Postoperative Regulatory T-Cells and Natural Killer Cells in Stage I Nonsmall Cell Lung Cancer Underwent Video-assisted Thoracoscopic Lobectomy or Thoracotomy

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Abstract

Background: Regulatory T-cells (Treg) play key roles in suppressing cell-mediated immunity in cancer patients. Little is known about perioperative Treg fluctuations in nonsmall cell lung cancer (NSCLC). Video-assisted thoracoscopic (VATS) lobectomy, as a minimal invasive procedure for treating NSCLC, may have relatively less impact on the patient's immune system. This study aimed to observe perioperative dynamics of circulating Treg and natural killer (NK) cell levels in NSCLC patients who underwent major lobectomy by VATS or thoracotomy.

Methods: Totally, 98 consecutive patients with stage I NSCLC were recruited and assigned into VATS or thoracotomy groups. Peripheral blood samples were taken on 1-day prior to operation, postoperative days (PODs) 1, 3, 7, 30, and 90. Circulating Treg and NK cell counts were assayed by flow cytometry, defined as $CD4^+CD25^+CD127^{low}$ cells in $CD4^+$ lymphocytes and $CD56^+16^+CD3^-$ cells within $CD45^+$ leukocytes respectively. With SPSS software version 21.0 (SPSS Inc., USA), differences between VATS and thoracotomy groups were determined by one-way analysis of variance (ANOVA), and differences between preoperative baseline and PODs in each group were evaluated by one-way ANOVA Dunnett t-test.

Results: In both groups, postoperative Treg percentages were lower than preoperative status. No statistical difference was found between VATS and thoracotomy groups on PODs 1, 3, 7, and 30. On POD 90, Treg percentage in VATS group was significantly lower than in thoracotomy group (5.26 ± 2.75 vs. 6.99 ± 3.60, $P = 0.012$). However, a higher level of NK was found on all PODs except on POD 90 in VATS group, comparing to thoracotomy group.

Conclusions: Lower Treg level on POD 90 and higher NK levels on PODs 1, 3, 7, 30 in VATS group might imply better preserved cell-mediated immune function in NSCLC patients, than those in thoracotomy group.

Key words: Natural Killer Cell; Regulatory T-cell; Surgical Invasiveness; Tumor Immunology; Video-assisted Thoracoscopic Lobectomy

INTRODUCTION

Video-assisted thoracoscopic (VATS) lobectomy has gradually gained popularity,[1-3] and been accepted as an alternative procedure to thoracotomy for resectable lung cancer by National Comprehensive Cancer Network (NCCN) since 2013.[4-6] Interest has developed on whether VATS preserves better immune status and benefits long-term survival.[7,8] Regulatory T-cell (Treg), known as a negative regulator of the cellular immunity, has been considered as a negative prognostic factor for cancer progression. Little is known[9] on the influence of lobectomy on Treg population in lung cancer patients. Natural killer (NK) cells are known to play a significant role in tumor immunosurveillance through their ability to recognize, target, and directly destroy tumor cells without prior sensitization.

In this study, we examined peripheral levels of Treg and NK cells in stage I nonsmall cell lung cancer (NSCLC) patients undergoing lobectomy by VATS or thoracotomy, to disclose perioperative fluctuation of Treg and NK, as well as the correlation between surgical invasiveness and postoperative cell-mediated immune functions.

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METHODS

Study population

Totally, 110 consecutive patients with clinical stage I NSCLC in our team were recruited from October 2012 to November 2013. Preoperative evaluations and TNM stage were performed according to 2014 NCCN clinical guideline for NSCLC. Exclusion criteria were as follows: History of malignancies; receiving surgical or intervene operation in recent 1-year; receiving immune therapy including traditional Chinese medicine within 6 months; concomitant immune-enhancing or immune-suppressing diseases including lupus erythematosus, Crohn's disease, and myasthenia gravis; allergic constitution; acquired human immune deficiency syndrome; and any known infection by microorganisms. Ethical approval was given by the clinical research ethics committee of the Second Affiliated Hospital of Zhejiang University, School of Medicine. Informed consent for the study was obtained from all patients.

Surgical techniques

The choice of VATS or open procedure was made by the patient himself/herself and was independent to enrollment. All patients received identical anesthesia with selective one lung ventilation and major lung resection, followed by lymphadenectomy (4, 5, 6, 7, 8, 9, 10 stations for left side cases and 2, 3, 4, 7, 8, 9, 10 for right side ones. Levels 11-13 nodes were dissected during anatomic lobectomy and proceeding *in vitro* dissection). All resected nodes were individually labeled and submitted entirely for pathological examination. Compared with conventional posterolateral thoracotomy, our three-ports VATS technique used one 3–4 cm utility thoracotomy in the fourth or fifth intercostal space near anterior axillary line without rib spreading. Exploring port was designed in the seventh or eighth intercostal space on the middle axillary line, and an 1–2 cm auxiliary operation hole was made below the inferior angle of the scapula. No artificial pneumothorax was used in VATS. Severe pleural adhesions and completely fused fissures were no longer contraindications of VATS on anatomic grounds. In conditions of lymph nodes (LNs) firmly adhering to pulmonary vessels which imply a high risk of life-threatening hemorrhage, VATS procedures were converted to thoracotomy for safety reasons. Those whose dissected LNs were positive for metastasis were excluded from the study because subsequent adjuvant therapy could impact immune function. Postoperative pain control was achieved by patient-controlled intravenous or paravertebral analgesia within first 48 h followed by oral analgesics if indicated.

Blood sample collection, cell isolation, and antibody reagents

Two milliliters of heparinized peripheral venous blood samples were collected on 10 AM 1-day before surgery as a baseline, and at the same time on postoperative day (POD) 1, 3, 7, 30 and 90. Samples were immediately processed. Mononuclear cells were isolated from the peripheral blood by centrifugation over Histopaque (Hettich UNIVERSAL 320R, Germany). A flow cytometry of Treg and NK subpopulations was performed using appropriate isotype controls and the following markers:

- CD4⁻FITC (Cat No. 340133, BD Biosciences, USA)
- CD25⁻PE (Cat No. 341009, BD Biosciences)
- CD45APC (Cat No. 340943, BD Biosciences)
- CD127⁻PerCP-Cy5.5 (lot: 90916, BD Pharmingen, USA)
- CD3⁻FITC/CD16⁺56⁻PE (Cat No. 340042, BD Biosciences).

All these antibodies were mouse anti-human. Cell membrane staining of CD127 was performed according to the product application.

Flow cytometry

Flow cytometry was performed on a FACSCanoII instrument (BD Biosciences). Data were obtained and analyzed using the FACSDiva6.0 software (BD Biosciences). In order to identify the Treg subsets, mononuclear cells were gated on side scatter versus CD4. CD4⁺ cells were then analyzed for CD25 high expression and low expression of CD127. Treg percentage was defined as CD4⁺CD25⁺CD127low cells in CD4⁺ lymphocytes.[10-13] NK percentage was defined as CD56⁺16⁺CD3⁻ cells within CD45⁺ leukocytes.

Statistical analysis

SPSS version 21.0 (SPSS Inc., USA) was used for statistical analysis. Differences of age (years), tumor diameter (cm), number of dissected LN, operation duration (min) and blood loss (ml) between VATS and thoracotomy groups were determined by independent *t*-test. Chi-square and nonparametric test (Kruskal–Wallis test) were used to compare the distribution of sex and tumor histopathology in these two groups. Time trend and variances in Treg/NK level with the change in postoperative time in either VATS or thoracotomy group were calculated by repeated measures test of general linear model (GLM) according to Mauchly's test of sphericity results. Differences of Treg/NK level between VATS and thoracotomy groups were determined by one-way analysis of variance (ANOVA). Comparison of Treg/NK level in various times after the operation with preoperative level as a control in each group was carried out by one-way ANOVA Dunnett *t*-test. Equality of variances was confirmed by Levene's test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Clinical outcomes

In total, 2284 dissected regional LNs, 37 LNs from 12 patients were pathologically diagnosed as positive for metastasis. Those 12 patients were then confirmed as advanced stage NSCLC and were excluded from the study. Two VATS procedures were converted to open thoracotomy after encountering LNs firmly adhere to pulmonary vessels, imposing the high risk of massive hemorrhage. Eventually

98 stage I patients were enrolled, with 70 patients in VATS group and 28 in thoracotomy group. Clinical information is summarized in Table 1. No demographic differences were found between the two groups. No blood transfusion or blood product was required for any patient. There was no perioperative mortality or major complications in each group. No patient withdrew, and no death or tumor recurrence was observed during the 3-month follow-up.

Dynamics of circulating regulatory T-cells and natural killer levels after major lung resection

Flow cytometric analysis of Treg cells and NK cells in the peripheral blood of VATS and thoracotomy groups was demonstrated in Figures 1 and 2. Repeated measures test of GLM results demonstrated that there were significant differences of Treg/NK level in various times in each group ($F = 35.34$, $P = 0.000$ of NK, $F = 6.284$, $P = 0.000$ of Treg). Time trend was obvious in NK ($F = 72.972$, $P = 0.000$) but not significant in Treg ($F = 16.74$, $P = 0.185$). Level of NK was found increasing with the extension of postoperative recovery time in both VATS and thoracotomy groups.

Comparison of Treg/NK level in various times after operation with preoperative level as a control in each group was demonstrated in Table 2. In VATS group, all postoperative Treg levels were found statistically lower than preoperative status. In thoracotomy group, differently, significant lower Treg percentages was only found on POD 30 (5.76 ± 2.71, $P = 0.042$). In both groups, on POD 1, NK percentages were significantly lower than preoperative status, and there was a clear rising time trend. In thoracotomy group, NK population did not recover to preoperative level until POD 7. Nevertheless, in VATS group, NK population recovered to preoperative status on POD 3 and kept rising, and peripheral NK levels on PODs 7, 30, and 90 were statistically higher than preoperative level [Table 2].

There was no significant difference on preoperative Treg or NK levels between VATS and thoracotomy groups (Treg: 7.61 ± 3.08 vs. 8.01 ± 4.20, $P = 0.603$; NK: 18.44 ± 6.19 vs. 19.28 ± 7.63, $P = 0.572$). Furthermore, in comparison between Treg levels in VATS and thoracotomy groups, no significant difference was confirmed on PODs 1, 3, 7 and 30 (POD 1: 5.88 ± 2.81 vs. 5.98 ± 2.59, $P = 0.874$; POD 3: 5.83 ± 3.39 vs. 6.43 ± 3.30, $P = 0.426$; POD 7: 5.32 ± 3.15 vs. 6.39 ± 2.70, $P = 0.117$; POD 30: 5.05 ± 3.13 vs. 5.76 ± 2.71, $P = 0.295$), unless that on POD 90, Treg percentages in VATS group were significantly lower than those in thoracotomy group (5.26 ± 2.75 vs. 6.99 ± 3.60, $P = 0.012$) [Figure 3a]. Meanwhile, compared with thoracotomy group, in VATS group, higher levels of NK were found on all PODs except on POD 90 (15.38 ± 6.39 vs. 12.60 ± 5.68 on POD 1, $P = 0.024$; 19.15 ± 6.23 vs. 12.90 ± 4.54 on POD 3, $P = 0.000$; 20.78 ± 6.62 vs. 16.16 ± 7.40 on POD 7, $P = 0.003$; 24.86 ± 8.03 vs. 20.86 ± 7.02 on POD 30, $P = 0.023$; 25.90 ± 8.52 vs. 22.72 ± 7.34 on POD 90, $P = 0.087$) [Figure 3b]. The results show that in stage I NSCLC patients, VATS approach indicates less inhibition and faster recovery of circulating NK cells, compared to

thoracotomy. In our observation, the benefit to NK counts from less invasive VATS approach diminished on POD 90.

Discussion

In spite of improvements on surgical skills, new chemical agents, evolution of radiation therapy, prognosis of lung cancer remains poor, the curing rate for NSCLC is
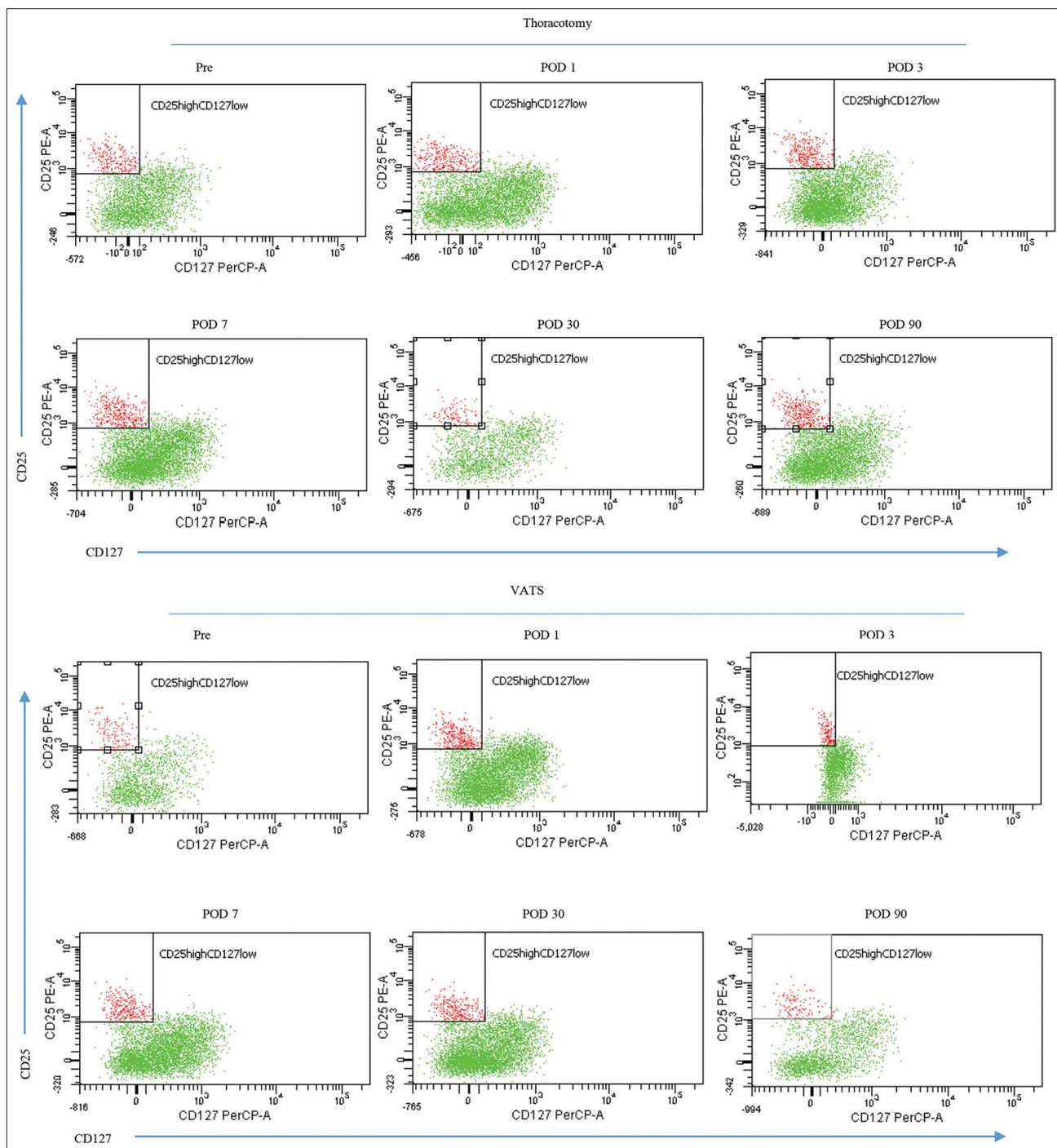
Table 1: Clinical demographics of patients in VATS and thoracotomy groups

Variables	VATS group (n=70)	Thoracotomy group (n=28)	P
Sex (male/female)	39/31	17/11	0.651
Age (years)*	63.1 ± 11.1	61.0 ± 7.8	0.294
Smoker/nonsmoker	44/26	18/10	0.895
Adeno/SCC/other†	34/32/4	15/12/1	0.613
Lobectomy/biolobectomy‡	68/2	27/1	1.000
Tumor diameter (cm)*	2.3 ± 1.2	2.6 ± 1.2	0.375
Number of dissected LN*	24.5 ± 6.7	22.9 ± 6.3	0.267
Operation duration (min)*	142.4 ± 22.3	134.1 ± 25.0	0.121
Blood loss (ml)*	74.7 ± 42.7	94.3 ± 49.0	0.055

*Mean ± SD; †Kruskal–Wallis test; ‡Chi-square tests-Fisher's exact test. Adeno: Adenocarcinoma; SCC: Squamous cell carcinoma; VATS: Video-assisted thoracoscopic; SD: Standard deviation; LN: Lymph node.

Table 2: Comparison of postoperative Treg/NK subpopulation with preoperative levels in VATS and thoracotomy groups by one-way ANOVA Dunnett t-test

Treg/NK		Sampling day	Value (%)	P
VATS				
	Treg	Preoperative	7.61 ± 3.08	
		POD 1	5.88 ± 2.81	0.004
		POD 3	5.83 ± 3.39	0.003
		POD 7	5.32 ± 3.15	0.000
		POD 30	5.05 ± 3.13	0.000
		POD 90	5.26 ± 2.75	0.000
	NK	Preoperative	18.44 ± 6.19	
		POD 1	15.38 ± 6.39	0.038
		POD 3	19.15 ± 6.23	0.961
		POD 7	20.78 ± 6.62	0.169
		POD 30	24.86 ± 8.03	0.000
		POD 90	25.90 ± 8.52	0.000
Thoracotomy				
	Treg	Preoperative	8.01 ± 4.20	
		POD 1	5.98 ± 2.59	0.079
		POD 3	6.43 ± 3.30	0.240
		POD 7	6.39 ± 2.70	0.219
		POD 30	5.76 ± 2.71	0.042
		POD 9	6.99 ± 3.60	0.645
	NK	Preoperative	19.28 ± 7.63	
		POD 1	12.60 ± 5.68	0.001
		POD 3	12.90 ± 4.54	0.002
		POD 7	16.16 ± 7.40	0.282
		POD 30	20.86 ± 7.02	0.848
		POD 90	22.72 ± 7.34	0.201

POD: Postoperative day; NK: Natural killer; VATS: Video-assisted thoracoscopic; Treg: Regulatory T-cells.

Figure 1: Representative dot plot of CD4+CD25+CD127low expression in the peripheral blood from patients undergoing thoracotomy (upper) or video-assisted thoracoscopic (lower). CD4+ cells were analyzed for CD25−PE and CD127−PerCP-Cy5.5 staining, CD25 high expression, and low expression of CD127 were then gated.

reported lower than 20%.[14] Treg is a subset of T-cells with immune-suppressive function,[15,16] defined[10,17-20] by CD4+CD25+FoxP3+/CD127low. Treg is considered to play key roles in suppressing cell-mediated immunity in lung cancer patients. In NSCLC patients, circulating Treg levels were elevated from an early stage,[9] and a higher level of Treg in tumor tissue, regional LNs, and peripheral blood is regarded as a negative prognostic factor.[16,21,22] Interestingly, peripheral blood Treg levels in advanced cancer patients

dropped significantly after effective chemotherapy or chemo-radiation therapy, parallel to clinical remission.[23-25]

As tumors may promote expansion, recruitment, and activation of Treg, surgical tumor removal may directly lead to a reduction of Treg population in cancer patients. After monitoring peripheral levels of Treg in gastric and esophageal cancer patients, Kono *et al.*[26] reported that elevated number of CD4+CD25+ Treg was significantly reduced after curative resection but increased again in
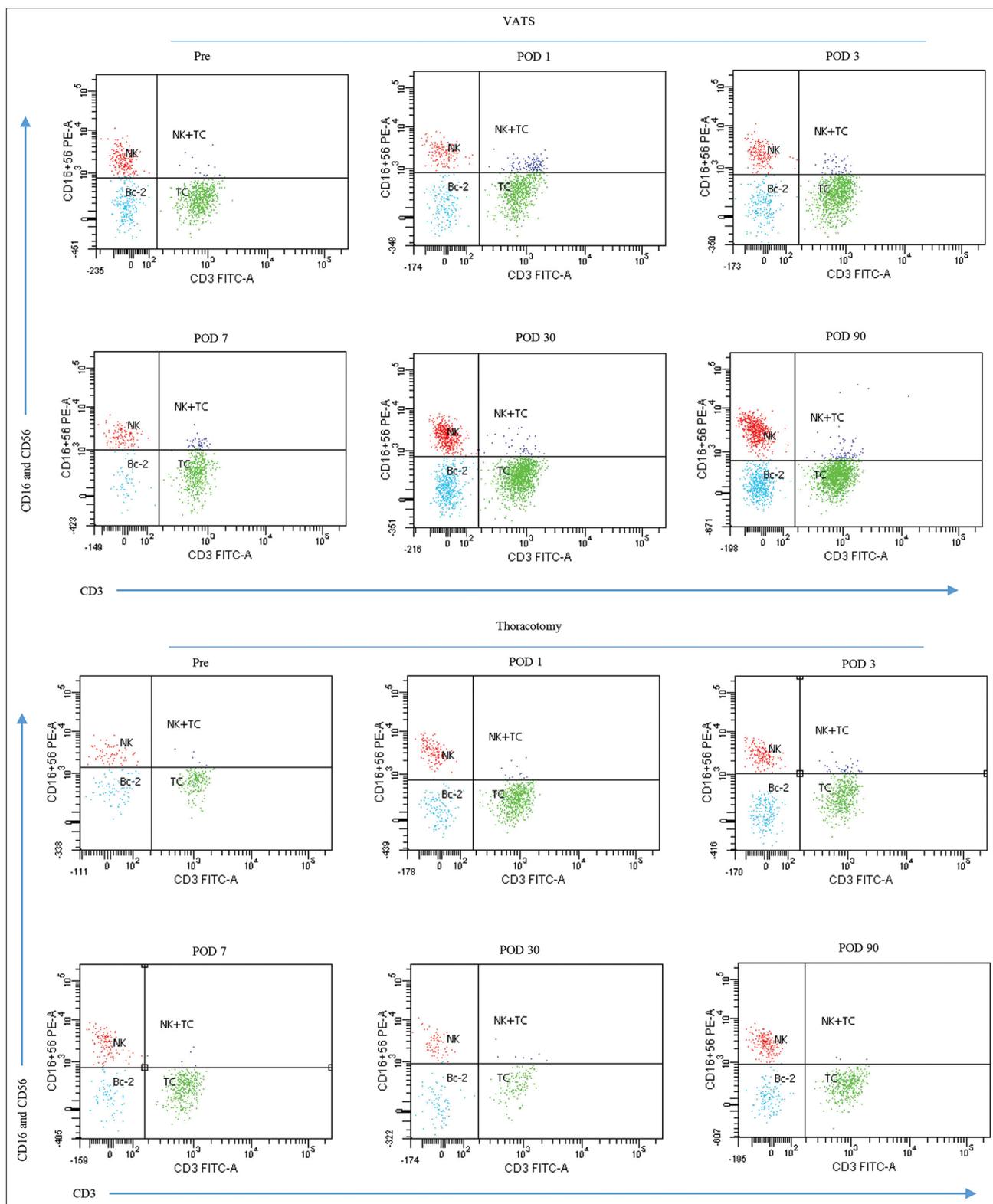
Figure 2: Representative dot plot of CD56$^+$16$^+$CD3$^-$ cells in the peripheral blood CD45$^+$ leukocytes from patients undergoing video-assisted thoracoscopic lobectomy (upper) or thoracotomy (lower). CD45$^+$ leukocytes were analyzed for CD3$^-$FITC/CD16$^+$56$^-$PE staining, subsequently CD16$^+$56 high expression, and negative expression of CD3 were gated.

the cases of cancer relapse. There are very few reports on perioperative Treg levels in lung cancer patients. Recently, Chen *et al.*[9] reported a direct assessment of the perioperative circulating Treg population in 36 NSCLC

patients, demonstrating decreased CD4$^+$CD25$^+$FoxP3$^+$ Treg percentages on POD 30 (preoperative vs. POD 30: 3.16% ± 1.32% vs. 2.46% ± 1.43%, P = 0.007). Similarly in our observation, in both VATS and thoracotomy groups,
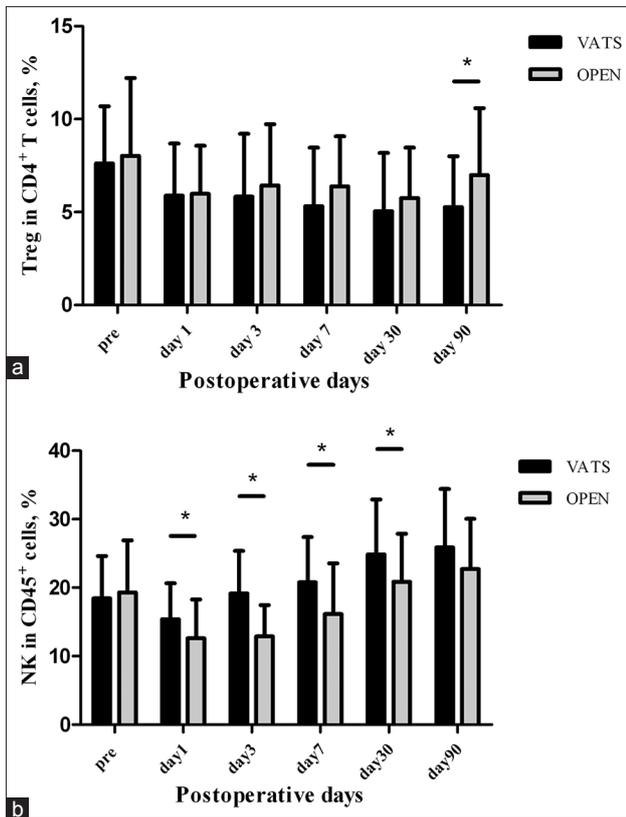
Figure 3: Comparison of peripheral regulatory T-cells (a) and natural killer cells (b) levels in patients undergoing video-assisted thoracoscopic lobectomy or thoracotomy by one-way ANOVA, *$P < 0.05$. Pre: preoperative.

$CD4^+CD25^+CD127^{low}$ Treg levels dropped significantly and remained below preoperative status within 30 days after surgery. Furthermore, it was suggested by Hanbo *et al*. that postoperative Treg values in early stage patients were not statistically different from Treg frequencies of control patients,[9] which implies that tumor mediated immune suppression by Treg may be completely eliminated after tumor removal in early NSCLC.

Meanwhile, although with controversy,[27] Saito *et al*.[28] suggested that Treg could also be an efficient indicative biomarker for surgical invasiveness. In their study, markedly lower levels of peripheral Treg were found after laparoscopic surgeries when compared to open ones. In this study, we examined circulating Treg in NSCLC patients undergoing major lung resection by VATS or thoracotomy. To minimize the influence of tumor progression on Treg level,[9,21,22,29] only stage I patients were enrolled. To our knowledge, it is a relatively early continuous observation on postoperative Treg population in stage I NSCLC cases, focusing on variances of surgical invasiveness by VATS or conventional thoracotomy.

Mechanisms of expansion, recruitment, and activation of Treg cells in postoperative patients are undoubtedly very complex, but tumor removal and surgical stress are believed to play important roles. Our study showed that peripheral $CD4^+CD25^+CD127^{low}$ Treg proportions significantly dropped

promptly after operations in both VATS and thoracotomy groups, but there was no significant difference between the two groups on PODs 1, 3, 7, and 30. This result suggests that within the 1st month postoperative, direct tumor removal ceases the tumor mediated Treg proliferation and release, and may be the most important factor on Treg reduction in early postoperative period. However, in VATS group, Treg proportion on POD 90 was found statistically lower than that in thoracotomy group. It may be explained that the diminished surgical trauma by VATS approach does not change the Treg level directly, but gradually induces less immune disturbance in multiple ways[7,8,30,31] and hence inhibits Treg for a longer time.

Less immune disturbance after VATS lobectomy was previously shown in several researches on plasma immunomodulatory mediators[7,8] and NK cells.[30,31] Notably, differences in nearly all of the postoperative cytokines and plasma immunomodulatory mediators measured thus far between VATS and thoracotomy groups are short-lived, in the order of hours to a couple of days.[7,8] In our study, on PODs 1, 3, 7, 30, NK cell numbers were found to be significantly higher following VATS lobectomy compared to the open approach, suggesting that VATS is associated with less inhibition and quicker recovery of NK cell than conventional thoracotomy. The rationale behind remains to be fully elucidated and actually encompasses numerous factors, including reduced acute-phase cytokine responses and postoperative pain, differences in patient expectations, and possible psychological effects.[30] On the contrary, the difference on Treg levels between VATS and thoracotomy group was not significant except on POD 90. This phenomenon suggests that, unlike plasma immune molecules and NK counts, difference of Treg levels between VATS and thoracotomy is a "delayed" and perhaps longer-lived marker for lesser immune disturbance. The mechanism is undoubtedly very complex and remains to be explored.[32]

Thus, VATS major lung resection for NSCLC is associated with less postoperative immune-suppression when compared with the thoracotomy approach. Nevertheless, whether a better-preserved postoperative immune status could be linked to survival benefit remains uncertain. Study including large-scale randomized trial and longer time follow-up recording multiple immune function markers after VATS and thoracotomy is needed for further evidence.[33] However, for the reasons of patients' intention[32] and 2015 NCCN recommendation for NSCLC, it's difficult to randomize patients into thoracotomy group. In addition, diversities in VATS technique may have additional effects on the postoperative immune markers.

The correlation between Treg proportions and histological subtypes in NSCLC was not taken into consideration in our study. Several reports claimed higher Treg proportions in adenocarcinoma than squamous carcinoma, but this difference seems existed in tumor and invaded LNs,[34] but not in peripheral blood.[9]

It is also noteworthy that identification of pure or naive immune-suppressive Treg population is still a challenge, explaining the varying results by different groups in clinical studies. Treg cells in cancer patients were initially determined by CD4 and CD25 co-expression.[35,36] Since the discovery of transcription factor FoxP3 in 2003, it became the most common marker for Treg cells. However, even the inclusion of FoxP3 assessment has been interpreted differentially when assessing frequencies of Treg cells in healthy individuals and cancer patients.[37,38] Furthermore, given that FoxP3 is an intracellular protein, detection of FoxP3 requires permeabilization of cells; hence this technique cannot be used to isolate viable Treg population.[39] Therefore, a unique or additional surface marker of Treg cells is needed. On basis of several reports,[10,12,13] the low cell-surface expression of CD127 allows for an accurate estimation of Treg numbers and the isolation of pure populations for *in vitro* studies, providing a flexible alternative to the transcription factor FoxP3. On the other hand, In contrast to $CD4^+CD25^+FoxP3^+$ cells, gating of a clear $CD4^+CD25^+CD127^{low/-}$ population was difficult.[39] Moreover, Klein *et al.*[39] demonstrated these two markers did not represent the same population of Treg cells. Recently, Beyer *et al.*[40] supported the superiority of combining CD127 and FoxP3 for further quantification of Treg cells in malignant diseases. In our study, $CD4^+CD25^+CD127^{low}$ were used to define Treg cells. The conclusion that VATS better preserves postoperative cellular immune function from our observation is not going to be confirmed unless the $CD4^+CD25^+CD127^{low}$ cells assessed in our study displayed immune depressive activity in future *in vitro* or even *in vivo* studies.

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