# Reciprocal $CD4^+$ T-Cell Balance of Effector $CD62L^{low}$ $CD4^+$ and $CD62L^{high}CD25^+$ $CD4^+$ Regulatory T Cells in Small Cell Lung Cancer Reflects Disease Stage

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**Abstract**

**Purpose:** Small cell lung cancer (SCLC) possesses high tendency to disseminate. However, SCLC patients with paraneoplastic syndrome mediated by immunity against onconeural antigens remain in limited-stage disease (LD) without distant metastases. Cumulative evidence regulates that a balance between immune and regulatory T (Treg) cells determines the magnitude of immune responses to not only self-antigens but also tumor-associated antigens. The purpose of this study was to elucidate the immunologic balance induced in SCLC patients.

**Experimental Design:** We analyzed T cells in the peripheral blood of 35 consecutive SCLC patients, 8 long-term survivors, and 19 healthy volunteers.

**Results:** Purified $CD4^+$ T cells with down-regulated expression of CD62L ($CD62L^{low}$) produced IFN-γ, interleukin (IL)-4, and IL-17, thus considered to be immune effector T cells (Teff). Significantly more Teff cell numbers were detected in LD-SCLC patients than that of extended-stage SCLC (ED-SCLC). By contrast, induction of $CD62L^{high}CD25^+$ $CD4^+$ Treg cells was significantly higher in ED-SCLC patients. Long-term survivors of SCLC maintained a high Teff to Treg cell ratio, whereas patients with recurrent disease exhibited a low Teff to Treg cell ratio. Teff cells in LD-SCLC patients included more IL-17–producing $CD4^+$ T cells (Th17). Moreover, dendritic cells derived from $CD14^+$ cells of LD-SCLC patients secreted more IL-23.

**Conclusion:** These results show that $CD4^+$ T-cell balance may be a biomarker that distinguishes ED-SCLC from LD-SCLC and predicts recurrence. This study also suggests the importance of inducing Teff cells, particularly Th17 cells, while eliminating Treg cells to control systemic dissemination of SCLC.

Small cell lung cancer (SCLC) is an aggressive disease with a strong tendency to disseminate. Approximately 15% to 20% of SCLC patients whose tumors are confined to the hemithorax and mediastinum and lack detectable distant metastases are considered to be limited-stage disease (LD). These LD-SCLC patients are often cured by treatment management. However, although no distant metastases are detected in LD-SCLC patients, regional treatments such as surgical resection or thoracic radiation therapy alone seldom result in a cure suggesting systemic micrometastases. Thus, repeated invasion by SCLC cells into the peripheral blood, some of which remain in the blood as circulating tumor cells, is considered to occur not only in extended-stage disease (ED) patients possessing prominent distant metastases but also in LD patients (1, 2). The reason why circulating tumor cells are unable to establish visible distant metastases in LD patients is unclear because no biological differences have been detected between tumor cells of LD-SCLC and ED-SCLC.

SCLC is considered to be a relatively immunogenic tumor because it occasionally causes paraneoplastic syndromes such as the Lambert-Eaton myasthenic syndrome (LEMS) mediated by an immunologic mechanism that recognizes shared onconeural antigens. Interestingly, SCLC patients suffering from LEMS tend to remain in long-term LD state and have favorable prognoses (3). Thus, it is postulated that the immune response, which attacks the neuromuscular system in LEMS patients, also fights the SCLC cells to constrain tumor progression.

In the immune system, regulatory $CD4^+$ T (Treg) cells with constitutive expression of the interleukin (IL)-2 receptor α chain (CD25) and the transcription factor forkhead box P3 (FOXP3) play a pivotal role in peripheral tolerance to self and

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### Translational Relevance

This study indicated that effector T cell to regulatory T cell ratio could be a useful biomarker to distinguish limited-stage disease from extended-stage small cell lung cancer and that therapy to increase Th17 and deplete regulatory T cells is a promising strategy.

non-self antigens, including tumor-associated antigens (4). It has been shown that Treg cell numbers increase in cancer-bearing patients and that the number of Treg cells correlates with the prognosis (5–10). We have reported that in addition to tumor-specific Treg cells, antitumor effector T (Teff) cells were generated in the same tumor-draining lymph nodes during tumor progression (11). T cells with down-regulated CD62L expression ($CD62L^{low}$) that were isolated from the tumor-draining lymph nodes mediated antitumor reactivity when infused i.v., resulting in the regression of established tumors (12, 13). When coinfused, Treg cells purified as $CD62L^{high}CD25^+$ $CD4^+$ from the same tumor-draining lymph nodes could inhibit the antitumor therapeutic efficacy of Teff cells. Importantly, the suppression of antitumor reactivity depended on the ratio of Teff to Treg cells (11). Taken together, it seems that the balance between induced $CD4^+$ Teff and Treg cells determines the immune responses against tumors. However, the $CD4^+$ T-cell balance in human malignancies has not been elucidated.

Here, we examined the peripheral blood mononuclear cells (PBMC) in 35 consecutive SCLC patients, 8 long-term survivors who had been disease-free for >3 years after treatment, and 19 healthy volunteers after obtaining a written informed consent. $CD62L^{low}$ $CD4^+$ T cells isolated from the peripheral blood exhibited primed effector T-cell function to secrete types 1 (Th1), 2 (Th2), and 17 (Th17) helper T-cell cytokines. On the other hand, the $CD62L^{high}CD25^+$ $CD4^+$ T-cell subpopulation showed cytosolic expression of FOXP3 and regulatory function to suppress cytokine production and inhibit the proliferation of Teff cells. A reciprocal balance was detected between the $CD62L^{high}CD25^+$ $CD4^+$ Treg cells and the effector $CD62L^{low}$ $CD4^+$ T cells. The former increased in ED-SCLC patients, and in contrast, the latter significantly increased without induction of Treg cells in LD-SCLC patients. Moreover, cytokine analyses revealed that the $CD62L^{low}$ $CD4^+$ T cells purified from LD-SCLC patients included more Th17 cells that produce preferentially IL-17 and that dendritic cells (DC) derived from $CD14^+$ cells of LD-SCLC patients produced more IL-23.

## Materials and Methods

***Patients.*** The present study comprised 35 consecutive SCLC patients, 8 long-term survivors, and 19 healthy volunteers from a single institution (Niigata University Medical and Dental Hospital, Niigata, Japan; Table 1). LD-SCLC patients who had been disease-free for >3 y after treatment were considered to be long-term survivors. Specimens were collected after obtaining written informed consent approved by the Niigata University Ethical Committee.

***Cell purification.*** PBMCs were obtained by centrifugation over Ficoll-Hypaque gradients. $CD4^+$ and $CD8^+$ T cells were purified by positive selection using $CD4^+$ or $CD8^+$ T-cell isolation kits (Dynal

**Table 1.** Patient characteristics

| Stage | Age | Gender |
|---|---|---|
| Healthy volunteer ($n$ = 19) | | |
| | 30 | M |
| | 29 | M |
| | 29 | M |
| | 32 | M |
| | 32 | M |
| | 36 | M |
| | 35 | M |
| | 31 | M |
| | 30 | M |
| | 64 | M |
| | 75 | M |
| | 67 | M |
| | 65 | M |
| | 56 | F |
| | 55 | M |
| | 57 | M |
| | 62 | F |
| | 59 | M |
| | 57 | M |
| Long-term survivors ($n$ = 8) | | |
| cT1N2M0 | 51 | M |
| cT4N2M0 | 62 | M |
| cT2N1M0 | 59 | M |
| cT4N2M0 | 64 | M |
| cT4N3M0 | 70 | M |
| pT1N0M0 | 80 | M |
| cT2N2M0 | 68 | M |
| pT1N0M0 | 68 | M |
| ED-SCLC ($n$ = 15) | | |
| cT1N3M1 | 66 | M |
| cT2N3M1 | 58 | M |
| cT1N3M1 | 61 | M |
| cT4N2M1 | 65 | M |
| cT4N3M1 | 62 | M |
| cT1N2M1 | 57 | F |
| cT1N3M1 | 58 | M |
| cT1N3M1 | 48 | M |
| cT4N2M1 | 62 | M |
| cT3N3M1 | 77 | M |
| cT1N3M1 | 63 | M |
| cT2N3M1 | 58 | M |
| cT1N2M1 | 59 | M |
| cT4N3M1 | 71 | F |
| cT1N3M1 | 58 | M |
| LD-SCLC ($n$ = 20) | | |
| cT2N2M0 | 72 | M |
| cT4N2M0 | 57 | M |
| cT4N3M0 | 55 | M |
| cT1N1M0 | 71 | M |
| cT1N3M0 | 62 | M |
| cT4N0M0 | 56 | M |
| cT3N2M0 | 63 | M |
| cT2N3M0 | 67 | M |
| cT2N0M0 | 64 | M |
| cT4N3M0 | 63 | M |
| cT1N2M0 | 66 | M |
| pT2N0M0 | 54 | M |
| cT1N2M0 | 69 | M |
| cT4N3M0 | 65 | M |
| cT1N0M0 | 57 | M |
| cT1N1M0 | 63 | M |
| cT4N3M0 | 71 | M |
| cT1N2M0 | 75 | M |
| cT1N1M0 | 67 | M |
| cT4N3M0 | 63 | F |

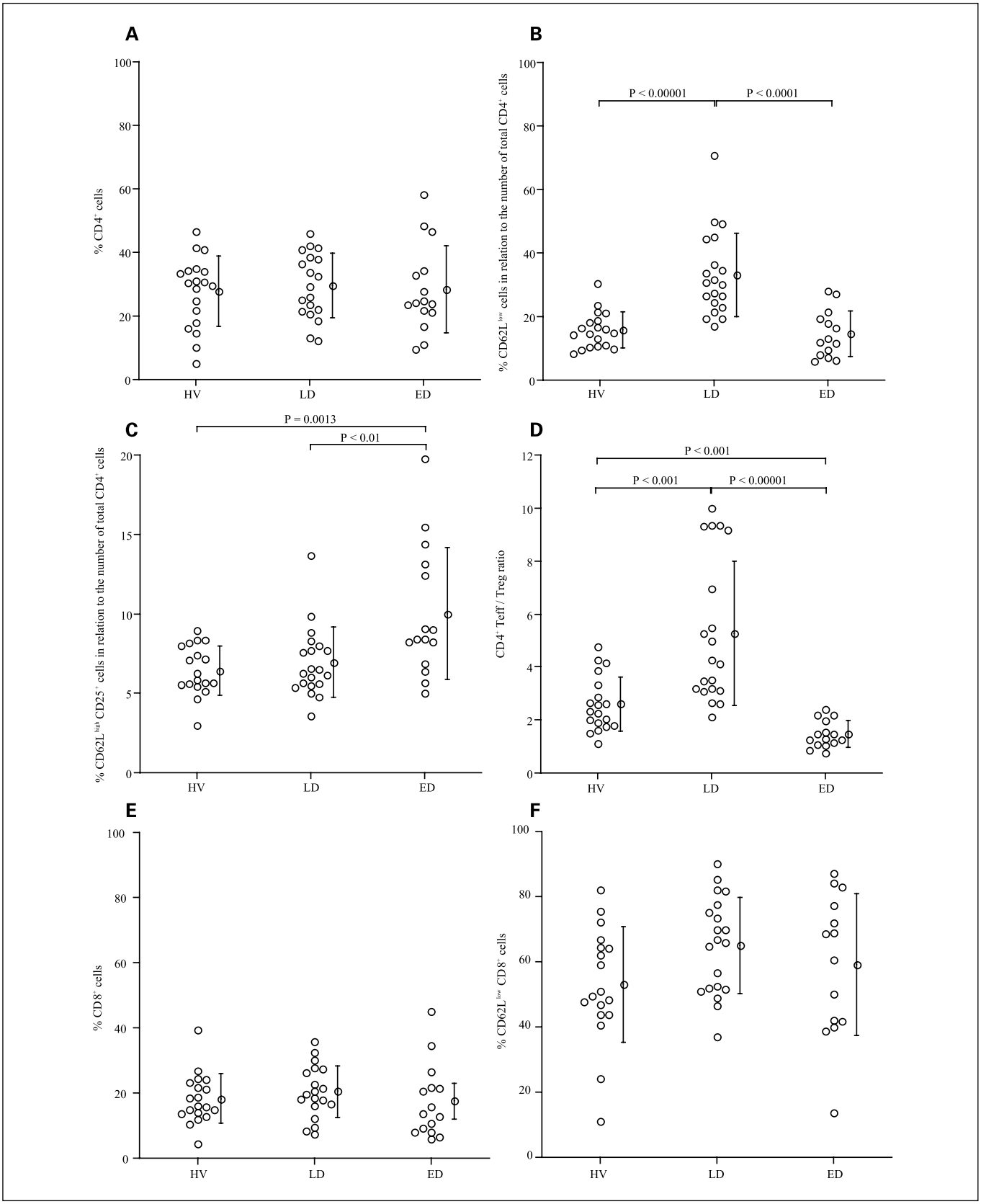


**Fig. 1.** PBMCs were stained with FITC-conjugated anti-CD62L, PE-Cy5–conjugated anti-CD25, PE-conjugated anti-CD4, or PE-conjugated anti-CD8 mAbs. *A* and *E,* percentages of $CD4^+$ and $CD8^+$ cells in relation to the total number of PBMCs in the lymphocyte region in forward and side scatter. *B* and *C,* percentages of $CD62L^{low}$ and $CD62L^{high}CD25^+$ cells in relation to $CD4^+$ cells in SCLC patients and healthy volunteers. *D,* ratio of $CD62L^{low}$ $CD4^+$ T cells to $CD62L^{high}CD25^+$ $CD4^+$ T cells. The ratios were calculated as follows: ratio = percentage of $CD62L^{low}$ cells/percentage of $CD62L^{high}$ $CD25^+$ cells. *F,* percentages of $CD62L^{low}$ in relation to $CD8^+$ cells.

Biotech). $CD4^+$ T cells were further separated into $CD62L^{high}$ and $CD62L^{low}$ cells. Cells were incubated with specific CD62L monoclonal antibody (mAb; 1H3) followed by a panning technique in T-25 flasks that were precoated with goat anti-mouse immunoglobulin antibodies (Jackson ImmunoResearch Laboratories). $CD62L^{high}$ T cells adhered to the plastic surface and could be easily obtained by scraping with a rubber policeman. To obtain highly purified $CD62L^{low}$ cells, the nonadherent cells were further depleted of CD62L-positive cells using anti-CD62L mAb and sheep anti-mouse immunoglobulin antibody-coated DynaBeads M-450 (Dynal Biotech) following the manufacturer's suggested procedure. $CD25^+$ cells were isolated by positive selection using anti-CD25 mAb-coated microbeads and autoMACS (Miltenyi Biotec). Cell purities were all >90%.

***Monocyte-derived DCs.*** DCs were generated from $CD14^+$ monocytes derived from peripheral blood. Briefly, $CD14^+$ cells were positively isolated using anti-CD14 mAb-coated microbeads (Miltenyi Biotec). The isolated $CD14^+$ cells were then cultured in complete medium (CM) supplemented with 80 ng/mL of recombinant human granulocyte macrophage colony-stimulating factor (a gift from Kirin) and 10 ng/mL IL-4 (eBioscience). On day 6, the nonadherent DCs were harvested by gentle pipetting. CM consists of RPMI 1640 supplemented with 10% heat-inactivated lipopolysaccharide-qualified FCS, 0.1 mmol/L

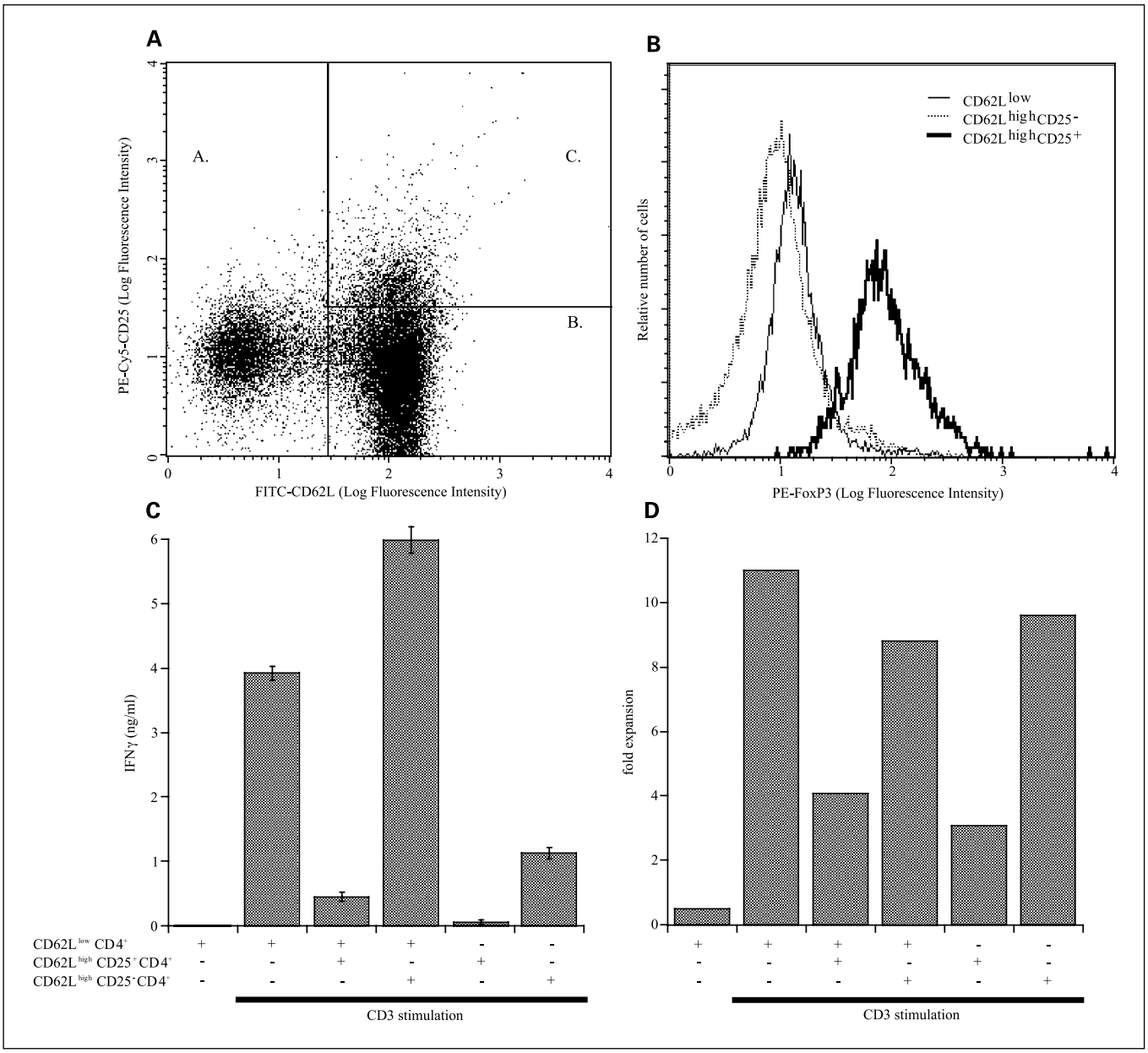


**Fig. 2.** $CD4^+$ T cells purified from PBMCs were stained with FITC-conjugated anti-CD62L, PE-Cy5–conjugated anti-CD25, and PE-conjugated anti-FOXP3 mAbs. *A,* CD62L and CD25 expression on the isolated $CD4^+$ T cells. Zones A, B, and C were defined as $CD62L^{low}$, $CD62L^{high}CD25^-$, and $CD62L^{high}CD25^+$, respectively. The percentages of $CD62L^{high}CD25^+$ $CD4^+$ T cells and $CD62L^{low}$ $CD4^+$ T cells in relation to the numbers of $CD4^+$ cells were analyzed. *B,* cytosolic FOXP3 expression of gated $CD62L^{low}$, $CD62L^{high}CD25^-$, and $CD62L^{high}CD25^+$ $CD4^+$ cells. *C,* IFN-γ production by $1 \times 10^5$ $CD62L^{low}$ $CD4^+$ T cells in the presence or absence of $5 \times 10^4$ $CD62L^{high}CD25^+$ $CD4^+$ T cells in 200 μL CM in a 96-well plate for 48 h. *D,* changes in the $CD62L^{low}$ $CD4^+$ T-cell counts; $1 \times 10^5$ $CD62L^{low}$ T cells stained with CFSE were stimulated overnight with immobilized anti-CD3 mAbs in 96-well plates in the presence or absence of $5 \times 10^4$ $CD62L^{high}CD25^+$ $CD4^+$ T cells. They were then cultured in CM supplemented with 10 units/mL of recombinant human IL-2 at a concentration of $1 \times 10^5$/mL for 48 h. The cells were counted and analyzed using a microfluorometer before and after culture. Fold expansion was calculated as follows: number of $CSFE^+$ cells after the 48-h culture period/number of $CFSE^+$ cells before the 48-h culture period.

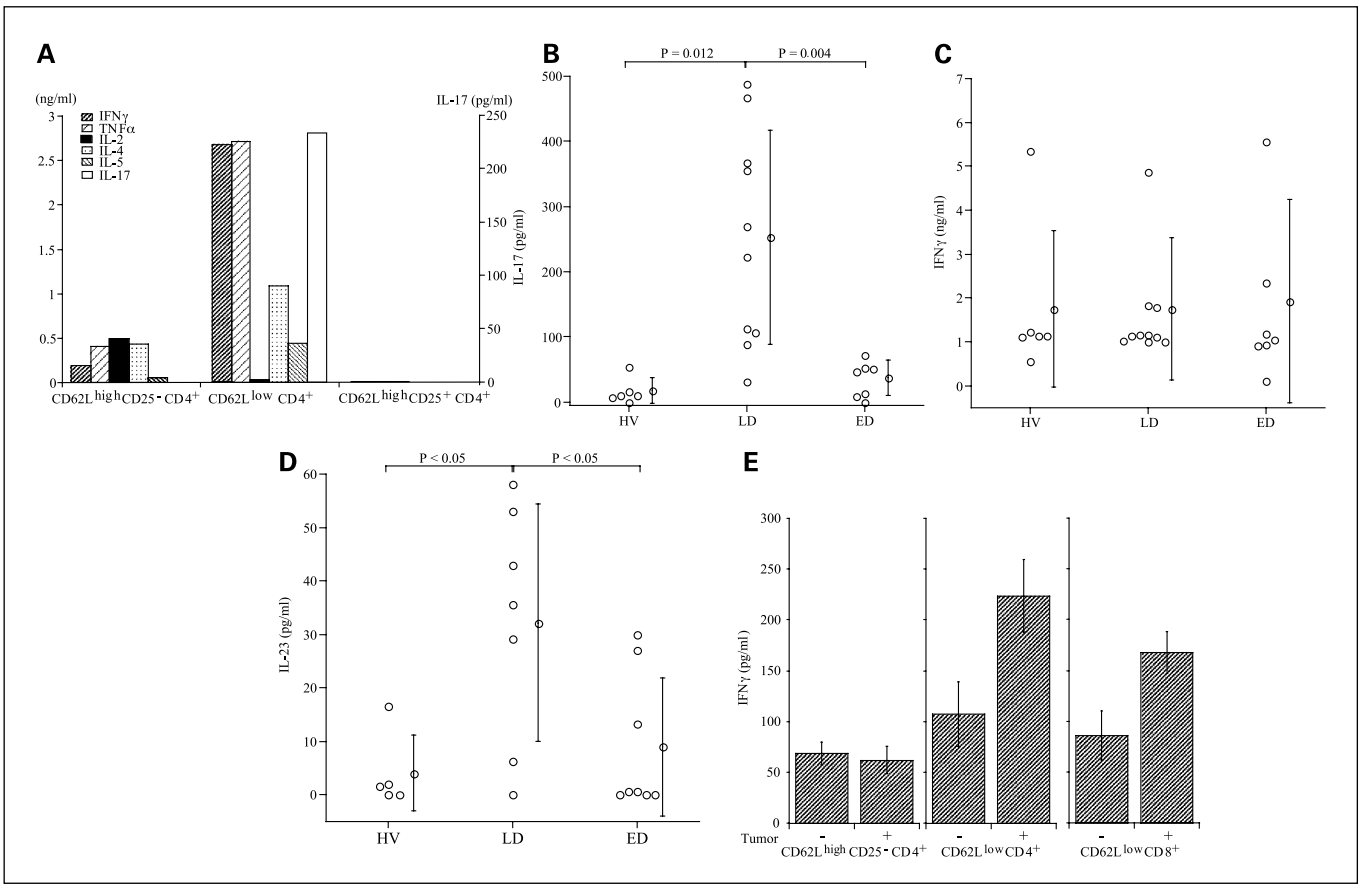


**Fig. 3.** In a 96-well plate, $2 \times 10^5$ $CD62L^{low}$ $CD4^+$ T cells, $CD62L^{high}CD25^-$ $CD4^+$ T cells, or $CD62L^{high}CD25^+$ $CD4^+$ T cells isolated from 10 LD-SCLC, 7 ED-SCLC, and 6 healthy volunteers were stimulated for 48 h with immobilized anti-CD3 mAbs in 200 μL CM. The supernatant was examined for IFN-γ, tumor necrosis factor-α (*TNFα*), IL-2, IL-4, IL-5, and IL-17 secreted by fractionated $CD4^+$ T cells using BD-CBA and ELISA. *A,* representative data obtained from one LD-SCLC patient. *B* and *C,* amounts of IL-17 and IFN-γ produced by $CD62L^{low}$ $CD4^+$ T cells obtained from LD-SCLC and ED-SCLC patients and healthy volunteers. *D,* amounts of IL-23 secreted in 200 μL CM by $2 \times 10^5$ monocyte-derived DCs on stimulation of 10 ng/mL lipopolysaccharide for 24 h. *E,* antigen-stimulated secretion of IFN-γ by $CD4^+$ T cells or $CD8^+$ T cells isolated from PBMCs of the SCLC patient p-T2N0M0 who had undergone surgical resection. In a 96-well plate, $1 \times 10^5$ $CD62L^{high}CD25^-$ $CD4^+$ T cells, $CD62L^{low}$ $CD4^+$ T cells, or $CD62L^{low}$ $CD8^+$ T cells were stimulated for 48 h with $5 \times 10^4$ $CD14^+$ cell-derived DCs in the presence or absence of $5 \times 10^4$ $CD62L^{high}CD25^+$ $CD4^+$ T cells in 200 μL CM. DCs were pulsed overnight with an equal number of 5,000 cGy – irradiated SCLC tumor cells, and they were purified with CD11c microbeads before coculture.

nonessential amino acids, 1 μmol/L sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate (all from Life Technologies, Inc.).

***Antibodies and phenotype analyses.*** The following mAbs were used: FITC-conjugated anti-CD3 (HIT3a) and CD4 (RPA-T4), phycoerythrin (PE)-conjugated anti-CD8 (RPA-T8) and CD25 (M-A251), PE-Cy7–conjugated anti-CD25 (M-A251), PE-Cy5–conjugated anti-CD62L (Dreg 56; all from BD PharMingen), and FITC-conjugated anti-CD62L (Dreg 56; eBioscience). Cell surface phenotypes were analyzed by direct immunofluorescence staining of $1 \times 10^6$ cells with conjugated mAbs. In each sample, 10,000 cells were analyzed using a FACScan flow microfluorometer (Becton Dickinson) and CellQuest software. Staining of cytosolic FOXP3 was done using PE-anti-human FOXP3 mAb (PCH101) and Staining set (eBioscience), according to the manufacturer's instructions.

***Cytokine analysis.*** Responder T cells were stimulated with immobilized anti-CD3 mAb or DCs pulsed with irradiated tumor cells for 48 h. The supernatants were harvested and assayed for cytokine concentrations by using a BD-Cytometric Bead Array (CBA; Becton Dickinson) or a quantitative "sandwich" enzyme immunoassay with an IFN-γ and an IL-17A ELISA kit (eBioscience), according to the manufacturer's instructions.

Responder $2 \times 10^5$ monocyte-derived DCs were cultured in the presence of 10 ng/mL lipopolysaccharide (Sigma-Aldrich) in 200 μL CM for 24 h. The amounts of IL-23 were determined by using ELISA kit (eBioscience), according to the manufacturer's instructions.

***Proliferation assay.*** The isolated T cells ($2 \times 10^5$) were stimulated in 200 μL CM for 24 h in 96-well plates precoated with anti-human CD3 (BD Biosciences). The ratio of $CD62L^{low}$ to $CD62L^{high}$ $CD4^+CD25^+$ T cells was 2:1. $CD62L^{low}$ T cells were labeled with 5 μmol/L 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE; Molecular Probes, Inc.) in HBSS at 37°C for 15 min and washed twice before CD3 stimulation. After stimulation for 24 h, the cells were counted and washed twice in HBSS. The T cells were then cultured in CM supplemented with 10 units/mL recombinant human IL-2 (a gift from Shionogi) at a concentration of $1 \times 10^5$/mL. Three wells were analyzed under each condition.

***Statistical analyses.*** *P* values were calculated by using two-sided Student's *t* test; $P < 0.05$ was considered to be statistically significant. For comparison of percentages of cells from the same patients before and after chemotherapy, a paired *t* test was used instead.

## Results

***Shifted $CD4^+$ regulatory and effector balance in patients with LD-SCLC and ED-SCLC.*** Before purification, PBMCs were analyzed for CD3, CD4, CD8, CD62L, and CD25 expression.

Table 1 shows characteristics of the healthy volunteers and patients included in this study. As shown in Fig. 1A, the percentages of $CD4^+$ T cells did not differ among the healthy volunteers and LD-SCLC and ED-SCLC patients. Figure 1B and C shows the percentages of $CD4^+$ cells that were $CD62L^{low}$ and $CD62L^{high}CD25^+$ cells. Approximately 5% to 10% of $CD4^+$ T cells belonged to the $CD62L^{high}CD25^+$ subpopulation in the healthy volunteers (Fig. 1C). The healthy volunteers included individuals both younger than (<40 years) and as old as the SCLC patients. No differences were observed between them with regard to the percentages of $CD62L^{low}$ and $CD62L^{high}CD25^+$ cells. Consistent with previous studies that showed that the Treg cell subpopulation increases in cancer-bearing patients (14–20), the $CD62L^{high}CD25^+$ subpopulation significantly increased in ED-SCLC patients compared with the healthy volunteers ($P = 0.0009$). In contrast, the percentages of the $CD62L^{high}CD25^+$ subpopulation in LD-SCLC patients showed no differences from those of the healthy volunteers. Further, a significantly larger $CD62L^{low}$ T-cell subpopulation was induced in the LD-SCLC patients compared with that in the healthy volunteers or ED-SCLC patients ($P = 0.000009$ and 0.00003, respectively; Fig. 1C). No significant difference was observed in the percentages of $CD62L^{low}$ $CD4^+$ T cells between the healthy volunteers and ED-SCLC patients. To illustrate the difference in the $CD4^+$ T-cell balance between the $CD62L^{low}$ $CD4^+$ T cells and $CD62L^{high}CD25^+$ $CD4^+$ cells, we calculated their ratio. As shown in Fig. 1D, a distinct $CD62L^{low}$ T-cell–dominant $CD4^+$ T-cell balance was induced in LD-SCLC patients, whereas a $CD62L^{high}CD25^+$ $CD4^+$ T-cell–dominant balance was observed in ED-SCLC patients. This study included four LEMS patients. All of them were LD-SCLC patients and exhibited effector-dominant $CD4^+$ T-cell balance. In contrast to the percentages of $CD4^+$ cells, the percentages of $CD62L^{low}$ primed effector $CD8^+$ T cells show no differences among LD-SCLC and ED-SCLC patients and healthy volunteers (Fig. 1E and F).

***$CD62L^{high}CD25^+$ $CD4^+$ T cells in SCLC patients expressed FOXP3 and possessed Treg cell functions.*** Figure 2A shows a representative expression pattern of CD62L and CD25 on $CD4^+$ cells. The $CD4^+$ cells were isolated from one of LD-SCLC patients. We defined zones A, B, or C as $CD62L^{low}$, $CD62L^{high}CD25^-$, and $CD62L^{high}CD25^+$, respectively. Expression of FOXP3, which is considered as the master switch for Treg cells, was analyzed in $CD4^+$ T cells. As shown in Fig. 2B, only the $CD62L^{high}CD25^+$ $CD4^+$ T cells expressed FOXP3. To determine whether these cells possessed regulatory properties, $CD62L^{low}$ T cells were cocultured with $CD62L^{high}CD25^+$ $CD4^+$ T cells in the presence of CD3 stimulation. $CD62L^{low}$ $CD4^+$ T cells, $CD62L^{high}CD25^-$ $CD4^+$ T cells, and $CD62L^{high}CD25^+$ $CD4^+$ T cells were purified from PBMCs with magnetic beads as described. Experiments were repeated with T cells isolated from LD-SCLC and ED-SCLC patients and healthy volunteers. Figure 2C and D shows the representative data obtained with T cells derived from an ED-SCLC patient. $CD62L^{high}CD25^+$ $CD4^+$ T cells inhibited cytokine production and the proliferation of $CD62L^{low}$ $CD4^+$ T cells. Thus, $CD62L^{high}CD25^+$ $CD4^+$ T cells represented Treg cells.

***Th1, Th2, and Th17 cells were in the $CD62L^{low}$ $CD4^+$ T-cell subpopulation.*** Next, we examined the cytokine production profile of each T-cell subpopulation. Sufficient number of purified T cells for cytokine analyses were obtained from 6 healthy volunteers, 10 LD-SCLC patients, and 7 ED-SCLC patients. Purified T cells were stimulated with immobilized anti-CD3 mAbs, and the supernatant was analyzed for IFN-γ, tumor necrosis factor-α, IL-2, IL-4, IL-5, and IL-17 by BD-CBA or ELISA according to the manufacturer's instructions. Figure 3A shows a representative cytokine production profile obtained from one of the LD-SCLC patients. $CD62L^{low}$ $CD4^+$ T cells secreted large amounts of Th1, Th2, and Th17 cytokines, such as IFN-γ, tumor necrosis factor-α, IL-4, IL-5, and IL-17, except for IL-2 that was mainly secreted by $CD62L^{high}CD25^-$ $CD4^+$ T cells. Thus, the $CD62L^{low}$ $CD4^+$ T cells included Th1, Th2, and Th17 cells, and most $CD62L^{high}CD25^-$ $CD4^+$ T cells were naive T cells.

***Greater IL-17 secretion by Teff cells from LD-SCLC and ED-SCLC patients.*** It has recently been described that a novel

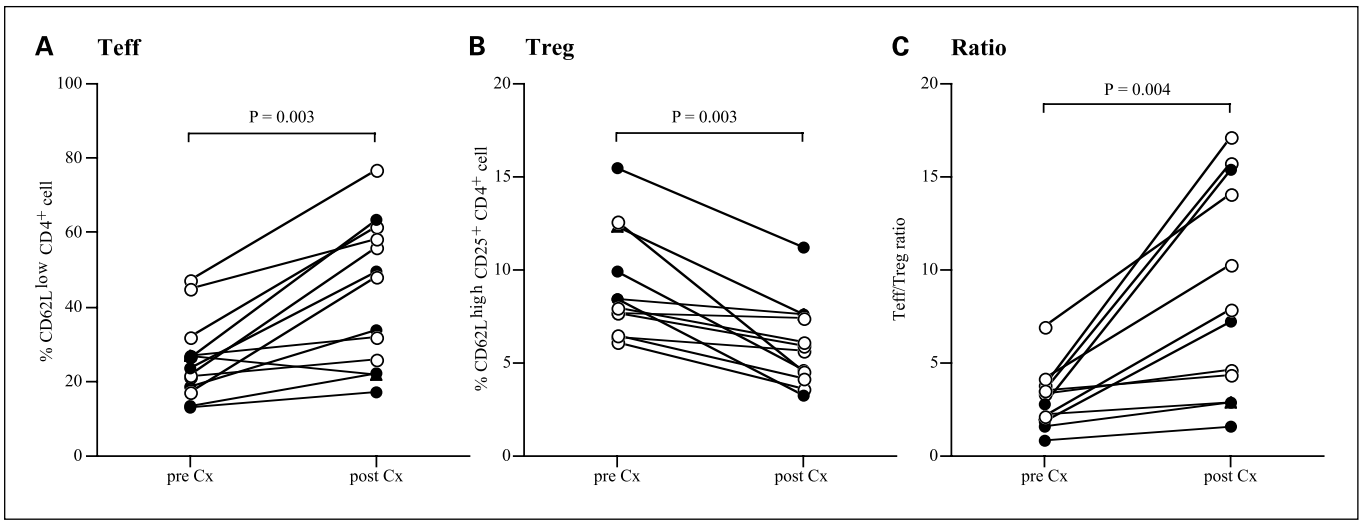


**Fig. 4.** Effect of chemotherapy on the $CD62L^{low}$ $CD4^+$ Tcells and $CD62L^{high}CD25^+$ $CD4^+$ Tcells was evaluated. The PBMCs were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD25, and PE-Cy5–conjugated anti-CD62L mAbs. *A* and *B*, percentages of $CD62L^{low}$ and $CD62L^{high}CD25^+$ cells in relation to the numbers of $CD4^+$ cells before and after chemotherapy. All these cells were subjected to two courses of platinum-based chemotherapy. *C*, ratio of the percentage of $CD62L^{low}$ $CD4^+$ Tcells to that of $CD62L^{high}CD25^+$ $CD4^+$ Tcells. ○, LD-SCLC patients; ●, ED-SCLC patients.

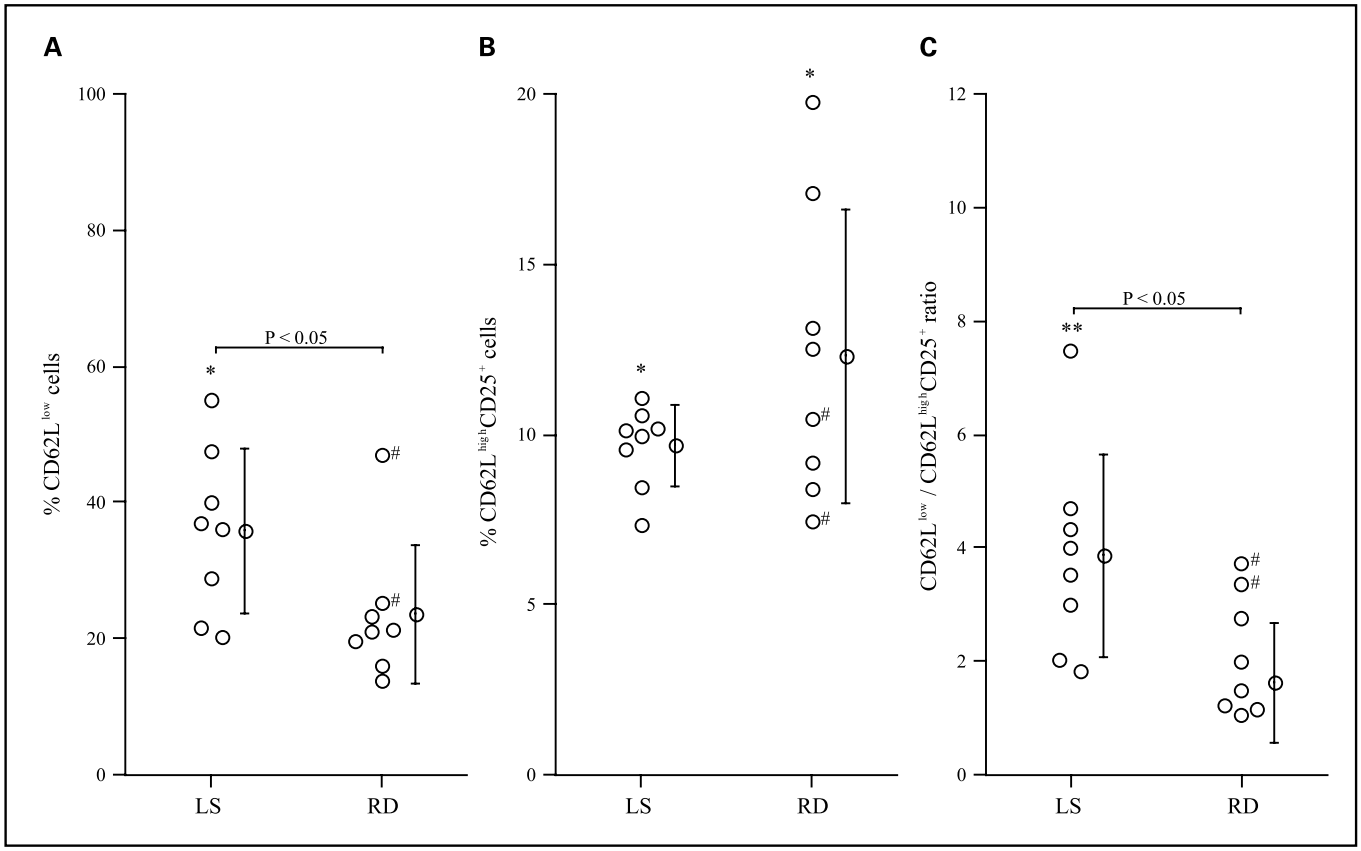


**Fig. 5.** *A* and *B*, percentages of $CD62L^{low}$ $CD4^+$ and $CD62L^{high}CD25^+$ $CD4^+$ T cells in relation to the number of $CD4^+$ cells in SCLC patients. *C*, ratio of $CD62L^{low}$ to $CD62L^{high}CD25^+$ T cells. LS, long-term survivors who had been disease-free for >3 y after treatment; RD, LD-SCLC patients who had recurrent disease after treatment. #, patients who had local recurrent diseases without distant, hematogenous metastases. *, $P < 0.01$; **, $P < 0.05$, compared with healthy volunteers.

subset of $CD4^+$ T cells producing IL-17A, IL-17F, and IL-22 (i.e., Th17 cells) is essential in several autoimmune diseases, such as multiple sclerosis (21–28). Moreover, recently, it was shown that Th17 cells mediated potent antitumor reactivity to eradicate large established tumor (29). Th1 cells also have been considered to play an important role in antitumor immunity. To determine the size of the $CD4^+$ T-cell subpopulation secreting IL-17, or IFN-γ, $CD4^+$ T cells isolated from SCLC patients and healthy volunteers were stimulated with immobilized anti-CD3 mAb, and the supernatants were quantified for IL-17A and IFN-γ using ELISA. As shown in Fig. 3B, $CD62L^{low}$ $CD4^+$ T cells isolated from LD-SCLC patients produced significantly more IL-17A than those isolated from ED-SCLC and healthy volunteers ($P = 0.012$ and 0.004, respectively). In contrast, levels of IFN-γ produced by the same number of $CD62L^{low}$ $CD4^+$ T cells showed no difference among the LD-SCLC and ED-SCLC patients and healthy volunteers (Fig. 3C). Thus, it is likely that Teff cells induced in LD-SCLC patients deviated to Th17 cells.

IL-23 produced by DCs is believed to play a critical role in expanding a Th17 subpopulation (30). It was reported that DCs derived from monocytes of the multiple sclerosis patients, in whom Th17 cells are highly pathogenic and essential, produced more IL-23 (24). Then, we tested if DCs derived from $CD14^+$ cells of the SCLC patients secrete IL-23. As shown in Fig. 3D, DCs derived from the LD-SCLC patients produced significantly more IL-23 on lipopolysaccharide stimulation.

***Autologous tumor antigen–specific IFN-γ production by $CD62L^{low}$ T cells derived from LD-SCLC patients.*** Next, to assess whether the $CD62L^{low}$ subpopulation comprised T cells that recognize antigens on autologous SCLC tumor cells, we evaluated cytokine production by T cells isolated from a stage IB LD-SCLC patient who had undergone surgical resection and adjuvant chemotherapy. The resected tumor was digested with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 units/mL hyaluronidase (Sigma) for 3 hours at room temperature. Tumor cells were cryopreserved by supplementation with 5% DMSO and 6% hydroxyethyl starch cryoprotectant mixture (CP-1; Kyokuto) at -80°C according to the manufacturer's instructions until they were used for coculture. $CD62L^{low}$ $CD4^+$ T cells, $CD62L^{high}CD25^{low}$ $CD4^+$ T cells, or $CD62L^{low}$ $CD8^+$ T cells isolated from the peripheral blood of the patient were stimulated with monocyte-derived DCs pulsed with 50 Gy–irradiated autologous tumor cells. One million monocyte-derived DCs were cocultured with $1 \times 10^6$ tumor cells in 2 mL of CM for 24 hours. $CD11c^+$ cells were further purified with CD11c microbeads and autoMACS after coculture for T-cell stimulation. In a 96-well plate, $1 \times 10^5$ $CD62L^{high}CD25^-$ $CD4^+$ T cells, $CD62L^{low}$ $CD4^+$ T cells, or $CD62L^{low}$ $CD8^+$ T cells were stimulated for 48 hours with $5 \times 10^4$ $CD14^+$ cell-derived DCs in the presence or absence of $5 \times 10^4$ $CD62L^{high}CD25^+$ $CD4^+$ T cells in 200 μL CM and the supernatants were measured for IFN-γ by using ELISA. As shown in Fig. 3E, $CD62L^{low}$ $CD4^+$ T cells secreted IFN-γ specific to autologous tumor antigens.

In contrast, the naive $CD62L^{high}CD25^{-}$ $CD4^+$ T-cell subpopulation did not show tumor antigen–specific IFN-γ release. Moreover, the addition of $CD62L^{high}CD25^+$ $CD4^+$ T cells inhibited tumor-specific IFN-γ secretion. $CD62L^{low}$ $CD8^+$ T cells also showed antigen-specific IFN-γrelease.

***Effect of chemotherapy on Teff to Treg cell ratio.*** It has been perceived that chemotherapy affects the general immune responsiveness in treated patients. However, recent studies indicated that certain cytotoxic agents may augment immune reactivities by reducing the number of Treg cells (31–33). To address the effects of chemotherapy on $CD4^+$ T-cell balance, T cells obtained from PBMCs were examined after two courses of platinum-based chemotherapy and analyzed using the paired Student's *t* test. Seven LD-SCLC and five ED-SCLC patients were included for the analyses. Eight patients were treated with cisplatin (CDDP) and etoposide; three patients, with carboplatin (CBDCA) and etoposide; and one patient, with CBDCA, ifosfamide, and etoposide. The peripheral blood was obtained from patients whose WBC count recovered to >3,000/μL 18 to 20 days after the last chemotherapy session. As shown in Fig. 4A, the percentages of $CD62L^{low}$ cells from the total number of $CD4^+$ T cells increased significantly ($P = 0.003$). In contrast, the percentage of Treg cells decreased ($P = 0.003$; Fig. 4B). Thus, platinum-based chemotherapy increased the Teff to Treg cell ratio ($P = 0.004$; Fig. 4C). It is probable that Treg cells is more sensitive to chemotherapeutic agents and that Teff cells possess a potent ability to proliferate. Thus, even in the ED-SCLC patients, the level of Treg cells significantly decreased and Teff cells readily proliferated after chemotherapy, resulting in an increase in the Teff to Treg cell ratio. During certain periods after chemotherapy, the LD-SCLC and the ED-SCLC patients exhibited high Teff to Treg cell ratio without significant differences.

***Effector CD4+ T cells dominant in long-term survivors.*** We analyzed whether the effector-dominant $CD4^+$ T-cell balance was maintained in SCLC patients who had been cured by treatment. As shown in Fig. 5, long-term survivors still retained significantly greater levels of $CD62L^{low}$ $CD4^+$ Teff cells compared with healthy volunteers ($P < 0.00001$). Although long-term survivors exhibited significantly but slightly greater levels of Treg cells than those in healthy volunteers ($P < 0.01$), their effector-dominant $CD4^+$ T-cell balance was the same as that in LD-SCLC patients before treatment (Figs. 1D and E and 5). In contrast, the percentages of $CD4^+$ Teff cells in LD-SCLC patients with recurrent disease were as low as those in ED-SCLC patients, although they had been diagnosed with LD-SCLC before treatment. In Fig. 5, the number indicates that recurrent disease was confined to the LD state. These patients without distant, hematogenous disease still maintained relatively effector-dominant balance.

## Discussion

In this study, we showed the presence of a distinct reciprocal balance between Treg and Teff cells in the peripheral blood of LD-SCLC and ED-SCLC patients. LD-SCLC patients exhibited a unique effector-dominant $CD4^+$ T-cell balance. Moreover, Teff cells induced in LD-SCLC patients included a larger Th17 cell subpopulation. In contrast, ED-SCLC patients exhibited Treg cell–dominant balance. The manner in which the distinct $CD4^+$ T-cell balance is generated in cancer-bearing hosts remains unclear. In several human malignancies, it was reported that Treg cells were induced in tumor microenvironments and tumor-draining lymph nodes (10, 14, 16, 17, 34, 35). Tumor cells themselves can convert immature DCs into transforming growth factor-β–secreting DCs that selectively induce Treg cells (36). Immature myeloid suppressor cells induced by the tumor are also thought to be responsible for Treg cell induction (37). We found that immature myeloid suppressor cells increased in ED-SCLC patients compared with healthy volunteers (data not shown). Thus, tumor cells tend to activate the peripheral tolerance machinery to induce Treg cells. Indeed, the number of Treg cells increased in the peripheral blood of ED-SCLC patients, who comprise 80% to 85% of SCLC patients.

The question arises why Teff cells were induced in the LD-SCLC patients. Mutated gene products can induce immunity by triggering the recognition of normally silent epitopes, including self and tumor-associated antigens (38). Subsequently, tumor cells themselves are likely to have the potential to induce immunity because they all possess gene mutations. It was shown that a certain type of mutation, such as truncation and amino acid substitutions that promote T helper cell responses, further enhanced immunity. Thus, one possible explanation is that LD-SCLC cells but not ED-SCLC cells have immunogenic gene mutations. However, this theory still cannot explain why Teff cells induced in LD-SCLC deviated to Th17 cells and how Treg cell generation was inhibited in LD-SCLC.

Th17 cells develop via a lineage distinct from the Th1 and Th2 lineages and play an essential role in a variety of autoimmune diseases, such as multiple sclerosis (21–26, 28). It was shown that monocyte-derived DCs from multiple sclerosis patients produced more IL-23 and that expansion of the pathogenic Th17 subpopulation was driven by IL-23 (30, 39). We detected that monocyte-derived DCs from LD-SCLC produced more IL-23 than that from ED-SCLC patients. Thus, it is likely that DCs in LD-SCLC patients have tend to expand a Th17 subpopulation. Recently, it was shown that Th17 and Treg cells are reciprocally induced depending on the cytokine balance (40). Transforming growth factor-β alone induces Treg cells; however, the addition of IL-6 to transforming growth factor-β results in Th17 cell differentiation and inhibits Treg cell induction. It is known that Th17 cells secrete not only IL-17 but also IL-6. Thus, it is possible that deviation to Th17 inhibits Treg cell development and facilitates priming of Th17 as a positive feedback mechanism in LD-SCLC patients.

It remains uncertain if the effector-dominant $CD4^+$ T-cell balance did in fact prevent distant metastases in LD-SCLC patients. In murine models, the adoptive transfer of tumor antigen–reactive effector $CD62L^{low}$ $CD4^+$ T cells tilted the Treg cell–dominant $CD4^+$ T-cell balance toward a dominant Teff cell environment that resulted in the regression of the growing tumor (13). Moreover, adoptive transfer of Treg cells isolated from tumor-draining lymph nodes promoted tumor growth (11). Recently, it was reported that Th17-polarized cells mediated eradication of large established tumor and that IL-23 worked as a cancer vaccine adjuvant to increase antitumor T cells and enhance effector T-cell function (29, 41). Thus, it is likely that the effector-dominant $CD4^+$ T-cell balance and Th17-polarized cells induced in LD-SCLC patients is not only a result but also plays a role in the defense mechanism against tumor cells. Consistent with this theory, the long-term survivors

maintained their effector-dominant $CD4^+$ T-cell balance, whereas LD-SCLC patients with recurrent disease due to distant metastases acquired Treg cell–dominant balance.

In contrast to $CD4^+$ T cells, the size of the effector $CD8^+$ T-cell population, which exhibited tumor antigen specificity, was not affected by disease stages or by distinct $CD4^+$ T-cell balance in SCLC patients. Although it has been reported that Treg cells can suppress either $CD4^+$ or $CD8^+$ effector T-cell proliferation (42–44), recent *in vivo* studies showed that antigen-specific $CD8^+$ T cells undergo essentially normal clonal expansion and effector differentiation in the presence of antigen-specific Treg cells (45–48). Our proliferation assays indicated that the Treg cells derived from SCLC patients delayed effector $CD8^+$ T-cell proliferation but did not inhibit it (data not shown). Thus, Treg cells may suppress a terminal effector function but not the size of the effector $CD8^+$ T-cell population (48).

The ratio of effector $CD4^+$ cells in relation to Treg cells could be a useful biomarker for assessing immunologic responses and distinguishing LD-SCLC patients from ED-SCLC patients. Further, the data in this article shed light on the significance of IL-23/Th17 axis for antitumor immunity. Furthermore, these results indicated that immunotherapy that increases tumor-reactive Teff cell levels and depletes Treg cells, thereby maintaining a Th17 cell-dominant $CD4^+$ T-cell balance, may be essential in establishing effective antitumor immunity.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Reciprocal $CD4^+$ T-Cell Balance of Effector $CD62L^{low}$ $CD4^+$ and $CD62L^{high}CD25^+$ $CD4^+$ Regulatory T Cells in Small Cell Lung Cancer Reflects Disease Stage

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