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Influence of chemotherapy on nitric oxide synthase, indole-amine-2,3-dioxygenase and CD124 expression in granulocytes and monocytes of non-small cell lung cancer

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There is no specific marker to evaluate the immuno-suppressive status of cancer patients. Several markers, such as CD124, latency-associated peptide (LAP), arginase I, indole-amine-2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS), are known to be associated with immune suppression. However, there is little research regarding the change in these parameters after chemotherapy. The present study enrolled 23 chemo-naïve non-small cell lung cancer (NSCLC) patients and 19 healthy donors. From the 23 NSCLC patients, 11 post-chemotherapy samples were collected. Surface and functional markers were analyzed by flow-cytometry. The mean fluorescence intensities (MFI) of iNOS were higher and the MFI of LAP were lower in NSCLC patient than in healthy donors ($P < 0.05$). In a comparison of pre-chemotherapy and post-chemotherapy groups with NSCLC, the MFI of iNOS on granulocytes and monocytes and IDO on monocytes were significantly lower in the post-chemotherapy group than in the pre-chemotherapy group ($P < 0.05$). In a serial analysis with 10 patients who had paired samples and who showed clinical benefits from chemotherapy, the MFI of iNOS for both cell types, and of IDO and CD124 for monocytes decreased significantly after chemotherapy, compared with those before chemotherapy (iNOS, 4.79 ± 1.75 *vs* 2.83 ± 0.77, $P = 0.005$, for granulocytes and 6.15 ± 2.94 *vs* 2.76 ± 1.05, $P = 0.005$ for monocytes; IDO, 6.81 ± 3.43 *vs* 4.64 ± 1.55, $P = 0.012$ for monocytes; CD124, 2.31 ± 0.39 *vs* 1.94 ± 0.43, $P = 0.008$ for monocytes). The changes in arginase I and LAP expression were not significant. The changes in iNOS, IDO and CD124 expression were significant after chemotherapy in NSCLC. Further evaluation of the possibility of immune status monitoring using these parameters is needed. (*Cancer Sci* 2012; 103: 155–160)

The role of the immune system in tumor occurrence, progression and inhibition has been debated for many years. Swann *et al.* (2007) has revealed that tumor cells can escape the immune reaction by various means; one of these is immune suppression, which could depend on various tumor and host factors, including tumor-induced immune suppressor cells.[1]

One of these cells is known as the myeloid-derived suppressor cell (MDSC). Human MDSC phenotypes have not been clearly established, although MDSC phenotypes have been established in mice models.[2] Currently, human MDSC is regarded as a heterogeneous group having various morphological or functional phenotypes.[3] In cancer patients, MDSC can be found in tumor tissues, lymph nodes, in the spleen and in peripheral blood.[4,5] It has been reported that removal of cancer results in a decreased number of MDSC and that the reduction in MDSC improves the immune response in various cancer models.[3,6–8]

These cells adopt various means of suppressing the function of effector lymphocytes against tumor cells. Some studies suggest that several enzymes, such as arginase I (ARGI), indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS), as well as surface markers, such as latency associated peptide (LAP) and CD124, are related to immune suppression and tumor progression.[2,3,9–11] However, although these markers are known to be associated with immune suppressive cells and the presence of tumors, few have been investigated with regard to changes in these parameters after chemotherapy. Here, we investigate the differences in immune parameters between healthy people and advanced non-small cell lung cancer (NSCLC) patients. We also evaluate the change in these parameters after chemotherapy, which might be associated with the immune suppressive cells in advanced NSCLC patients.

Patients and Methods

Patients and healthy controls. The patients enrolled in this study had been diagnosed with stage IIIB/IV or recurrent NSCLC and had shown good performance from March 2008 to February 2009 at Seoul National University Hospital. Patients who had had a recent infection, who showed prior malignancy or who had received chemotherapy, including adjuvant treatment before blood sampling, were excluded. The healthy controls consisted of healthy volunteers who had had no recent infections and who were not taking any regular medication at the time of the evaluation.

Disease evaluation and blood samples. Peripheral blood samples were collected from patients and normal healthy volunteers. Baseline blood samples were collected within the 1 week before the beginning of chemotherapy. Post-treatment blood samples were collected after two cycles of chemotherapy and within 4 weeks after a chest computed tomography for response evaluation, which was done after cell count recovery from chemotherapy-related hematologic toxicity (white blood count ≥4000/uL, absolute neutrophil count >1500/uL, platelets ≥100 000/uL).

The TNM stage was evaluated according to the sixth edition of the American Joint Committee on Cancer staging system. Treatment response was evaluated after two cycles of chemotherapy according to the Response Evaluation Criteria in Solid Tumors Committee.[12]

The chemotherapy regimens involved a platinum-based combination. If the patient received granulocyte colony-stimulating

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factor or radiation therapy during the chemotherapy, the post-chemotherapy blood sample was excluded from the analysis. Patients who experienced neutropenic fever were also excluded as candidates in the post-treatment cell analysis.

Antibodies and flow cytometric analysis.

Cell surface staining. Whole blood from NSCLC patients and healthy donors was collected, and 100 μL of each aliquot was mixed with each antibody set. These were the CD3-FITC/CD20-PE/CD56-APC, CD4-FITC/CD8-PE/CD3-APC, CD15-FITC/CD124-PE/CD14-APC and CD15-FITC/LAP-PE/CD14-APC sets (Becton Dickinson, Franklin Lakes, NJ, USA). In addition, one aliquot was used as a negative control. The mixtures were incubated at room temperature in the dark for 20 min, except the LAP antibody, which was incubated for 45 min. Then, 2 mL of BD FACS lysing solution (Becton Dickinson) was added for red blood cell lysis. The aliquots were centrifuged at 140*g* for 5 min at 4°C after which a BD cytofixation buffer was added. The cells were analyzed by FACS.

Intracellular staining. The 100 uL of each aliquot was mixed with 2 mL of BD FACS lysing solution and this was incubated for 10 min. The aliquots were centrifuged at 1600 rpm for 5 min and BD Cytofix/Cytoperm solution (Becton Dickinson) was then added. They were then incubated for 20 min at 4°C. For intracellular staining, the antibody sets were used, which were CD15-FITC/CD14-APC, CD15-FITC/iNOS/CD14-APC, CD15-FITC/IDO/CD14-APC and CD15-FITC/ArgI/CD14-APC (anti-iNOS Ab: [R&D Systems, Minneapolis, MN, USA] MAB9502, clone 2DA-BA [aa 781-798], mouse IgG1; anti-IDO Ab: [Chemicon, Temecula, CA, USA] MAB5412, clone 10.1, mouse IgG3; anti-ArgI Ab: [Hycult Biotechnology, Plymouth Meeting, PA, USA] HM2162, mAb clone 6G3 and mouse IgG1). One aliquot was used as an isotype control.

For staining the iNOS, IDO, ARGI and secondary antibody (PE-Donkey anti-mouse IgG, Jackson 715-116-150; Jackson, West Grove, PA, USA) were used.

Statistical analysis. The probability of differences between the NSCLC group and the healthy donor group were assessed using the Mann–Whitney *U*-test. The subsequent analysis of the difference between pre-chemotherapy and post-chemotherapy paired samples was conducted using the Wilcoxon signed-rank test. Statistical significance was defined as $P < 0.05$.

Ethics. The study protocol was reviewed and approved by the Institutional Review Board of Seoul National University College of Medicine and Hospital. The recommendations of the World Medical Association Declaration of Helsinki for Ethical Principles for Medical Research Involving Human Subjects were also followed.

Results

Patients' characteristics. The present study enrolled 23 patients with histologically proven NSCLC and 19 healthy volunteers. Post-chemotherapy blood samples were obtained from 11 out of 23 patients. The median age of the healthy control group was lower than that of the cancer patient group (29.1 *vs* 59.7 years, $P < 0.001$). The median age was 59.7 years for the pre-treatment patient group ($n = 23$) and 65.2 years for the post-treatment patient group ($n = 11$). Adenocarcinoma was confirmed in 11 patients and squamous cell carcinoma in seven patients.

In the pre-chemotherapy group, the treatment responses were a partial response (PR) in seven patients, stable disease (SD) in 13 patients and progressive disease (PD) in three patients. In the post-chemotherapy group, the response was PR in four, SD in six, and PD in one patient (Table 1).

Comparison of immune parameters. Expression levels of iNOS, IDO, ARGI, CD124 and LAP were analyzed in cancer patients by age, gender and smoking status, and there was no significant difference in immune markers (Table 2).

Table 1. Characteristics of patients ($n = 23$) with advanced non-small cell lung cancer and healthy controls

Characteristics	Number (%)		*P*-value
	Chemotherapy patients ($n = 23$)	Healthy control ($n = 19$)	
Age			
Median	59.7	29.1	<0.001
Range	39–76	25–54	
Gender			
M	15 (65.2)	15 (78.9)	1.0
F	8 (34.8)	4 (21.1)	
Smoking			
Smoker	6 (26.1)	2	0.076
Ex-smoker	7 (30.4)	2	
Never-smoker	10 (43.5)	15	
Histology			
Adenocarcinoma	11 (47.8)		
Squamous cell carcinoma	7 (30.4)		
Others	5 (21.8)		
ECOG performance			
0	6 (26.1)		
1	15 (65.2)		
2	2 (8.7)		
T stage			
1	1 (4.4)		
2	8 (34.8)		
3	3 (13.0)		
4	11 (47.8)		
N stage			
0	4 (17.3)		
1	1 (4.4)		
2	8 (34.8)		
3	10 (43.5)		
Stage			
IIIB	4 (17.4)		
IV or recurred	19 (82.6)		
Response			
Partial response	7 (30.4)		
Stable disease	13 (56.5)		
Progressive disease	3 (13.1)		
Post-chemotherapy samples	11 (47.8)		

ECOG, Eastern Cooperative Oncology Group.

Lymphocyte subsets and intracellular enzyme expressions between advanced non-small cell lung cancer patients and healthy donors. The lymphocyte subsets were not significant, except for the B cell subset. The mean of the B cell subset in the NSCLC group was higher than in the healthy donors (14.95 *vs* 11.74), which was significant ($P = 0.03$) (Table 3).

In a comparison of the enzyme expression, iNOS expression for granulocytes and monocytes was higher in the NSCLC patients than in the healthy donors ($P = 0.028$ for granulocytes and $P = 0.037$ for monocytes). However, LAP expression for both granulocytes and monocytes was significantly lower in the NSCLC patients than in the healthy donors ($P = 0.018$ for granulocytes and $P = 0.006$ for monocytes). There were no significant differences in the other parameters (Table 4).

Lymphocyte subsets and intracellular enzyme expression between pre-chemotherapy and post-chemotherapy groups in paired samples. There were no significant changes in the lymphocyte subsets between the pre-chemotherapy and post-chemotherapy samples. In a comparison of the intracellular enzyme expression levels, iNOS expression levels were 4.69 ± 1.69 for granulocytes and 5.85 ± 2.97 for monocytes in

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Table 2. Immune marker expression analysis by clinical factors in cancer patients

	Age		*P*-value	Sex		*P*-value	Smoking		*P*-value
	≤60 (*n* = 11)	>60 (*n* = 12)		M (*n* = 15)	F (*n* = 8)		Smoker (*n* = 6)	Non-smoker (*n* = 17)	
Monocyte									
iNOS	4.64 ± 2.79	4.76 ± 2.44	0.902	4.35 ± 2.41	5.35 ± 2.86	0.401	3.56 ± 1.44	5.10 ± 2.77	0.256
IDO	5.74 ± 2.74	5.24 ± 3.09	0.498	4.88 ± 2.67	6.61 ± 3.07	0.156	4.63 ± 1.87	5.78 ± 3.15	0.658
ARGI	2.58 ± 0.63	2.38 ± 0.73	0.518	2.39 ± 0.65	2.64 ± 0.74	0.498	2.36 ± 0.64	2.52 ± 0.71	0.562
CD124	2.29 ± 0.45	2.15 ± 0.47	0.417	2.28 ± 0.40	2.12 ± 0.54	0.697	2.19 ± 0.56	2.23 ± 0.43	0.969
LAP	30.95 ± 19.52	36.51 ± 26.36	0.833	35.30 ± 20.89	31.53 ± 27.34	0.500	38.19 ± 26.71	32.13 ± 22.06	0.640
Granulocyte									
iNOS	3.46 ± 1.49	4.24 ± 1.77	0.295	3.78 ± 1.57	4.02 ± 1.90	1.000	3.08 ± 0.96	4.14 ± 1.78	0.263
IDO	4.41 ± 2.00	4.19 ± 1.92	0.926	3.86 ± 1.54	5.12 ± 2.37	0.272	3.58 ± 1.24	4.55 ± 2.07	0.462
ARGI	9.52 ± 2.32	9.75 ± 2.80	0.580	9.31 ± 2.33	10.26 ± 2.92	0.439	9.04 ± 1.25	9.85 ± 2.84	0.401
CD124	1.63 ± 0.28	1.64 ± 0.58	0.438	1.48 ± 0.13	1.89 ± 0.66	0.059	1.46 ± 0.07	1.70 ± 0.52	0.185
LAP	12.09 ± 9.71	10.95 ± 10.76	0.622	11.43 ± 8.61	11.59 ± 12.67	0.612	9.92 ± 5.40	12.12 ± 11.48	0.876

ARGI, arginase I; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; LAP, latency associated peptide.

Table 3. Comparison of percentage of lymphocyte subsets between non-small cell lung cancer (NSCLC) patients and healthy donors

Lymphocytes subset (%)	NSCLC patients (*n* = 23)			Healthy donors (*n* = 19)			*P* value
	Mean	Range	Median	Mean	Range	Median	
NK cell	18.43 ± 8.91	6.72–33.57	17.63	18.57 ± 7.33	4.08–31.09	21.05	0.715
B cell	14.95 ± 5.39	7.33–28.99	13.29	11.74 ± 4.28	5.36–19.94	10.63	0.034
$CD8^+$ T cell	26.27 ± 6.98	17.29–37.00	25.07	27.45 ± 6.58	11.31–43.21	26.73	0.361
$CD4^+$ T cell	39.90 ± 10.32	21.66–63.66	38.76	37.16 ± 8.89	22.24–57.26	38.13	0.351

Table 4. Comparison of intracellular enzyme expressions between non-small cell lung cancer (NSCLC) patients and healthy donors

Cell subset	Enzymes	NSCLC patients (*n* = 23)			Healthy donors (*n* = 19)			*P*-value
		Mean ± SD	Range	Median	Mean ± SD	Range	Median	
Granulocytes	iNOS	3.87 ± 1.66	1.40–8.59	3.5	2.58 ± 1.33	0.80–4.76	2.49	0.028
	IDO	4.30 ± 1.92	1.90–7.85	3.69	2.89 ± 0.34	2.58–4.02	2.81	0.079
	ARGI	9.65 ± 2.53	5.60–14.90	9.56	11.41 ± 2.88	7.05–16.50	10.48	0.107
	CD124	1.64 ± 0.47	1.15–3.29	1.64	1.47 ± 0.17	1.22–1.81	1.45	0.310
	LAP	11.49 ± 10.03	2.04–40.66	8.44	18.81 ± 13.54	3.39–61.34	18.44	0.018
Monocytes	iNOS	4.71 ± 2.56	1.03–11.69	3.29	2.95 ± 1.84	0.78–5.55	3.29	0.037
	IDO	5.48 ± 2.88	1.40–12.45	4.90	4.03 ± 0.50	3.28–5.05	4.08	0.130
	ARGI	2.48 ± 0.68	1.40–4.19	2.40	2.73 ± 0.41	2.03–3.44	2.78	0.113
	CD124	2.22 ± 0.46	1.38–2.84	2.30	2.27 ± 0.44	1.41–3.46	2.17	0.735
	LAP	33.87 ± 22.98	5.81–79.30	33.00	52.51 ± 14.60	29.73–82.47	50.61	0.006

ARGI, arginase I; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; LAP, latency associated peptide.

the pre-chemotherapy group and 2.77 ± 0.77 for granulocytes and 2.71 ± 1.02 for monocytes in the post-chemotherapy group (mean ± SD). The expressions decreased significantly in the post-chemotherapy group, compared with those in the pre-chemotherapy group ($P < 0.05$). The decrease in IDO expression was also significant for monocytes (6.50 ± 3.43 in the pre-chemotherapy group and 4.46 ± 1.58 in the post-chemotherapy group, $P = 0.008$), but not for granulocytes. The change in ARGI expression was not significant for both cell types. The differences in the CD124 expression levels for monocytes was marginal between the pre-chemotherapy and post-chemotherapy groups (2.22 ± 0.47 in the pre-chemotherapy group and 1.94 ± 041 in the post-chemotherapy group, $P = 0.059$).

Among the 11 patients who submitted both pre-chemotherapy and post-chemotherapy samples, only one patient showed a progressive disease response. When the data were analyzed for the 10 patients who had clinical benefits from chemotherapy (PR and SD), in addition to iNOS and IDO expression, the expression of CD124 for monocytes also showed a significant change ($P = 0.008$). The difference in LAP expression for granulocytes was marginal ($P = 0.051$) (Tables 5 and 6). The serial changes in the immune parameters between the pre-chemotherapy and post-chemotherapy results are plotted in Figure 1.

Discussion

The results of this study showed that there were significant differences and changes in several parameters, iNOS, IDO and CD124, which were associated with MDSC between advanced NSCLC patients and healthy donors and between pre-chemotherapy and post-chemotherapy status.

Altered immune status plays an important role in the development of cancer, and most patients with advanced cancer are immune-suppressed.[1] Studies have shown that various cells are

Table 5. Comparison of percentage of lymphocyte subsets between pre-chemotherapy and post-chemotherapy groups with paired samples

Lymphocytes subset	Pre-chemotherapy (n = 10)			Post-chemotherapy (n = 10)			P-value
	Mean	Range	Median	Mean	Range	Median	
NK cell	19.11 ± 11.35	7.25–33.57	14.36	24.56 ± 20.90	1.78–64.02	14.16	1.00
B cell	12.11 ± 3.31	7.3–17.26	12.45	14.72 ± 6.85	4.35–21.63	16.45	0.123
$CD8^+$ T cell	25.84 ± 7.82	17.66–37.00	24.54	23.34 ± 7.83	11.45–36.32	24.21	0.161
$CD4^+$ T cell	39.38 ± 8.07	26.76–48.78	41.81	40.44 ± 10.06	24.43–56.53	39.83	0.401

Table 6. Comparison of intracellular enzyme expressions between pre-chemotherapy and post-chemotherapy groups with paired samples

Cell subset	Enzymes	Pre-chemotherapy (n = 10)			Post-chemotherapy (n = 10)			P-value
		Mean ± SD	Range	Median	Mean ± SD	Range	Median	
Granulocytes	iNOS	4.79 ± 1.75	3.30–8.59	4.02	2.83 ± 0.77	1.70–4.42	2.68	0.005
	IDO	5.04 ± 2.12	2.40–7.85	5.18	4.59 ± 2.54	1.90–9.55	4.55	0.285
	ARGI	9.54 ± 2.75	5.60–13.31	9.90	10.73 ± 4.14	5.36–17.96	10.73	0.575
	CD124	1.62 ± 0.64	1.15–3.29	1.50	1.62 ± 0.37	1.08–2.10	1.65	0.575
	LAP	10.27 ± 10.14	2.67–35.88	7.40	11.52 ± 23.15	1.94–77.04	3.23	0.051
Monocytes	iNOS	6.15 ± 2.94	3.20–11.69	5.21	2.76 ± 1.05	0.73–4.36	2.92	0.005
	IDO	6.81 ± 3.43	2.77–12.45	5.95	4.64 ± 1.55	2.20–6.89	4.44	0.012
	ARGI	2.57 ± 0.81	1.57–4.19	2.50	2.51 ± 0.84	1.49–4.40	2.38	0.799
	CD124	2.31 ± 0.39	1.52–2.75	2.30	1.94 ± 0.43	1.23–2.50	2.03	0.008
	LAP	28.53 ± 17.07	8.86–79.30	17.07	21.28 ± 12.59	3.11–36.97	21.51	0.214

ARGI, arginase I; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; LAP, latency associated peptide.

involved in tumor-induced immune suppression. Among the various cells, MDSC are considered to be the major culprit in immune perturbation, as they are known to be associated with innate and adaptive immunity inhibition.[13]

Although many trials have sought to activate patients' immune function against cancer, no effective result has emerged so far.[13] For effective immune activation against cancer, it is necessary to attenuate immune suppression and to monitor the extent of suppression.

In a laboratory model, MDSC are known to be a heterogeneous population. However, MDSC can be classified as either granulocytic or monocytic.[14,15] The role of the two MDSC subsets is not clear, but it has been reported that granulocytic MDSC requires close cell-to-cell contact with T cells in the lymphoid organ and that monocytic MDSC suppresses the immune reaction by producing various cytokines without direct cell-to-cell contact.[3] In our study, all of the immune parameters were measured in the peripheral blood. This explains the high expression of several markers, including IDO and CD 124 for monocytes.

The surface markers of MDSC have not been defined clearly in the human model. However, recent studies have suggested several surface or functional markers. CD124 (IL-4Rα) expression is a known surface marker of MDSC that is associated with producing transforming growth factor (TGF)-β via IL-13 stimulation from natural killer T cells.[16] LAP expression cells are also known to be associated with immune suppressive function through TGF-β.[11] Moreover, iNOS, ARG and IDO are well-known functional markers of MDSC, which suppress the immune function by producing reactive oxygen species and depleting arginine or tryptophan.[8,17] In our study, there was lower expression of iNOS, IDO and CD124 in the post-chemotherapy than in the pre-chemotherapy samples.

This might be associated with the immune suppressive status of the patients. Most of the responses to the treatment were SD or PR. Although there was no definite change in the size of lesions as a result of chemotherapy, these patients obtained a clinical benefit from the treatment. The decreased tumor burden might have improved the immune status. Another hypothesis is that immune suppressor cells are reduced by chemotherapy. This can alter the immune status. As a result, the immune parameters might change. This might be an implication of the improved immune status after chemotherapy.

Dendritic cells with high LAP expression rates have been reported to have immune an suppressive function.[11] In our study, LAP expression on myeloid cells was lower in cancer patients than in healthy donors. Furthermore, LAP expression has a tendency to increase after chemotherapy.

Immune functions of the patients are influenced by various factors (e.g. age, gender and smoking status) in addition to tumor status. Our observation is focused mainly on changes in MDSC-related markers with anti-cancer chemotherapy. Interpretation of the results is limited by the fact that other confounding variables can influence the immune parameters. Thus, further study with a large number of cancer patient samples is warranted.

This study has several limitations. First, this study did not involve a comparison of the changes in the immune parameters between the partial response or stable disease group and the progressive disease group. Therefore, it is not clear whether these changes were caused by the clinical benefits from chemotherapy. Second, the immune parameters were evaluated in peripheral blood, which does not reflect the local immune status around the tumor.

However, there have been few studies regarding the presence of MDSC and its relationship with cancer in humans.[18–21] Moreover, most of the data concerns the correlation between cancer and MDSC counts in cross-sectional analyses. Our study showed serial changes in various markers which were associated with MDSC for both pre-chemotherapy and post-chemotherapy conditions. This suggests the possibility of immune status monitoring using immune parameters that are associated with MDSC.

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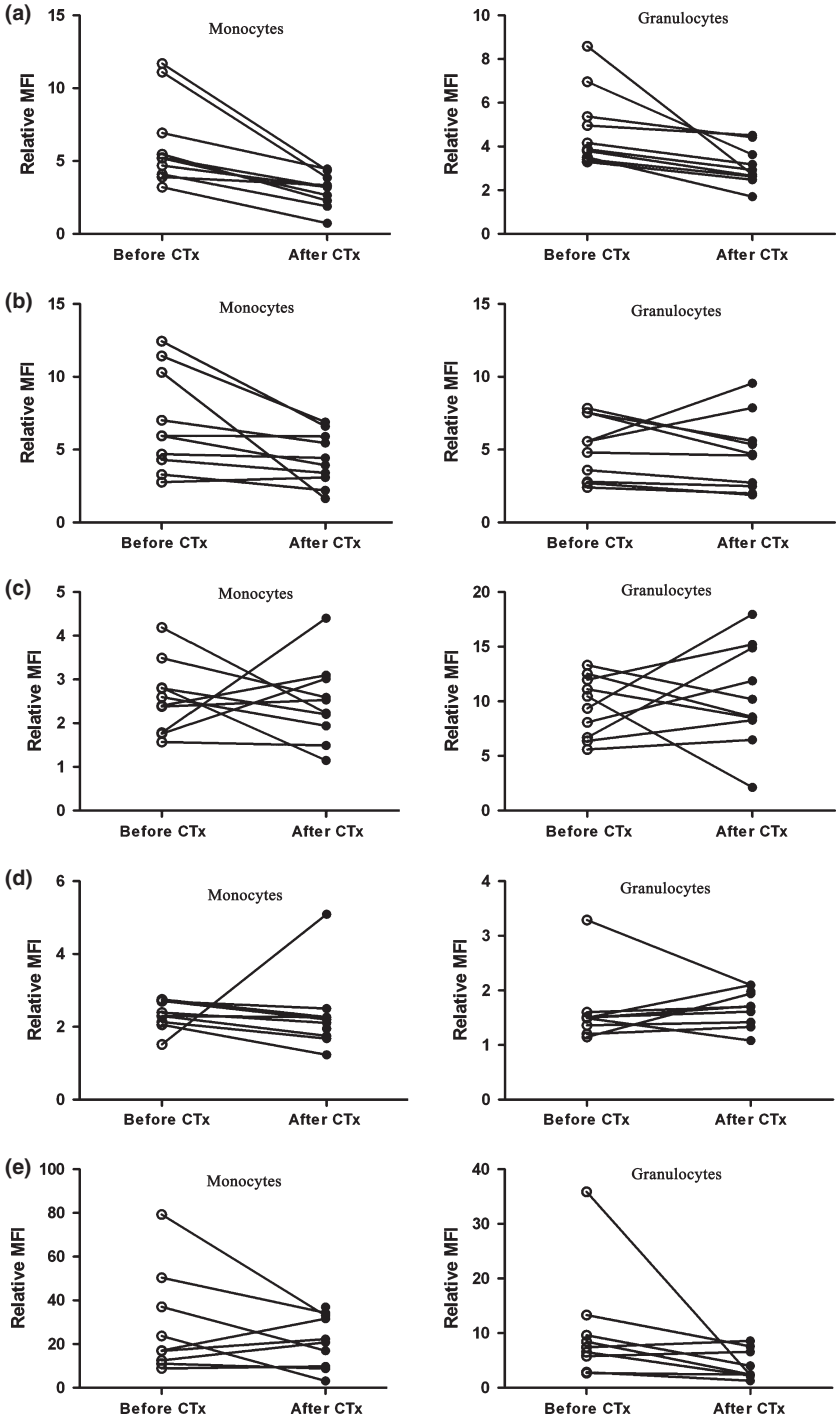

Fig. 1. Changes in the various immune parameters in the patients who had clinical benefits from chemotherapy: (a) inducible nitric oxide synthase expression for monocytes and granulocytes, (b) indole-amine-2,3-dioxygenase expression for monocytes and granulocytes, (c) arginase I expression for monocytes and granulocytes (d) CD124 expression for monocytes and granulocytes and (e) latency-associated peptide expression for monocytes and granulocytes. MFI, mean fluorescence intensities.

One recent report notes a change in the MDSC count after surgical treatment in NSCLC patients.[22] In that study, a specific subpopulation of mononuclear cells decreased after surgical removal of the tumor. This also suggests that specific cell subpopulations associated with MDSC are correlated with tumor burden.

In summary, several markers associated with MDSC showed significant differences between NSCLC patients and healthy donors. In a serial analysis between pre-chemotherapy and post-chemotherapy results, iNOS, IDO and CD124 expression decreased significantly after chemotherapy. Further study on the usefulness of monitoring these markers is warranted.

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Disclosure Statement

The authors have no conflict of interest

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