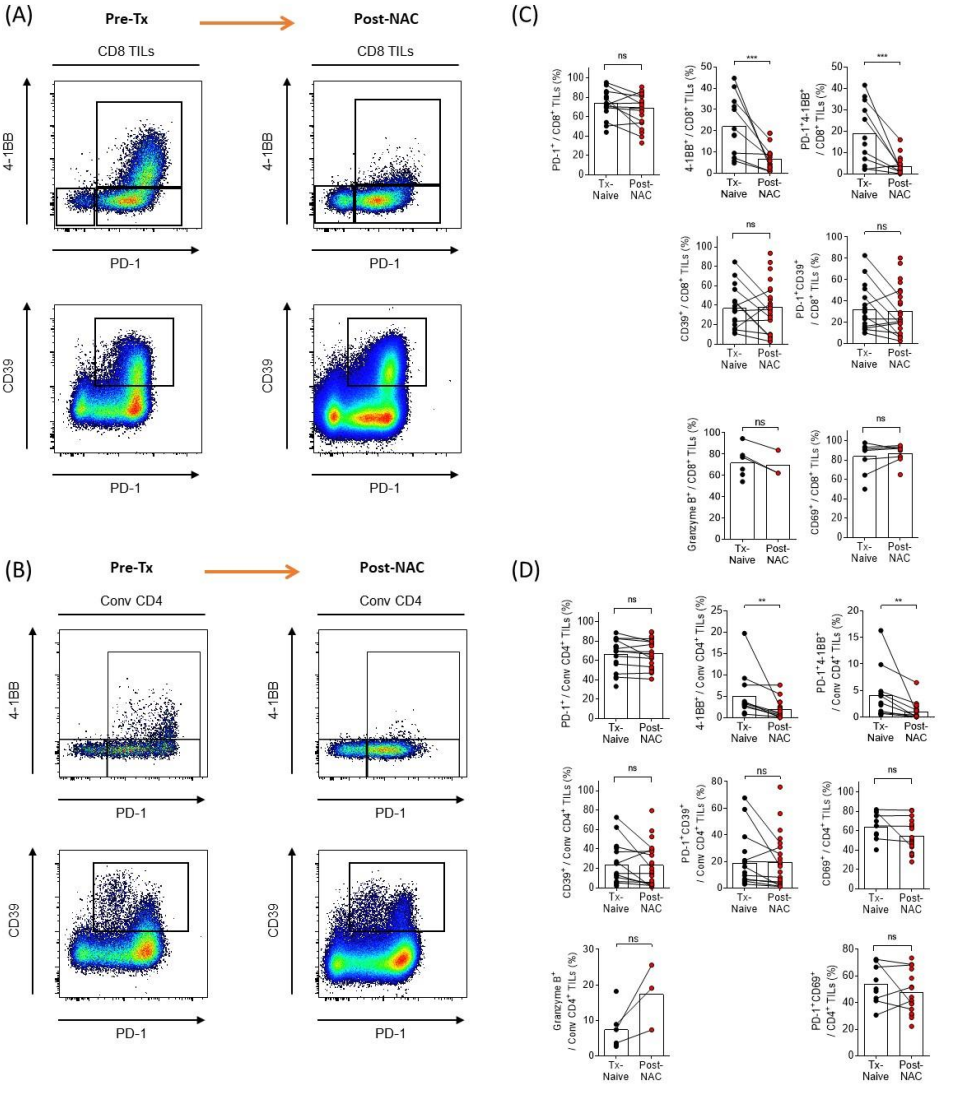

Figure 4. Expression of 4-1BB were decreased, but other immune checkpoints were not changed in CD4 and CD8 TILs after NAC

Expression of immune checkpoint receptors (PD-1), proliferation marker(4-1BB), exhaustion marker (CD-39) on CD8+ TILs (C) and CD4+ TILs (D) was compared between treatment naive and post NAC patients. Representative plots are presented in (A) and (C). Expression of 4-1BB were decreased, but other immune checkpoints were not changed in CD8 TILs and CD4 TILs after NAC. Statistical analysis was performed using the independent samples t-test or Mann-Whitney U-test. ns, non-significant; ** $P < 0.01$; *** $P < 0.001$

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B. Proliferative Ki-67 decreased in CD8 TILs

Ki-67 was first identified as an antigen in Hodgkin lymphoma cell nuclei that is highly expressed in cycling cells but strongly down-regulated in resting G_0 cells. This characteristic has made Ki-67 a clinically important proliferation marker for grading multiple types of cancers.(19)

In CD8 TILs, ki-67 decreased after NAC. Unlike CD8 TILs, CD4 TILs did not show any significant change after NAC.

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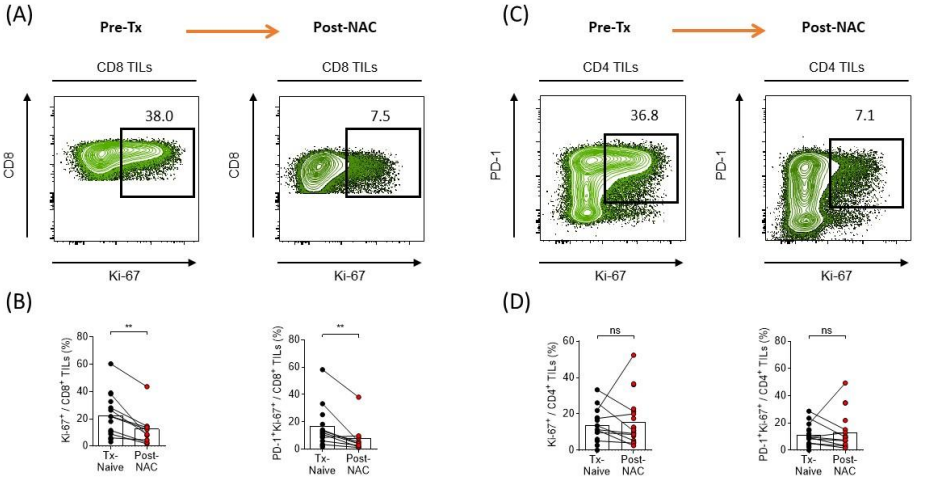

Figure 5 Proliferative Ki-67+ TILs were decreased after NAC.

Expression of proliferation marker Ki-67 on CD8+ TILs (B) and CD4+ TILs (D) was compared between treatment naive and post NAC patients. Representative plots are presented in (A) and (C). Statistical analysis was performed using the independent samples t-test or Mann-Whitney U-test. ns, non-significant; ** $P < 0.01$

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C. CD4+ TILS and CD8+ TILs were less exhausted after NAC.

Percentage of TCF1+/CD8+ TILs, PD-1+TCF-1+/CD8+TILS, PD-1+CD39+TCF-1+/CD8+ TILs increased in NAC patients. Percentage of TOX+/CD8+ TILs, PD-1+TOX+/CD8+TILS, PD-1+CD39+TOD+/CD8+ TILs decreased in NAC patients.

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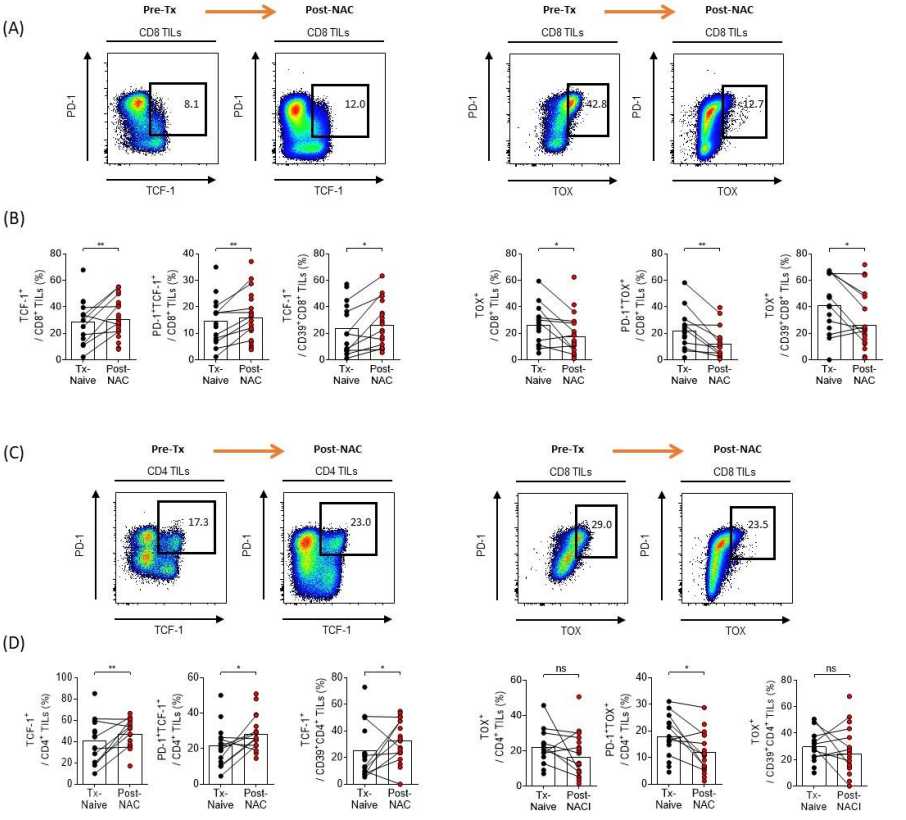

Figure 6. PD-1+CD8 TILs and PD1+CD4 TILs became less exhausted after NAC.

Expression of stemness marker TCF1+ and exhaustion marker TOX+ on CD8+ TILs is presented in (B). Expression of stemness marker TCF1+ and exhaustion marker TOX+ on CD4+ TILs is presented in (D). Representative plots are presented in (A) and (C). It was compared between treatment naive and post NAC patients. Statistical analysis was performed using the independent samples t-test or Mann-Whitney U-test. * $P < 0.05$; ** $P < 0.01$

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5. Analyzing NAC effects on TIL Treg

A. Immunophenotype change of TIL Treg

Regulatory T cells in tumor tissue changes after neoadjuvant chemotherapy. Immune checkpoint receptor PD-1, CTLA-4 and proliferation marker 4-1BB, exhaustion marker CD39 and granzyme B expression was analyzed by FACS. Expression of 4-1BB and CTLA-4 were decreased, but other immune checkpoint inhibitors were not changed in TIL Tregs after NAC. Different from 4-1BB, proliferation marker, expression of Ki-67+TIL Treg and PD-1+Ki67+TIL Treg increased after NAC.

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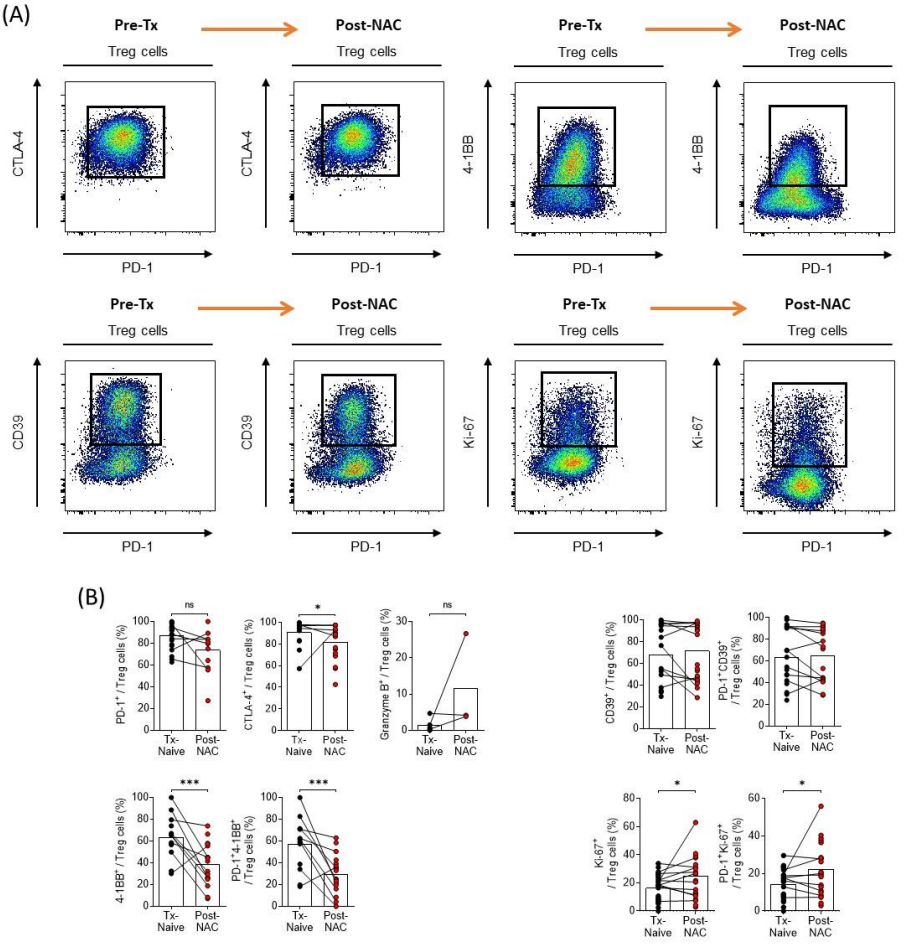

Figure 7. Expression of CTLA-4 & 4-1BB on TIL Treg cells were decreased and Ki-67 increased after NAC.

Expression of immune checkpoint receptors (PD-1, CTLA-4), proliferation marker (4-1BB, Ki-67), exhaustion marker (CD-39) on TIL Tregs were compared between treatment naive and post NAC patients. (B) Representative plots are presented in (A). Expression of 4-1BB and CTLA-4 were decreased, but other immune checkpoints were not changed in TIL Tregs after NAC. Statistical analysis was performed using the independent samples t-test or Mann-Whitney U-test. ns, non-significant; * $P < 0.05$; *** $P < 0.001$

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B. Expression of chemokine receptors decreased in TIL Treg

Chemokines and chemokine receptors orchestrate cell migration and homing in the body. Chemokine receptor 4 (CCR4) is the receptor for two CC chemokine ligands (CCLs)-CCL17 (also called thymus- and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine). Among the various T-cell subsets, CCR4 is predominantly expressed by Th2 cells, cutaneous lymphocyte antigen-positive skin-homing T cells and Treg cells.(20) CCR8 is a chemokine receptor expressed principally on Tregs and is known to be critical for CCR8+ Treg-mediated immunosuppression. Recent studies have demonstrated that CCR8 is uniquely upregulated in human tumor-resident Tregs of breast, colon, and lung cancer patients when compared to normal tissue-resident Tregs.(21)

Expression of CCR 4 was mostly analyzed by unpaired set. The trend seemed CCR4 was higher in Treg after NAC. For CCR8, the trend seemed CCR8 decreased after NAC.

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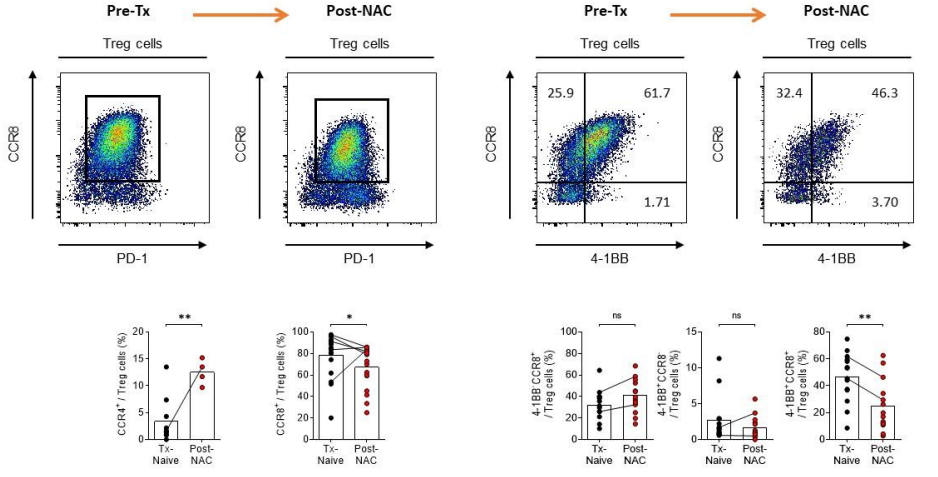

Figure 8. Expression of chemokine receptor CCR4 and CCR8 on intratumoral Treg were decreased after NAC.

Expression of chemokine receptors on TIL Treg was compared between treatment naive and post NAC patients. Expression of chemokines decreased. Statistical analysis was performed using the independent samples t-test or Mann-Whitney U-test. ns, non-significant; * $P < 0.05$; ** $P < 0.01$

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6. Cytokines were more produced after NAC

Cytokines released by T cells, such as IFN-γ and TNF-α, can have antitumor activities by inducing cancer cell senescence. (22) The composition of immune infiltrates is shaped by the expression of cytokines, chemokines, antigens, major histocompatibility complex molecules, and costimulatory molecules. (23)

IFN-γ is a pleiotropic molecule with associated antiproliferative, pro-apoptotic and antitumor mechanisms. Interferon-gamma is secreted predominantly by activated lymphocytes such as CD4 T helper type 1 (Th1) cells and CD8 cytotoxic T cells, γδ T cells, and NK cells and, to a less extent, by natural killer T cells (NKT), B cells , and professional antigen-presenting cells (APCs) (24)

The clinical significance of IFN-γ expression in human cancer has been observed. (25) IFN-γ plays a key role in activation of cellular immunity and subsequently, stimulation of antitumor immune-response. Based on its cytostatic, pro-apoptotic and antiproliferative functions, (26)

TNF-α is a multifunctional cytokine involved in apoptosis, cell survival, inflammation, and immunity acting via two receptors. (27) TNF-α could be a cancer killer. The property of TNF-α in inducing cancer cell death renders it a potential cancer therapeutic, although much work is needed to reduce its toxicity for systematic TNF-α administration. (28) In patients with advanced cancer, treatment with the TNF-α–specific antibody infliximab reduced plasma IL-17 levels. (29)

IL-17A has been shown to recruit myeloid derived suppressor cells (MDSCs) to dampen anti-tumor immunity. IL-17A can also enhance tumor growth in vivo through the induction of IL-6, which in turn activates oncogenic transcription factor signal transducer and activator of transcription 3 (STAT3) and upregulates pro-survival and pro-angiogenic genes in tumors. Data from ovarian

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cancer suggest that Th17 cells are positively correlated with NK cell–mediated immunity and anti-tumor CD8 responses (30)

Cytokine secreting capacity showed increasing tendency after NAC but was not statistically significant.

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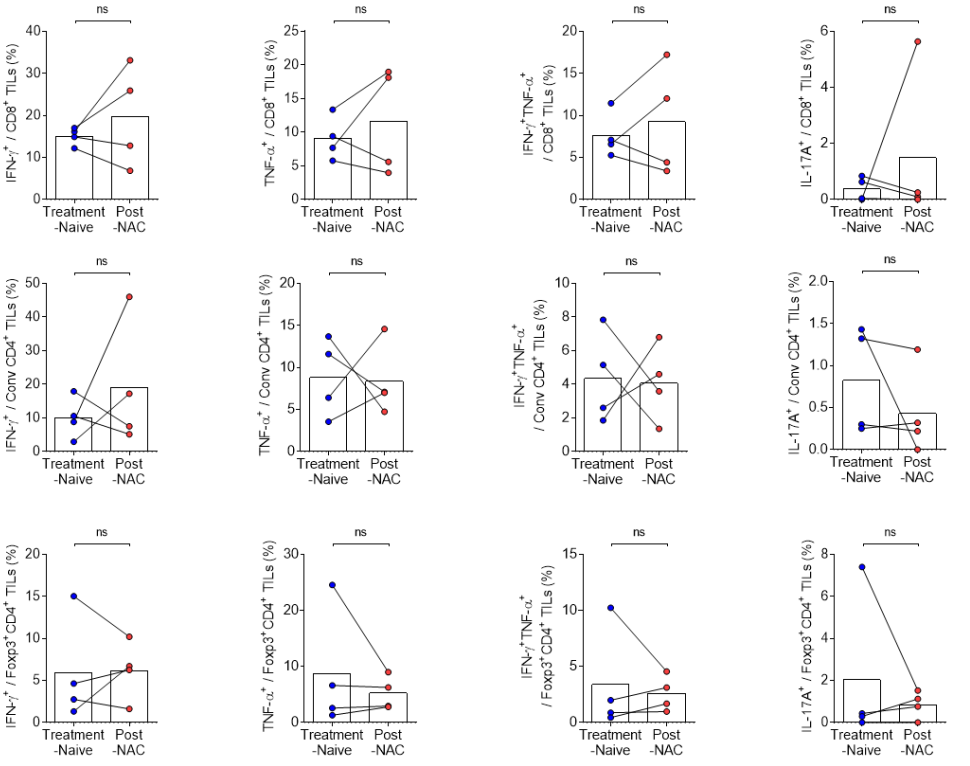

Figure 9. Cytokine secreting capacity change is not significant after NAC by intracellular staining.

Cytokine secreting capacity (IFN-γ, TNF- α, IL-17A) on CD8+ TILs, CD4+ TILS and TIL Treg. It was compared between treatment naive and post NAC patients. Statistical analysis was performed using the independent samples t-test or Mann-Whitney U-test. ns, non-significant

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IV. DISCUSSION

Like previous studies, T cell population changed after NAC. CD4 and CD8 TILs increased and Treg decreased. Fraction 2 Treg, which is active Treg decreased, leading to more active tumor immune microenvironment. It can be inferred that by neoadjuvant chemotherapy, tumor burden decreases. The signal pathway underlying this change is yet unknown. Possible explanation is that by reduced tumor bulk, loading of cytotoxic T cell decrease and leading to better function of T cell. It is plausible that proliferation marker increased after NAC. Decreased tumor burden may lead to decreased Treg relative frequency and decreased antigen exposure and may result in increasing proliferation marker. However, future studies are needed to find signal pathways underlying these changes.

Unlike previous studies which was done by immunohistochemical staining (3), this study was the first study that was done by flowcytometry. Flowcytometry enabled examine multiple immunophenotypes at once. For relation evaluation, I tried intracellular cytokine staining and exhaustion was noted.

This study was performed with newly diagnosed advanced ovarian cancer patients. Due to its short period of time, most patients achieved complete response and were free from progression. Evaluating survivals-progression free survival or overall survival does not seem statistically significant. The cohort needs careful follow up observation period to see whether the results are truly different. Data concerning prognosis, such as age, chemotherapy regimen, BRCA mutation status, histopathologic type, stages, and CRS were all collected so careful follow up is needed.

Because this study was done with cryopreserved samples, samples with enough number of cells could only be done. It was hard to perform more

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samples with intracellular staining or T cell proliferation assay due to limited numbers of cells. Collecting paired sample was not easy as well. Diagnosing ovarian cancer was not only done with diagnostic laparoscopy, but also by fine needle aspiration biopsy or peritoneal fluid sampling so that collecting sample itself was impossible. If effect of NAC was pronounced, it was difficult to get enough post NAC samples.

For future studies, cooperating with clinical filed, more specimen is needed. With more specimen, analyzing intracellular staining and T cell proliferation assay is possible that we can see some more of results.

KGOG 3046 (TRU-D) is a clinical investigation which is comparing neoadjuvant immuno-chemotherapy (NACI) with NAC. It is A single-arm phase II study of neoadjuvant chemotherapy plus Durvalumab and Tremelimumab in the treatment of advanced-stage ovarian cancer.(31) It will be very interesting to compare the results with current study. Preliminary studies showed that cytokine secreting capacity increased after NACI.

Due to its small population of existence, cytokine secreting capacity showed no statistically significant change before and after the chemotherapy. Collecting samples both before and after chemotherapy is difficult. In advanced cases, it is usual to diagnose by fine needle aspiration biopsy, which is hard to get enough samples. In some cases, response to neoadjuvant chemotherapy is so excellent that tumor tissue is very small to collect. Although statistically not significant, 3 out of 4 samples of NACI showed increasing cytokine secreting capacity. Insignificancy may be attributed to small sample number. Other possible explanation is tumor tissue. 1 sample showed decrease in cytokine secreting capacity. The sample was lymph node tissue, unlike other tissues which were collected from tumor bulk.

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V. CONCLUSION

T-cell exhaustion is characterized by the stepwise and progressive loss of T-cell functions and can culminate in the physical deletion of the responding cells. TCF-1 and TOX are important markers of T-cell exhaustion. Increasing TCF-1 and decreasing TOX shows TILs become less exhausted after neoadjuvant chemotherapy. Cytokines in T cells generally increased after NAC including NAC. But was not statistically significant.

In conclusion, neoadjuvant chemotherapy makes T cell less exhausted and restores cytokine secreting capacity, leading to better tumor microenvironments.

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ABSTRACT (IN KOREAN)

난소암 환자의 수술 전 항암 화학요법에 따른 종양 침윤 림프구의 면역학적 변화 탐구

<지도교수 이정윤>

연세대학교 대학원 의학과

김엽

난소암은 모든 부인암 중 가장 치사율이 높다. 조기 발견할 수 있는 방법이 없으며 75% 이상은 진행된 상태에서 진단되어 예후가 나쁘다. 대부분의 진행된 병기에서는 선행항암화학요법 후 종양감축술을 시행한다. 항암화학요법은 골수억제를 통해 면역억제효과를 보이는 것으로 생각되었으나 최근 연구결과에 의하면 종양미세환경을 변화시키는 것으로 밝혀졌다. 본 연구는 선행항암화학요법에 의한 종양 침윤 림프구의 변화가 있는지, 있다면 어떤 변화가 있는지 보기 위해 시행되었다.

선행항암화학요법 시행 전후로 수집된 15쌍의 샘플 및 쌍을 이루지 않은 샘플들을 선택하였다. 쌍을 이루지 않은 샘플은 14개의 항암요법을 받지 않은 샘플과 11개의 항암요법을 받은 샘플이었다. 샘플을 녹인 후 나누어 일부는 세포 내 사이토카인 자극 후 에, 일부는 자극을 주지 않고 염색하여 유세포분석을 시행하였다. 자극을 주지 않은 종양 침윤

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림프구에서 TCF1는 선행항암화학요법 후 증가하였다. TOX는 선행항암화학요법 후 감소하였다. 세포 내 사이토카인 자극 후 염색하여 분석한 경우 선행항암화학요법 전후로 유의한 차이를 보이지 않았다.

T세포 탈진은 점진적인 T세포의 기능상실과 반응하는 세포의 물리적 소멸을 동반한다. TCF1과 TOX는 T세포 탈진의 중요한 표지자이다. 증가하는 TCF1과 감소하는 TOX는 종양 침윤 림프구가 선행항암화학요법 이후 덜 탈진된다는 것을 보여준다. 선행항암화학요법 전후로 IFN-γ, TNF-α 등의 사이토카인 분비능은 차이가 유의하지는 않으나 증가하는 추세를 보였다.

결론적으로, 선행항암화학요법은 T세포 탈진을 감소시키고 사이토카인분비능을 증가시켜 면역항암화학요법에 대한 반응을 증가시킬 것으로 예상된다.

핵심 되는 말: 진행성 난소암, 선행항암화학요법, 면역관문저해제