의학석사 학위논문

Cellular Biomarker for the Responding of Anti-PD-1 Therapy in Non-Small Cell Lung Cancer Patients

비소세포성 폐암환자에서 anti-PD-1 therapy 약물반응의 cellular biomarker 발굴 연구

2018 년 2 월

서울대학교 대학원

의과학과 면역학 전공

박 수 명

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February 2018

The Department of Biomedical Sciences in Seoul National University College of Medicine Graduate School

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A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine(Immunology)

at Seoul National University College of Medicine

February 2018

Approved by Thesis Committee:

February 2018

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지도교수 연제인

이 논문을 의학석사 학위논문으로 제출함

2018 년 2 월

서울대학교 대학원

의과학과 면역학 전공

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박수명의 의학석사 학위논문을 인준함

2018 년 2 월

위원장 _____ (인)

부위원장 _____ (인)

위원 _____ (인)

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Abstract

Cellular Biomarker for the Responding of Anti-PD-1 Therapy in Non-Small Cell Lung Cancer

Immune checkpoint inhibitors which have been actively used and studied in recent years opened a new era in cancer immunotherapy. The strategy that targeting Programmed cell death protein1 (PD-1) and its ligand Programmed cell death protein ligand1 (PD-L1) to block their function showed us surprising results in many clinical and pre-clinical studies. Nivolumab and Pembrolizumab which are fully humanized anti-PD-1 therapeutic antibody have been used for cancer immunotherapy in many types of cancers. Because of their powerful anti-tumor effect, long duration time and acceptable side effects, they could achieve big success in pharmaceutical market. However, high cost and low drug response rate limit their practical and common usage of the drugs. Despite of many trials to find out predictive biomarkers, there are no reliable markers found so far. For the biomarker of anti-PD-1 therapy, tumor expression status of PD-L1 by immunohistochemistry (IHC) staining has been used, but it has many problems concerning the accuracy of prediction and unstandardized protocols. Here we found cellular biomarker for anti-PD-1 therapy which can powerfully predict the drug response with better accuracy and simplified way compared to the conventional IHC-based tumor PD-L1 expression marker. We checked lymphocytes as immune active cells and myeloid-derived suppressor cell (MDSC) for the suppressive immune cells based on our hypothesis that the drug response for the immune

checkpoint blockade depends on the systemic immune status of the individual patient. Peripheral blood regulatory T cell (Treg)/ Lectin-type oxidized LDL receptor-1 (Lox-1)+ PMN-MDSC ratio after the first infusion of anti-PD-1 drug significantly higher in responder group. Its cut-off value gives us the outstanding prediction accuracy for the patient prognosis, so we can expect it as a strong biomarker for anti-PD-1 therapy.

Keywords : Checkpoint inhibitor, Anti-PD-1 therapy, Non-small cell lung cancer, Biomarker, Myeloid-derived suppressor cell, regulatory T cell, Lectin-type oxidized LDL receptor-1

I. Introduction

Cancer immune evasion and checkpoint blockade

Tumor-induced immune suppression is one of the main mechanisms of the cancer immune evasion[1, 2]. Tumor cells express various immunosuppressive cytokines and chemokines to recruit suppressive cells, and they also directly work as a suppressor by expressing the inhibitory signaling molecules[1-4]. In tumor microenvironment, tumor undergoing immune escape secrete various suppressive molecules such as Transforming growth factor-β (TGF-β), IL-10, Indoleamine 2,3-dioxygenase1 (IDO) and express excess of inhibitory signaling molecules like PD-L1 to suppress the anti-tumoral immune cells including CD4+T cell, CD8+ cytotoxic T lymphocyte (CTL) and NK cells[2]. Overexpressed Programmed cell death protein ligand1 (PD-L1) and other coinhibitory receptors on tumor cells enable them to expand and avoid from the immune surveillance[3, 4]. Immune checkpoint molecules, which mediate the immune inhibitory signaling, such as Cytotoxic T-Lymphocyte Associated Protein4 (CTLA-4) and Programmed cell death protein1 (PD-1) have been used as effective target for the anti-cancer immunotherapy[4, 5]. PD-1 on activated T cells can bind with its ligand, PD-L1 and Programmed cell death protein ligand2 (PD-L2) on tumor cells or antigen-presenting cells (APCs) to transfer the inhibitory signaling[3]. Many types of checkpoint inhibitors were developed by pre-clinical and clinical studies; not only with inhibitory molecules like CTLA-4 and PD-1/PD-L1 but also T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) and Lymphocyte-activation gene 3 (LAG-3)[6]. Specially for anti-PD-1 therapy, their dramatic effects and little side effects

made a big paradigm shift in cancer immunotherapy area[7]. Nivolumab (commercial name Opdivo) and pembrolizumab (commercial name Keytruda), both humanized IgG4 anti-PD-1 monoclonal antibodies, which targeting the PD-1/PD-L1 axis showed outstanding clinical benefits in many types of cancers[8].

Anti-PD-1 blockade in non-small cell lung cancer and its biomarker

Lung cancer is one of the most common and high-mortality cancers in worldwide[9]. Lung cancer can be divided by two types; non-small cell lung cancer (NSCLC), which is the major type(over 85%), and the minor type, small cell lung cancer (SCLC)[10]. The first-line chemotherapy for advanced NSCLC is mostly based on the platinum-based chemotherapy including cisplatin and oxaliplatin[11, 12]. Antiangiogenic therapies like anti-vascular endothelial growth factor (VEGF) and various targeted therapy including EGFR tyrosine kinase inhibitors (TKIs), MET, BRAF and HER3 inhibitors have been introduced depend on the genetic mutation of the patient as a second or subsequent lines of therapy[12, 13].

Nivolumab and Pembrolizumab has been widely used for the treatment of NSCLC since its first approval for non-small cell lung cancer by Food and Drug Administration (FDA) in each 2015 and 2016[8, 14-17] and also for other types of cancers such as melanoma[17-20] and renal cell carcinoma (RCC)[17]. Both of the drugs showed promising effect with less side effects, increased overall survival (OS), progression-free survival (PFS) and long duration of response[14, 15, 21].

All patients (n)	Tumor PD-L1 expression (n)		Objective response		Reference
			Yes	No	
41	positive	23 (56%)	9 (39%)	14 (61%)	Janis M. Taube et al., Association of PD-1, PD-1 Ligands, and Other Features of the Tumor Immune Microenvironment with Response to Anti-PD-1 Therapy, 2014, Clin Cancer Res; 20(19)
	negative	18 (44%)	1 (6%)	17 (94%)	
44	positive	12 (27%)	8 (67%)	4 (33%)	Jeffrey S. Weber et al., Safety, Efficacy, and Biomarkers of Nivolumab With Vaccine in Ipilimumab-Refractory or -Naïve Melanoma
	negative	32 (73%)	6 (19%)	26 (81%)	
44	positive	23 (52%)	9 (39%)	14 (61%)	Jeffrey S. Weber et al., Safety, Efficacy, and Biomarkers of Nivolumab With Vaccine in Ipilimumab-Refractory or -Naïve Melanoma
	negative	21 (48%)	5 (24%)	16 (76%)	
52	positive	25 (48%)	9 (36%)	16 (64%)	Suzanne L. Topalian et al., Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer, 2012, NEJM; 366(26)
	negative	27 (52%)	0 (0%)	17 (100%)	

Table 1. Objective response rate of anti-PD-1 therapy based on PD-L1 expression in tumor tissue

Average response rate for Nivolumab and Pembrolizumab in NSCLC is around 19~21%[22], but the treatment costs 5,040$ and 12,432$ each in every 2 or 3 weeks, in case of 60kg adult[23]. Thereby several predictive biomarkers for these anti-PD-1 therapy have been found; PD-L1 expression status and tumor infiltration lymphocytes (TILs) in patient tumor tissue based on the immunohistochemistry (IHC) staining[17, 24-26]. PD-L1 status in tumor tissue is type of biomarker that most widely accepted and used. On October 2016, US FDA approved Pembrolizumab for the metastatic NSCLC patients with high PD-L1 expression (Tumor proportion score, TPS≥ 50%)[27]. However prediction accuracy of PD-L1 status is not strong enough to solidify the diagnosis, because there are some controversial results about the diagnostic biomarker used PD-L1 expression status. Several reports found that most of the patients that express PD-L1 in tumor tissue showed improved objective response rate (36~67%)[17, 25] (table2). But the problem is, even though the patients have PD-L1 expression, 33~64% of the patients do not have any drug responses[22, 24] (table2) and several PD-L1 negative tumor patients (~24%) also showed the drug response[17, 24, 25] (table2). Thus the patient diagnosis for anti-PD-1 therapy in NSCLC is still remains unstandardized. In addition, Its low response rate (table1) in most of the cancer types and excessively high market price (table2;[23]) have limited its common usage[22, 23].

Myeloid-derived suppressor cell as a biomarker for checkpoint blockade

Myeloid-derived suppressor cell (MDSC) is a group of immature myeloid progenitor cells that represent immunosuppressive activity[28]. There are two major types; Polymorphonuclear MDSC (PMN-MDSC or G-MDSC),

	Nivolumab	Pembrolizumab
Cost per mg($)	28	103.6
Dose & Schedule	3mg/kg every 2wks	2mg/kg every 3wks
PFS (months)	3.5	6.3
Per patients cost ($) (months)	12,600	20,716
Per patients cost ($) (PFS)	44,100	130,511
1-Year cost (billion $)	47.2	83.9

Table 2. Effect and cost of anti-PD-1 drug in NSCLC

the one that represent granulocytic phenotype; and monocytic MDSC (M-MDSC), which has a monocytic phenotype[28]. M-MDSC that express high levels of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) as their suppressive function characterized by CD11b+Ly6GlowLy6Chigh in mouse and HLA-DR-CD11b+CD14+ in human[28, 29]. Whereas PMN-MDSC that majorly exert its suppressive mechanism by high expression of NADPH and ROS characterized by HLA-DR-CD11b+Ly6G+Ly6Clow in mouse and CD14-CD11b+ CD33+CD15+ in human[28, 29]. They activate and expand their population under the pathogenic environment such as inflammation, trauma and tumor[28, 30]. Along with regulatory T cell (Treg), MDSC function as a powerful immune suppressive cell in tumor microenvironment during the tumor immune escape[1, 28, 31]. Many studies revealed that MDSC can be applied as one of the promising target or biomarker for the cancer immunotherapies[31-33] and some of them are target MDSC to enhance the efficacy of checkpoint inhibitors[34-36].

Highfill et al. proved that the disruption of MDSC trafficking by CXCR2 knockout in rhabdomyosarcoma (RMS) tumor enhances the efficacy of anti-PD-1 therapy in mice and they also found that increased serum level of CXCL1 and CXCL8, which are the ligand for CXCR2, is correlated with the poor prognosis of the pediatric sarcoma patients[35]. Yana G. Najjar et al. also demonstrate that the combination therapy of CXCR2 antagonist with PD-1 blockade gives the better prognosis in murine and human RCC model[36]. In case of studies for metastatic melanoma patient who treated with Ipilimumab, which is CTLA-4 monoclonal antibody, peripheral MDSC has been proposed as a strong biomarker for the better prognosis in many studies[37-42]. But the MDSC as a biomarker for anti-PD-1 therapy in NSCLC have never been proposed yet, only one paper that divide patient

cohort depend on the peripheral MDSC level for the patients of Ipilimumab-refractory or naïve melanoma treated with Nivolumab was published in 2016[43].

In this study, we investigated the cellular biomarker at early timepoint of the anti-PD-1 drug administration cycle in advanced (stageⅢ-Ⅳ) NSCLC patients. Peripheral blood mononuclear cells (PBMCs) of the patient was collected and analyzed before the anti-PD-1 drug administration (baseline sample) and every cycle, which takes 2~3 weeks, before they got new administration (Figure 1) to monitor the patient's immune cell profiles and compare with their real prognosis. We hypothesized that immune suppressive populations such as MDSC or Treg can reflect the immune status of the patients, so that act as an indicator of the patient immune system; It would be downregulated in the positive drug responders after the drug administration, whereas increased in case of the non-responders. Lymphocytes such as B cell, NK cell, NKT cell, T cell, CD4+ or CD8+ T cells also analyzed as indicators of the immune activity of the patients, and they were expected to show the opposite result with MDSC. We also checked the expression of Lectin-type oxidized LDL receptor-1 (Lox-1) in PMN-MDSC population. In human, any specific markers have been found so far that can distinguish between PMN-MDSC and neutrophil (polymorphonuclear, PMN) except the mechanical isolation methods by density gradient separation using reagents like Ficoll or Histopaque. Lox-1 is the novel marker found by Gabrillovich group in 2016, can distinguish PMN-MDSC and PMN cells in human[44]. As a result, we could find out the optimal ratio between immune-active lymphocytes and immune-suppressive MDSC group; Treg/Lox-1+PMN-MDSC gives us the

best drug response and patient prognosis. Treg/Lox-1+PMN-MDSC ratio is expected as a reliable biomarker for positive responder of anti-PD-1 therapy. It highlights the significance of immune suppressive cells as a biomarker in context of the immune checkpoint blockades.

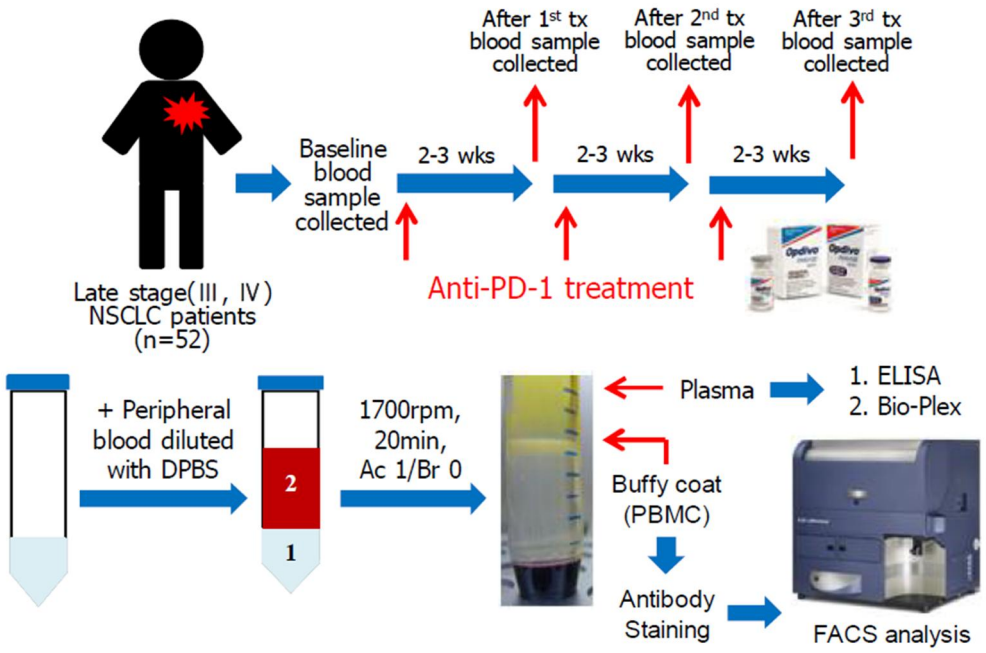


Figure 1. Scheme of study design and experimental flow

II. Materials and methods

Patients, treatment, and clinical evaluation

This study included 53 advanced NSCLC patients who failed to platinum-based chemotherapy and received Nivolumab(Bristol-Myers Squibb) or Pembrolizumab(Merck) in the Yonsei Cancer Center in Seoul, Korea. Patients' inclusion criteria included; 1) over 20 years old, 2) had a confirmed diagnosis of recurrent/metastatic NSCLC patients, 3) failed to previous platinum based chemotherapy, 4) received at least one cycle of nivolumab over 90 minutes at a dose of 3mg/kg of body weight every 2 weeks and, 5) written with informed consent. Exclusion criteria were the presence of an autoimmune disease, HIV, hepatitis B or C, pregnancy, symptomatic brain metastases, or concomitant systemic therapy for NSCLC. Asymptomatic or pretreated brain metastases were allowed to be included. Treatment efficacy was assessed using contrast-enhanced CT at around 8 weeks after the first drug infusion and clinical response defined as complete response (CR), partial response (PR), stable disease (SD) and progressive disease(PD). Patients who showed increased tumor mass ≥20% by CT scan after drug infusion defined as PD, which indicate no clinical benefit(non-responder). Patients with CR, ≥30% decreased(PR) or -29~+19%(SD) tumor mass were defined as clinically beneficial patients (responder).

Isolation of PBMC

The peripheral blood was taken in EDTA tube within 5 days before the first

drug treatment (point 1: baseline) as well as 0 to 3 days before the second (point 2: after 1st tx), before the third (point 3: after 2nd tx), and before the fourth infusion (point 4: after 3rd tx). The PBMCs were isolated by density gradient centrifugation using Ficoll(GE Healthcare) and freshly analyzed by flow cytometry right after the isolation and antibody staining. The plasma was collected, aliquoted, and stored at -80℃ for ELISA and multiplex chemokine and cytokine assays.

Flow cytometry and antibodies

Flow cytometry was performed by FACS LSR Fortessa (BD Biosciences) and the data were analyzed with FlowJo software (Treestar). The following fluorescence-conjugated monoclonal antibodies were purchased and used for the surface staining: BD Biosciences: anti-CD45 (HI30)-BV650, CD15 (HI98)-PerCP-Cy5.5, CD14 (MΦP9)-APC-Cy7, HLA-DR (G46-6)-V500, CD56 (B159)-FITC, CD19 (SJ25C1)-PE-Cy7, CD8 (SK1)-APC-H7, CD4 (L200)-PerCP-Cy5.5, CD3 (UCHT1)-V500, CD274 (MIH1)-PE, CD25 (M-A251)-PE-Cy5, CD33 (P67-6)-PE-Cy7, CD11b (ICRF44)-APC, CD103 (Ber-ACT8)-FITC, CD279 (EH12.1)-PE, mouse IgG1,k Isotype (MOPC-21)-PE, mouse IgM,k Isotype (G155-228)-FITC; Biolegend: Lox-1 (15C4)-PE; eBioscience: CD127 (eBioRDR5)-APC; Bio-Rad: CD273 (MIH14)-FITC. ROS production was measured by staining with 2',7'-dichlorofluorescin diacetate(DCFDA, from Invitrogen). Singlet cells were selected based on the scatter profiles and dead cells were stained with 4',6-Diamidino-2-Phenylindole(DAPI, Molecular Probes) and excluded from the analysis.

Multiplex chemokine and cytokine assays

Plasma samples were detected and quantified for forty human chemokine levels by Bio-Plex Pro™ Human Chemokine 40-plex Panel(Bio-Rad) according to the manufacturer's protocol.

ELISA

Serum level of S100A8/9 was determined by ELISA assay for S100A8/9 (R&D) according to the manufacturers' protocol.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software package (GraphPad Software, Inc., San Diego, USA). Graphs represent mean values ± SD. P values were calculated in each respective figure using Student's t test for comparing like groups or log-rank statistics for survival analyses. P-values less than 0.05 were considered as being statistically significant and is illustrated with an asterisk (*). Progression free survival (PFS) was defined as the time from starting Nivolumab or Pembrolizumab therapy to progression of disease or death due to any cause. PFS was estimated by the Kaplan–Meier method.

III. Results

Peripheral Treg and MDSC showed significant difference between non-responder and responder group in early time point of the therapy

To find out the cellular biomarker for anti-PD-1 therapy, peripheral blood from 52 of non-small cell lung cancer patients who treated with anti-PD-1 therapy (Table 3) were collected and freshly analyzed by flow cytometry before the therapy(baseline) and after each cycle of the therapy (Figure 1). For lymphocyte group, CD19+ B cell, NK cell, NKT cell, CD3+ total T cell, CD4+ T cell, CD8+ T cell and CD127low CD25+ Treg cell out of CD45+ leukocytes were analyzed (Figure 2). HLA-DR-CD11b+CD14+ M-MDSC, CD14-CD11b+CD33+CD15+ PMN-MDSC and Lox-1+ PMN-MDSC were analyzed for the MDSC group (Figure 2). Before the therapy(Baseline), B cell and CD4+ T cell were slightly higher (Figure 3A) and overall MDSC populations were showed decreased pattern in responder group, but it did not show significant differences (Figure 3B). Whereas Treg was significantly higher in responder group compare to the non-responder group (Figure 3A). In addition to the Treg, NK cell was also showed significantly higher pattern in responder group at 2~3 weeks after the first therapy(After 1^{st} tx) (Figure 4A). In MDSC group, Lox-1+ PMN-MDSC from non-responders was markedly decreased (Figure 4B).

Table 1. Clinical characteristics of patients treated with nivolumab

Variables	n (%)
Age, years	
Median (range)	53 (39-81)
Sex	
Male	34 (64.2)
Female	19 (35.8)
ECOG performance status	
0	14 (26.4)
1	31 (58.5)
2	8 (15.1)
Smoking	
Never/light smoker	20 (37.7)
Ever smoker	33 (62.3)
Histology	
Adenocarcinoma	29 (73.6)
Non-adenocarcinoma	14 (26.4)
Mutation type	
EGFR mutation	5 (9.4)
ALK or *ROS1* rearrangement	2 (3.8)
Wild type	46 (86.8)
Best response to nivolumab	
SD/PR	14 (26.4)/ 12 (22.6)
PD	26 (50.9)
Previous treatment	
Chemotherapy	52 (98.1))
Targeted therapy	17 (32.1)
Immunotherapy	1 (1.9)
No. of prior therapy	
N=1	20 (37.7)
N=2	16 (30.2)
N>2	17 (62.3)

Table 3. Clinical characteristics of patients

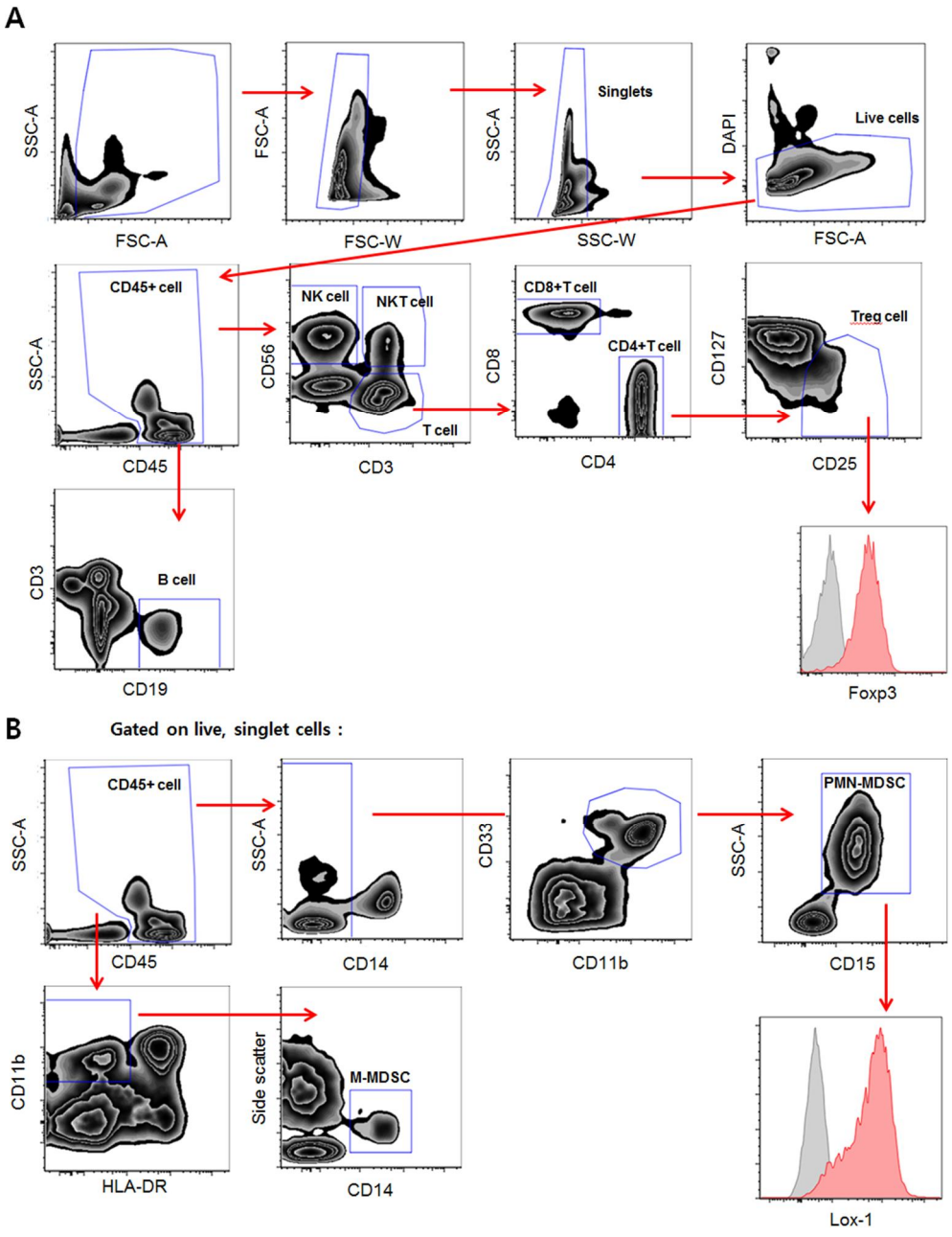


Figure 2. Gating strategy for lymphocytes and MDSC. Gating strategies for

peripheral blood of non-small cell lung cancer patients treated with anti-PD-1 therapy in lymphocytes (A); CD19+ B cell, NK cell, NKT cell, CD3+ total T cell, CD4+ T cell, CD8+ T cell, CD127lowCD25+ Treg cell and MDSC (B); HLA-DR-CD11b+CD14+ M-MDSC, CD14-CD11b+ CD33+CD15+ PMN-MDSC and Lox-1+ PMN-MDSC. Singlet cells were selected and dead cells were removed by scatter plot.

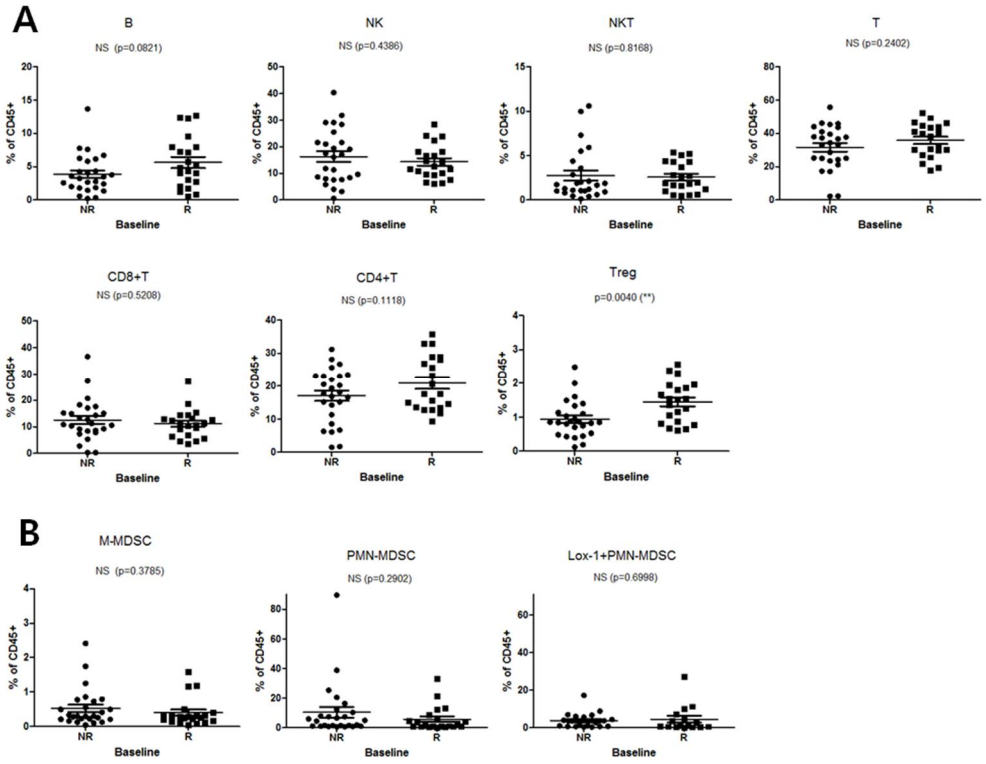


Figure 3. Comparison of peripheral immune cell profile between non-responder and responder in baseline. Baseline peripheral level of lymphocytes (A); B cell, NK cell, NKT cell, T cell, CD8+ T cell, CD4+ T cell, Treg and MDSC (B); M-MDSC, PMN-MDSC, Lox-1+ PMN-MDSC out of CD45+ leukocytes were analyzed by multi-color staining flow cytometry in non-responder and responder of anti-PD-1 therapy before the first therapy. Dot graphs represent frequency of immune cells and small horizontal lines indicate the mean(±SD). Single dot indicates single patient, *p<0.05 (two-tailed Student's *t*-test).

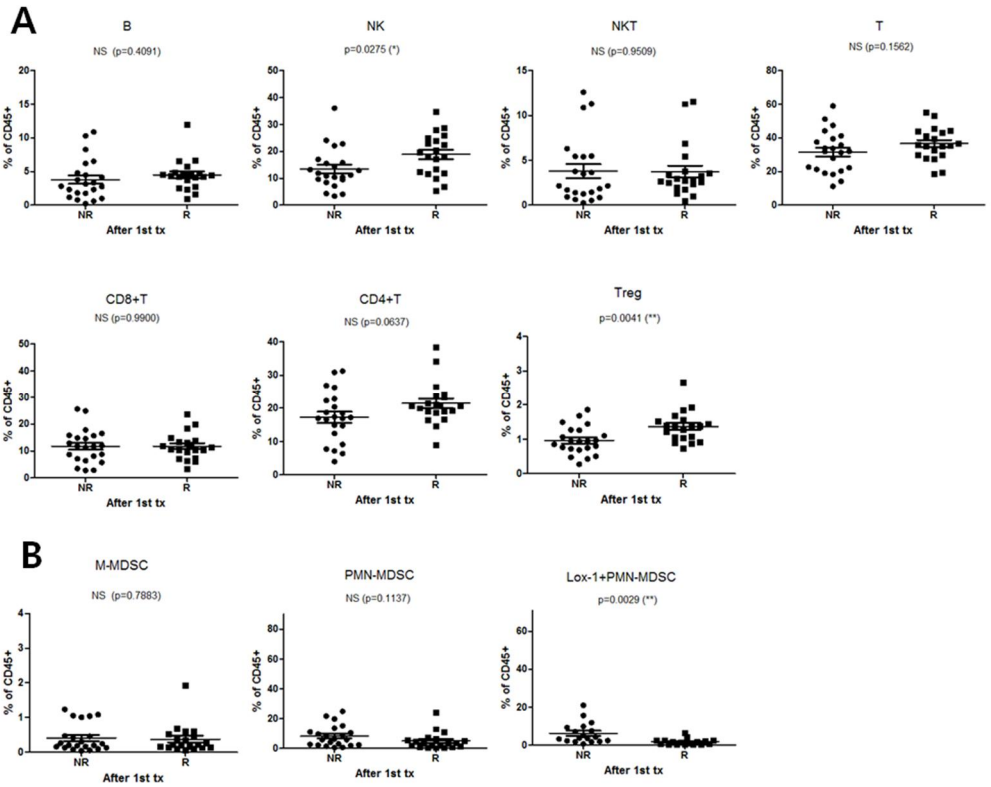


Figure 4. Comparison of peripheral immune cell profile between non-responder and responder group after first therapy. Peripheral level of lymphocytes (A); B cell, NK cell, NKT cell, T cell, CD8+ T cell, CD4+ T cell, Treg and MDSC (B); M-MDSC, PMN-MDSC, Lox-1+ PMN-MDSC out of CD45+ leukocytes were analyzed by multi-color staining flow cytometry in non-responder and responder of anti-PD-1 therapy after 2~3 weeks from the first therapy. Dot graphs represent frequency of immune cells and small horizontal lines indicate the mean(±SD). Single dot indicates single patient, *$p<0.05$.

Peripheral Treg/Lox-1+ PMN-MDSC ratio showed significant difference between non-responder and responder group better than the PD-L1 tumor expression and NLR

To examine and compare the prediction degree of anti-PD-1 drug responding, 3 types of clinical prognostic factors were analyzed and compared; PD-L1 tumor expression, neutrophil to lymphocyte ratio(NLR) and peripheral Treg/Lox-1+ PMN-MDSC ratio that we have newly found as a biomarker. PD-L1 expression in tumor tissue was obtained by IHC analysis and it does not have a big difference between non-responder and responder group (Figure 5A). NLR has been using as a prognostic marker for cancer and increased NLR is related to the poor patient prognosis[45]. NLR, which is determined by complete blood count(CBC) machine showed significantly higher level in non-responder group after the first and second infusion (Figure 5B). However, peripheral Treg/Lox-1+ PMN-MDSC ratio showed more difference between non-responder and responder group than the NLR (Figure 5C). It showed dramatically higher pattern in all time point of the responder group, whereas almost all of the non-responders exclude 1 case in baseline showed decreased level (Figure 5C).

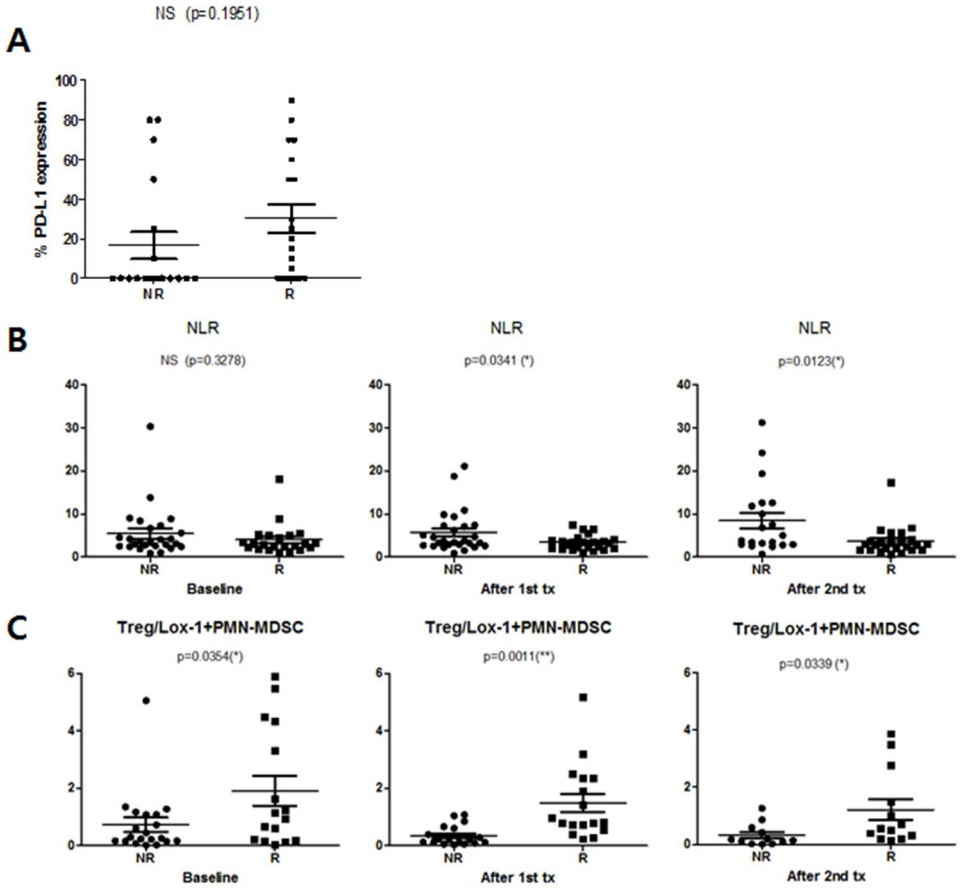


Figure 5. Peripheral Treg/Lox-1+PMN-MDSC ratio can predict the drug response better than the other clinical markers. Tumor tissue expression of PD-L1 was analyzed by IHC analysis in tumor biopsy of each non-responder and responder group before the anti-PD-1 therapy (A). NLR of each responding group were analyzed by CBC machine and checked in baseline, after the first therapy and the second therapy (B). Peripheral Treg/Lox-1+ PMN-MDSC ratio was analyzed by flow cytometry and also checked in baseline, after the first therapy and the second therapy for each responding

group (C). Small horizontal lines indicate the mean(±SD). Single dot indicates single patient, *p<0.05.

Peripheral Treg/Lox-1+PMN-MDSC ratio showed decreased pattern according to the progression of therapy

To look over the trend of peripheral Treg/Lox-1+PMN-MDSC ratio in each response group, we checked the ratio from the time point of baseline to after third therapy. Mean values of the ratio showed the decreased trend according to the progression of therapy in both non-responder and responder group.

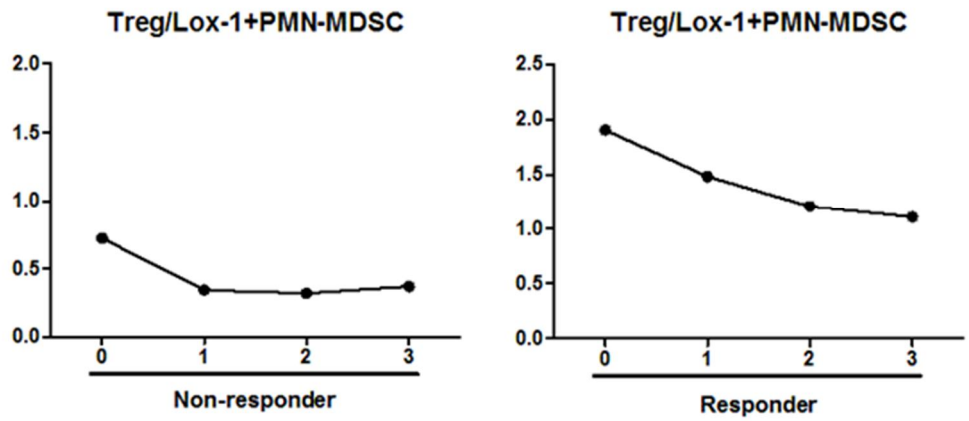
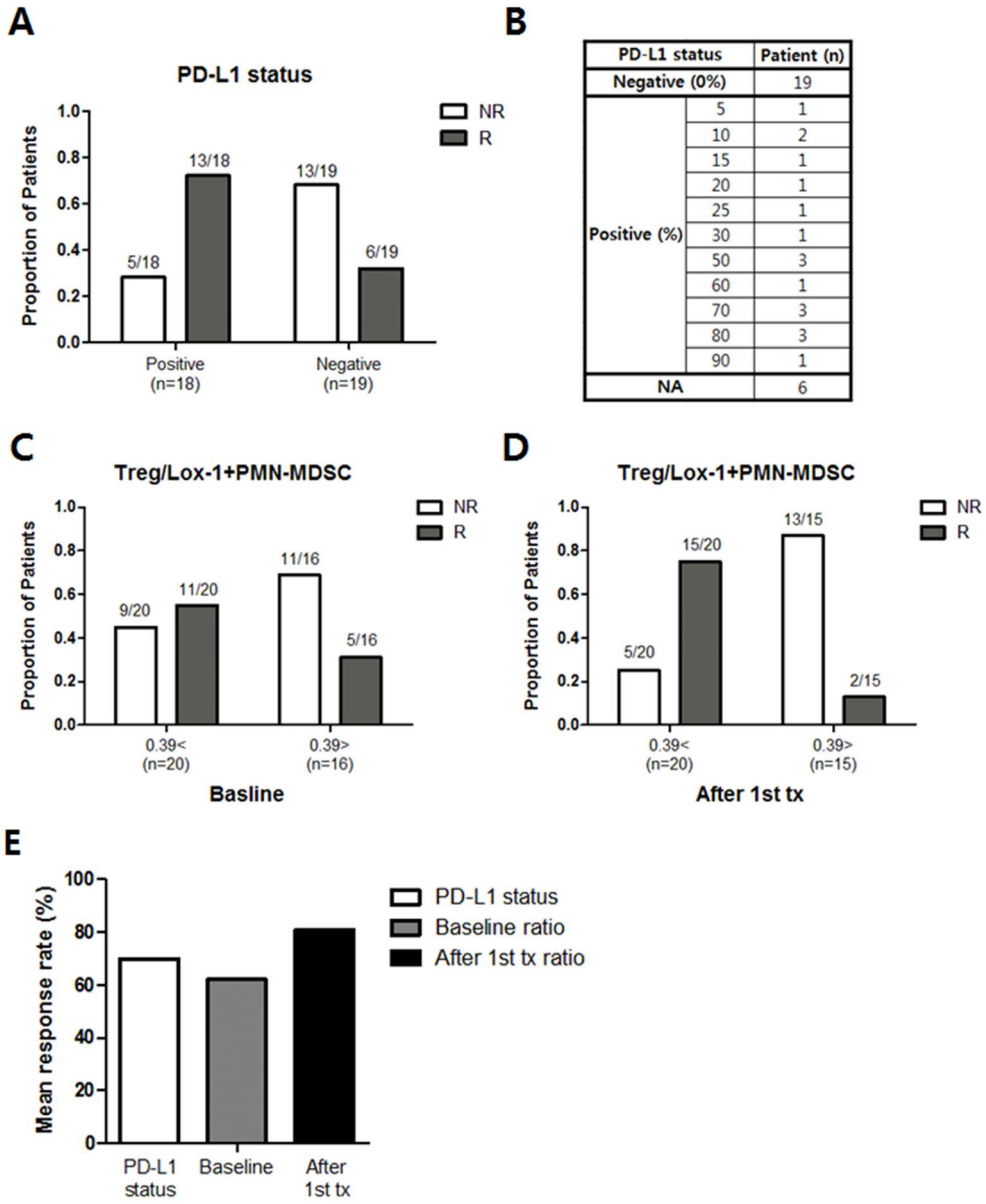


Figure 6. Mean-pattern of peripheral Treg/Lox-1+PMN-MDSC profile in non-responder and responder group. The trend of mean values of peripheral

Treg/Lox-1+PMN-MDSC ratio in non-responder and responder group was analyzed at the time point of baseline, after first therapy, after second therapy and after third therapy(Each value of x axis indicate followings; 0=baseline, 1=after first therapy, 2=after second therapy, 3=after third therapy).

Peripheral Treg/Lox-1+PMN-MDSC ratio after first therapy showed better prediction accuracy for anti-PD-1 therapy compare to the ratio in baseline timepoint or PD-L1 tumor expression marker

For further comparative analysis of each biomarker, we checked PD-L1 tumor expression and peripheral Treg/Lox-1+PMN-MDSC ratio at the time point of baseline and after first therapy. Based on the each criterion of the therapy, predictive positive responder and negative responder populations were divided and from each of them, the real drug responses were represented (Figure 7A, 7C, 7D). PD-L1 status divided responding group based on the expression of PD-L1 in tumor tissue using IHC assay (Figure 7A, 7B). In case of peripheral Treg/Lox-1+PMN-MDSC ratio in baseline and after first therapy divided responding group based on the 0.39 cut-off value which gives the best prediction efficacy (Figure 7C, 7D). Peripheral Treg/Lox-1+PMN-MDSC ratio after first therapy showed the best prediction accuracy compare to the other two markers (Figure 7E).



PD-L1 status		Patient (n)
Negative (0%)		19
Positive (%)	5	1
	10	2
	15	1
	20	1
	25	1
	30	1
	50	3
	60	1
	70	3
	80	3
	90	1
NA		6

Figure7. Comparative analysis for the prediction accuracy of PD-L1 status and peripheral Treg/Lox1+PMN-MDSC ratio. Responding group divided by PD-L1 positivity (A) or 0.39 cut-off value of peripheral Treg/Lox-1+ PMN-MDSC ratio (C- D) were analyzed based on the real drug responding. Each

percentages of positive PD-L1 expression status were represented in (B). Peripheral Treg/Lox-1+PMN-MDSC ratio after first therapy showed the best response rate among the other prediction markers (E).

The poor anti-PD-1 drug response of non-responder might cause by the PMN-MDSC with high Lox-1 expression and ROS production

Gabrilovich and his colleagues supposed lectin-type oxidized LDL receptor-1 (Lox-1) as a marker of PMN-MDSC which can specifically distinguish it from neutrophil in peripheral blood of human[44]. Based on their study, we confirmed that peripheral PMN-MDSC of non-small cell lung cancer patients has much higher Lox-1 expression and ROS production level than the neutrophil (Figure 8A-D).

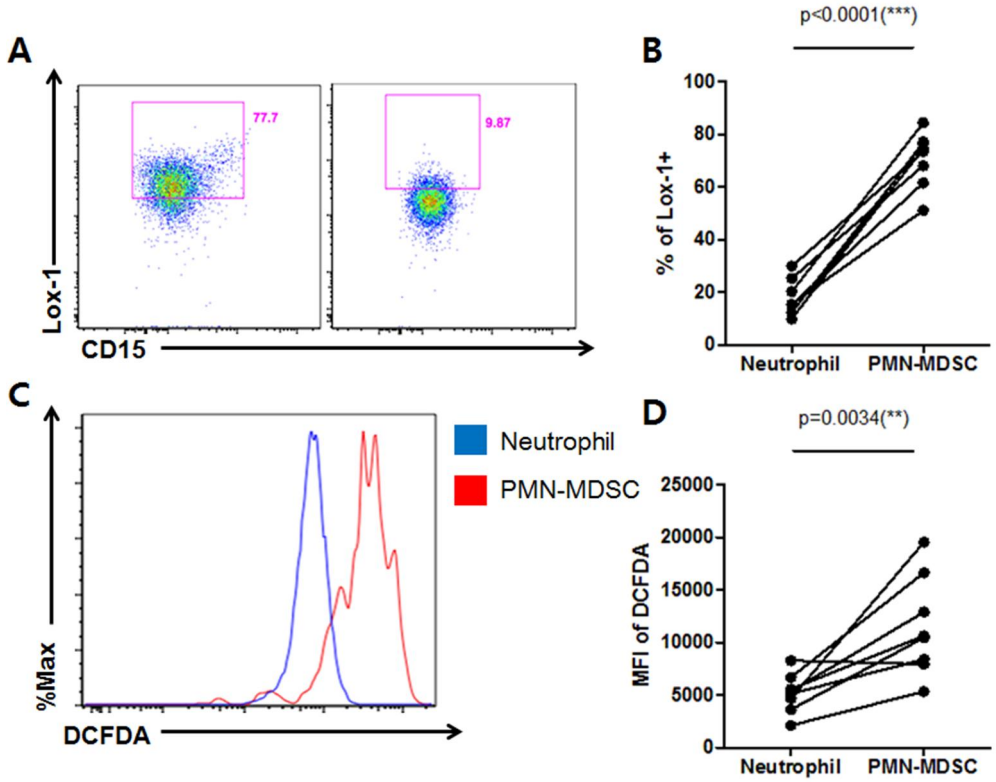


Figure 8. PMN-MDSC has higher Lox-1 and ROS production level compare to the neutrophil. Flow cytometric plot of Lox-1+ neutrophil and PMN-MDSC population (A). PMN-MDSC showed higher Lox-1+ population level then the neutrophil (B). ROS production in neutrophil and PMN-MDSC were detected by DCFDA and were quantified by Geom.MFI of DCFDA (C). PMN-MDSC showed higher ROS production than the neutrophil (D). Single dot indicates single patient, *p<0.05.

High expression of MDSC-related chemokines and cytokines might block the beneficial effect of the drug in non-responders of anti-PD-1 therapy

To further investigate the systemic effect of the anti-PD-1 drug response, we checked 40 kinds of chemokines and cytokines in plasma of non-small cell cancer patients before the therapy (Figure 9A) and 2~3 weeks after the first therapy (Figure 9B). As a result, 9 out of 40 molecules showed significant differences between non-responder and responder group. Chemokines and cytokines such as IL-1β, IL-6, CXCL8(IL-8), CCL2 and CCL20 which are related with the differentiation, activation, expansion and suppressive function of MDSC were upregulated in non-responder group compare to the responder group. Inhibition of CCL23 is known to decrease the Treg migration[46]. CXCL8 and CCL20 were both downregulated in baseline and after the first therapy (Figure 9A, B). CXCL8 is the major ligand of CXCR2, which strongly contribute to the MDSC recruitment[35] and its plasma level was dramatically higher in non-responder group than the responder group (Figure 9A, B). CCL27 is the only one chemokine that showed upregulated pattern in responder group than the non-responder group after the first therapy (Figure 9B). We additionally checked plasma level of S100A8/9 heterodimer using ELISA (Figure 10). S100A8/9 is well-known as a factor that related to the function of MDSC, and it also showed significantly higher pattern in non-responder whereas decreased pattern in responder (Figure 10).

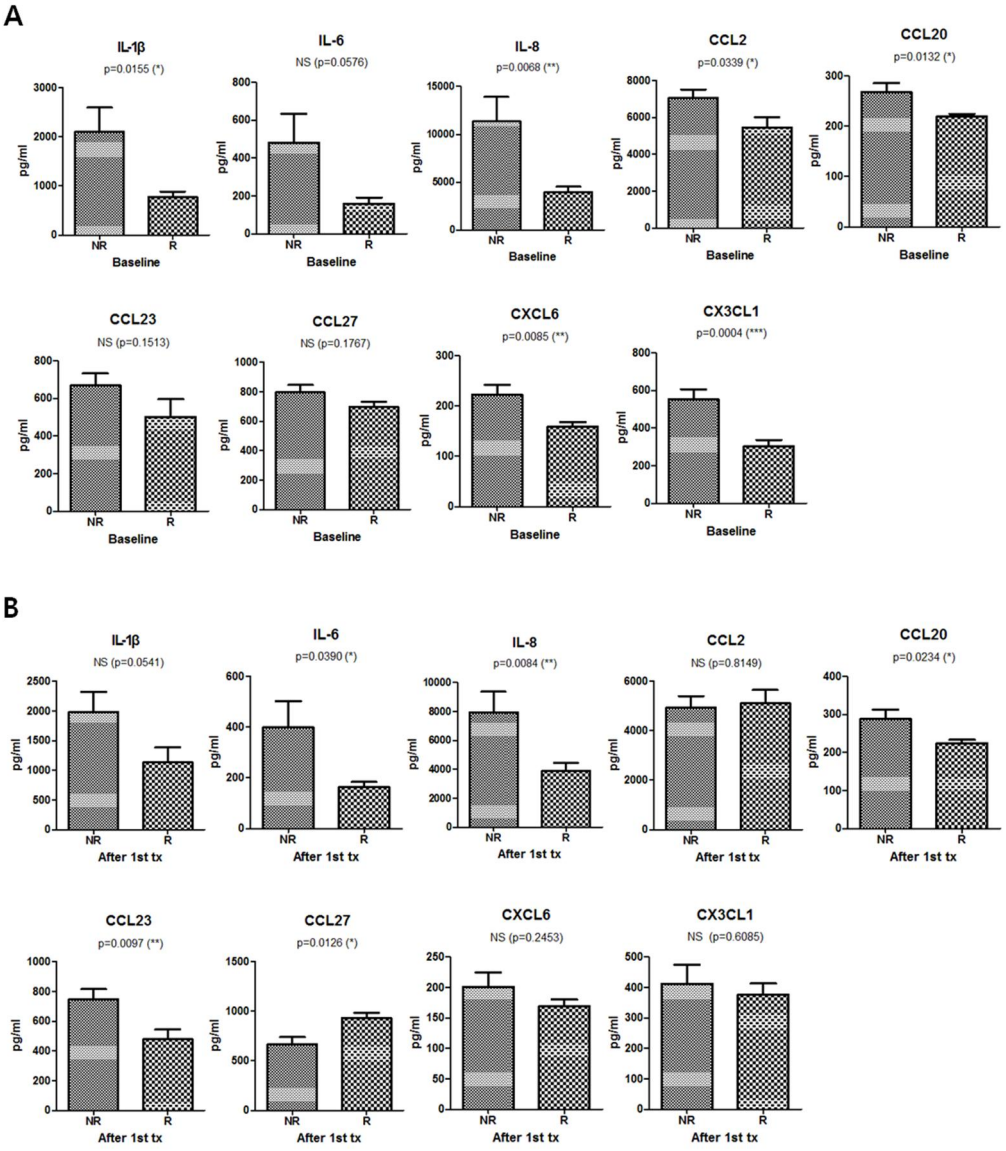


Figure 9. MDSC-related chemokines and cytokines were significantly upregulated in plasma of non-responder group compare to the responder group. Multi-plex chemokine/cytokine assay for the plasma of non-small cell lung cancer patient before the therapy (A) and 2~3 weeks after the first

therapy (B). Any molecules that showed significant difference between non-responder and responder group in either baseline or after first therapy are represented. Bar graphs represent concentration of target molecules(pg/ml) in plasma and small horizontal lines indicate the mean(±SD), *$p<0.05$.

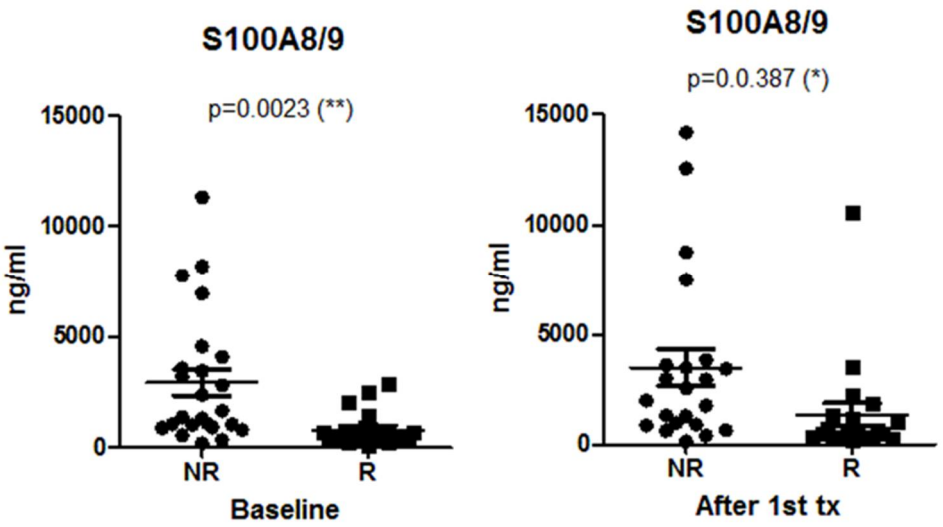


Figure 10. Plasmic S100A8/9 heterodimer level was upregulated in non-responder group compare to the responder group. Soluble S100A8/9 level in plasma of the each non-responder and responder group were detected by ELISA in baseline and after the first therapy. Dot graphs represent concentration of S100A8/9(ng/ml) in plasma and small horizontal lines indicate the mean(±SD). Single dot indicates single patient, *$p<0.05$.

IV. Discussion

In this study, we monitored and evaluated the profiles of circulating lymphocyte and MDSC in patients with NSCLC receiving anti-PD-1 (Nivolumab or Pembrolizumab) at baseline and every cycle of the therapy. We analyzed PBMC by multicolor-flow cytometry for B cell, NK cell, NKT cell, T cell, CD4+ T cell, CD8+ T cell and Treg cell for lymphocyte group, CD14-CD11b+CD33+CD15+PMN-MDSC, Lox-1+PMN-MDSC and HLA-DR-CD11b+CD14+ M-MDSC for MDSC group. The goal of this study was to find the reliable biomarker for anti-PD-1 therapies that can predict the drug response before the administration or at the early cycle of the therapy. Because of their highly expensive drug cost, relatively low response rate in NSCLC and other solid tumors compare to the Hodgkin lymphoma[22] and big individual variation of drug effect cause difficult situations to both physicians and patients.

Expression status of PD-L1, the binding ligand of PD-1, in tumor tissue has been conventionally used as a predictive biomarker for the anti-PD-1 therapy but has many problem issues. Even though the NSCLC patients who have PD-L1+ tumor and treated with pembrolizumab showed 45.2% of objective response rate (ORR)[21], but still the problems are remained. PD-L1 negative patients also showed the drug response and in case of Nivolumab, the prediction accuracy is still low; 18% ORR for squamous NSCLC[15], 31% ORR for non-squamous NSCLC[14] patients with PD-L1+ tumor. In addition, dynamic expression of PD-L1 during the T cell recognition or activation, heterogeneous expression pattern within same individual, and

unstandardized detection methods that using three different primary antibodies and protocols[7, 47] are the main obstacles[7].

Here we found Treg/Lox-1+PMN-MDSC ratio as a peripheral biomarker for anti-PD-1 therapy in advanced NSCLC patients. Treg/Lox-1+PMN-MDSC ratio was significantly high in responder at baseline and after 1st tx, and also showed better sensitivity and specificity compare to the IHC-based PD-L1 tumor cell expression biomarker (data not shown). In concordance with our hypothesis, PMN-MDSC and Lox-1+PMN-MDSC showed decreased pattern in responder. We further confirmed that % of Lox-1+ and the ROS production in PMN-MDSC were significantly higher than the neutrophil (Figure 6). In addition, various chemokines and cytokines such as IL-1β, IL-6, CXCL8, CXCL6, CCL2, CCL20 and CCL23 that have suppressive function or related to the MDSC activation, survival and trafficking were significantly upregulated in plasma of non-responders compare to the responders (Figure 7A-B).

IL-1β and IL-6 are well known as a regulator of MDSC accumulation and activation[28, 29, 48-50]. IL-1β and IL-6 act as a suppressive cytokines by driving the expression of TGF-β, arginase1 (ARG1) and iNOS each through the JAK1/STAT1 and JAK1, JAK3/STAT6 signaling axis[28, 48]. CXCR2 (IL-8Rβ) is known as the regulator of recruitment of neutrophils into inflammatory site[51, 52] or MDSC tumor trafficking[35, 53]. Many studies have found that the elevated serum level of CXCR2 and its ligands in cancer patients are related to the poor prognosis[35, 51, 53-56]. According to our multi-plex chemokine/cytokine assay, the level of CXCL8 (IL-8) and CXCL6, which are the ligand of CXCR2, were higher in serum of non-responders. Tumor-derived CXCL8 is known to chemoattracts both PMN-

MDSC and M-MDSC, and induce the formation of neutrophil extracellular traps (NETs) in PMN-MDSC[57] and also attract tumor-associated neutrophils(TANs) to the tumor site in Ras+ cancer[53, 58, 59]. CXCL8 showed elevated pattern in serum level of patients with poor prognosis in various cancer types; metastatic sarcoma[35], colon cancer[53, 54], NSCLC[53, 60, 61] and many other cancer types[53]. CXCL6 is known to stimulate the accumulation and recruitment of bone marrow-derived fibroblast and collagen I expression in renal fibrosis[62]. They also help invasion and proliferation of cancer through the β-catenin-mediated pathway in fibroblast[63].

CCL2 is the well-known key regulator for the chemotaxis of monocyte and MDSC to the tumor site and deficiency of CCR2, which is the receptor of CCL2, on MDSC cause defective MDSC migration and tumor progression [29, 64-66]. We found that the serum level of CX3CL1 was significantly higher in non-responders at baseline. CX3CL1 is expressed on lymphocytes, neurons, microglial cells and osteoblasts[67] and associated with T cell infiltration[68]. The soluble form of CX3CL1 can released after the cleavage by disintegrin and metalloproteinase domain-containing protein 17 (ADAM17)[69], and interestingly, ADAM17 is related to the suppressive function of MDSC[70]. ADAM17 expressed on MDSC decreases CD62L expression of naïve CD4+ and CD8+ T cells to inhibit the recirculation of T cells into draining lymph nodes[70]. In addition, Herbst et al. demonstrated that high expression level of CX3CL1 in pretreatment tumor is correlated with prognosis of cancer patient treated with anti-PD-L1, so that it has strong potential to be applied as a biomarker of anti-PD-L1 therapy[68]. There was a report that the cross-talk between M2 type macrophage and cancer cell via upregulated CCR2 and CX3CR1, which is the receptor of CX3CL1, drives

the lung cancer carcinogenesis and metastasis[65]. Collectively, upregulated serum level of CCL2 and CX3CL1 in non-responders may explained by their high peripheral MDSC that highly express CCR2, CX3CR1 and ADAM17. CCL20, which also showed higher serum level in non-responder, from glioma can induce the production of CCL2 from macrophages to recruits Treg and differentiate monocytes into M-MDSC and help their accumulation[66].

CCL23 did not show any big difference between non-responders and responders in baseline phase, whereas it was highly downregulated in responder group after the first infusion. Yan et al showed that peripheral CCL23 expression of breast cancer patients is correlated with poor survival and metastatic status[71]. The blocking of CCL23 significantly decreased the frequency of Treg migration and increased the CD4+/CD8+ T cell ratio[46].

We additionally checked serum level of S100 calcium-binding protein A8 (S100A8) and S100A9 using ELISA and it was also upregulated in non-responders compare to responders at both point of baseline and after first therapy (Figure 8). The pro-inflammatory S100A8/9, which expression is induced by STAT3, is the well-known regulator of MDSC expansion and recruitment as reported in a number of studies about MDSC[28, 29, 48, 70]. These results indicate that the poor drug response and prognosis of non-responders were may induced by the high suppressive function of ROS-producing Lox-1+PMN-MDSC and MDSC-related chemokines that promote tumor progression.

However, the peripheral level of Treg was totally opposite from our first

expectation; it was in high level in responder, rather than the lower level. We could find several reports showed that peripheral Treg showed higher pattern in responding group of anti-PD-1 (Nivolumab) with metastatic melanoma[72] and high peripheral Treg group represent better overall survival(OS) in anti-CTLA-4 (Ipilimumab) treated advanced melanoma[37], but the mechanism or rationale is unknown. As a result of the 40-pannel of chemokine/cytokine assay, 9 molecules showed the significant difference between non-responders and responders, and among that 9 molecules, only CCL27 showed higher pattern in responder after the first treatment which is the same pattern as Treg. Several reports demonstrate the antitumor activity of CCL27; tumor cells infected with recombinant adenoviral vector encoding CCL27 showed suppressed tumor growth in CD4+ and CD8+ T cell dependent manner and promote the infiltration and accumulation of CD3+ lymphocytes and NK cells in tumor[73]. Another study of the same group also found that alone or co-transduction of CCL27 gene with IL-2 into tumor cells can improve CD4+ and CD8+ T cell infiltration and tumor-infiltration immune cells in mouse tumor model[74].

In conclusion, we demonstrated that the frequency of peripheral Treg is higher in responder, whereas Lox-1+ PMN-MDSC is decreased in anti-PD-1 treated non-small cell lung cancer patients. Upregulation of MDSC-related chemokines and cytokines in non-responder group strongly support the poor drug responses of non-responders. Thereby we proposed that peripheral Treg/Lox-1+ PMN-MDSC ratio can serve as a promising biomarker for anti-PD-1 therapy.

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국문 초록

비소세포성 폐암에서 anti-PD-1 therapy 약물반응의 cellular biomarker 발굴 연구

최근 몇 년 사이에 활발하게 사용되고 연구되고 있는 면역관문억제제는 항암 면역치료의 새로운 시대를 열었다. Programmed cell death protein1 (PD-1)과 그것의 리간드인 Programmed cell death protein ligand1 (PD-L1)을 타깃으로 하여 그것의 기능을 막으려는 전략은 많은 임상 및 전임상 연구에서 놀라운 결과들을 보여주었다. 완전히 인간화된 항-PD-1의 치료용 항체인 Nivolumab과 Pembrolizuman은 다양한 암 종에서 항암 면역치료제로 사용되어 왔다. 그것들의 강력한 항암효과, 긴 지속시간 그리고 허용 가능한 만큼의 부작용 때문에 제약시장에서 큰 성공을 거둘 수 있었다. 하지만, 환자가 부담해야 하는 높은 비용과 낮은 약물 반응률은 약의 실질적이고 통상적인 사용을 제한하였다. 약물반응을 미리 예측할 수 있는 생체표지자들을 발견하려는 수많은 시도들에도 불구하고, 지금까지 믿을 만 한 표지자는 발견되지 않았다. 항-PD-1 치료의 생체표지자로서 면역조직화학염색법에 의한 종양내의 PD-L1 발현 상태가 이용되어 왔으나, 예측의 정확성이나 표준화되지 않은 프로토콜들에 관한 문제들이 계속 제기되어 왔다. 여기에 우리는 기존의 면역조직화학 염색법을 기반으로 하는 종양 PD-L1 발현 표지자 보다 더 정확하고 간단한 방법으로 항-PD-1 약물 반응을 예측할 수 있는 세포성 생체표지자를 발견하였다. 우리는 면역관문억제제에 대한 약물 반응은 환자 개인의 전신 면역체계 상태에 의존적이라는 가설 하에 면역 활성 세포집단으로써 림프구들을, 면역 억제 세포집단으로써는 골수유래면역억제세포를 확인하였다. 항-PD-1 약물의 첫 투여 이후 말초 혈액의 조절

T 세포/Lectin-type oxidized LDL-receptor-1 (Lox-1) PMN-MDSC 비율은 약물 양성반응 그룹에서 유의하게 증가되었다. 그것의 cut-off 값은 환자 예후 예측에 있어서 뛰어난 정확성을 보였고, 따라서 우리는 그것을 항-PD-1 치료의 강력한 생체표지자로 기대하고 있다.

중심 단어 : 면역관문억제제, 항-PD-1 치료, 비소세포성 폐암, 생체표지자, 골수유래면역억제세포, 조절 T 세포, Lectin-type oxidized LDL receptor-1