Dynamic changes in immune cell profile in head and neck squamous cell carcinoma: Immunomodulatory effects of chemotherapy

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Tumor cells have evolved sophisticated means of escape from the host immune system. To date, several important immunological phenomena have been revealed in peripheral blood as well as within tumors. In the present study, we first investigated the proportion and activation status of peripheral immune regulatory cells and CD8$^+$ T-cell subsets in patients with head and neck squamous cell carcinoma (HNSCC) using a multicolor flow cytometer, and then evaluated how therapy with docetaxel, cisplatin, and 5-fluorouracil modulated the immune cell profile in peripheral blood. The proportion of naïve T cells was lower and that of effector memory T cells (T$_{EM}$) was higher in HNSCC patients than in healthy donors. Moreover, the proportions of activated T$_{EM}$ cells and effector T cells (T$_{EFF}$) were dramatically increased in patients with advanced stage disease. The proportion of regulatory T cells and CD14$^+$HLA-DR$^-$ myeloid-derived suppressor cells was elevated in HNSCC patients. Of note, after therapy, in addition to the transient reduction in immune regulatory cells, decreases in central memory T cells and increases in T$_{EFF}$ cells were observed among CD8$^+$ T-cell subsets, suggesting differentiation from central memory T cells into T$_{EFF}$ cells. Our results suggested that, despite the immunosuppressive status in HNSCC patients, tumor-specific immune responses mediated by CD8$^+$ T cells might be induced and maintained. Moreover, chemotherapy can trigger not only a transient reduction in immune regulatory cells but also further activation of CD8$^+$ T cells.

Antitumor immunity plays an important role in protection against the development of malignancy. However, tumor cells have evolved sophisticated means of escape from the host immune system through the downregulation of key molecules necessary for the immune response to proceed and/or interference with the functions and survival of immune cells.[1,2] Such tumor-induced immune suppressive networks within the tumor microenvironment lead to further systemic immunosuppression in cancer patients. Indeed, in peripheral blood from a variety of malignancies, several important immunological phenomena have been uncovered, such as those involving increases in immune suppressive cells,[3,4] an imbalance in Th1/Th2 ratio,[5] and dysfunction of effector T cells and dendritic cells.[6,7]

In head and neck squamous cell carcinoma (HNSCC), which is known to be highly immunosuppressive, we and others have indicated an increase in immune regulatory cells including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs),[3,4] lower absolute numbers of T cells,[8] and dysfunction of effector CD8$^+$ T cells.[6] Notably, this immune cell profile is also more closely related to the efficacy of treatment as well as the prediction of prognosis. In the present study, we investigated not only the proportion but also activation status of peripheral immune cells, including immune regulatory cells

and CD8$^+$ T-cell subsets in patients with HNSCC using multicolor flow cytometry. For evaluation of the activation status in immune cells, CD38 was used as an activation marker in T cells. CD38 is a multifunctional protein that serves as both an antigen and an enzyme,[9] and it has been used as a marker for T and B cell activation and differentiation.[10,11]

CD8$^+$ T cells, which can specifically recognize and kill tumor cells, are essential cells for cell-mediated antitumor immune responses and comprise several subsets, naïve T cells, central memory T cells (T$_{CM}$), effector memory T cells (T$_{EM}$), and effector T cells (T$_{EFF}$).[12,13] CD8$^+$ T cells clonally expand in response to antigenic stimulation, and they differentiate into T$_{EFF}$ that work for the elimination of tumor cells, whereas some cells differentiate into memory T cells that have the ability to rapidly generate effector functions. Central memory T cells have greater proliferative potential than T$_{EM}$ cells, whereas T$_{EM}$ cells have certain effector functions compared with T$_{CM}$ cells.

Turksma et al.[14] reported a significant shift from naïve to T$_{EM}$ in patients with HNSCC. Moreover, Kuss et al.[6] revealed that the frequency of T$_{EFF}$ is significantly increased in the circulation of HNSCC patients; however, these CD8$^+$ T cells have signaling defects and are sensitive to apoptosis. Thus, in the peripheral circulation of cancer patients, evidence

has suggested that dysfunction and/or imbalance of each subset of $CD8^+$ T cells might be responsible for or contribute to promoting tumor immune evasion.

The impact of chemotherapy on the host immune system is complicated and there are benefits and disadvantages. Chemotherapeutic agents have major immunosuppressive adverse effects such as lymphopenia and favored selection of treatment-resistant tumor cell variants, whereas they increase susceptibility of tumor cells to immune attack[15,16], induce immunogenic cell death[17,18], and reduce immunosuppressive cells[19,20] As a component of multimodal therapy for HNSCCs, patients with locally advanced disease often receive induction chemotherapy followed by definitive radiotherapy or chemoradiotherapy.[21] The addition of docetaxel to the cisplatin and 5-fluorouracil (5-FU) induction regimen (TPF) improved efficacy with manageable toxicity in comparison with the cisplatin and 5-FU alone.[22] To date, each chemotherapeutic agent in the TPF regimen is well known to exert various immunomodulatory effects *in vitro* and/or *in vivo*. Any three chemotherapeutic agents selectively decreased the proportion and immunosuppressive function of MDSCs, resulting in the enhancement of antitumor immune responses.[23–25] Moreover, docetaxel enhances $CD8^+$ T cell responses to CD3 cross-linking, and 5-FU upregulates the expression of tumor-associated antigen and HLA molecules in cancer cell lines.[26,27] However, the influence of TPF chemotherapy, the most popular chemotherapy regimen used to treat advanced HNSCC, on immune cells in peripheral blood has not been investigated in patients with HNSCC. Another important aim of the present study was to evaluate how TPF chemotherapy modulated the immune cell profile in peripheral blood in these patients. Our data provide new insights into the clinical significance of immune cell profile and the possibility of chemo-immunotherapy in patients with HNSCC.

Materials and Methods

Patients and controls. Peripheral blood samples were collected from 60 patients with HNSCC treated at the Department of Otolaryngology–Head and Neck Surgery, Gunma University Hospital (Maebashi, Japan). Patients who were receiving oral/i.v. steroids or who had known autoimmune disease were excluded. Samples from 44 patients treated with surgery, radiotherapy, or concurrent chemoradiotherapy were collected before the start of treatment. Samples of 16 patients treated with TPF therapy were collected before cycle 1 day 1 (day 0), 5 days after the start of treatment (day 6), and at the end of cycle 1 (day 21). Samples at baseline were obtained before dexamethasone premedication. Control blood samples were collected from 20 age-matched healthy donors (HDs). The characteristics of patients and healthy volunteers are summarized in Table 1. All patients and controls provided written informed consent to participate in the study, which was approved by the Institutional Review Board of Gunma University and was carried out in accordance with the Declaration of Helsinki.

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on a Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA, USA) gradient in accordance with the manufacturer's instructions. Nonspecific binding of antibodies to Fc receptors on PBMCs was blocked using BD Fc Block (BD Bioscience, San Jose, CA, USA) in accordance with the manufacturer's instructions. PBMCs (0.5×10^6) were then stained for expression of

Table 1. Characteristics of 60 patients with head and neck squamous cell carcinoma (16 treated with docetaxel, cisplatin, and 5-fluorouracil [TPF]), and 20 healthy volunteers

	Healthy donors (n = 20)	All patients (n = 60)	Patients without TPF (n = 44)	Patients with TPF (n = 16)
Age, years, mean (range)	64 (50–79)	68 (45–84)	68 (48–84)	65 (45–81)
Gender, n (%)				
Male	10 (50)	53 (88)	39 (89)	14 (88)
Female	10 (50)	7 (12)	5 (11)	2 (12)
Tumor stage, n (%)				
I		3 (6)	3 (7)	0 (0)
II		11 (19)	11 (24)	0 (0)
III		7 (13)	6 (15)	1 (9)
IV		39 (62)	24 (54)	15 (91)
Site of origin, n (%)				
Oral cavity		8 (13)	8 (17)	0 (0)
Oropharynx		13 (19)	6 (15)	7 (36)
Hypopharynx		10 (17)	5 (12)	5 (36)
Larynx		22 (38)	19 (44)	3 (19)
Paranasal cavity		7 (13)	6 (12)	1 (9)

surface markers using specific anti-human mAbs against the following molecules: CD3 (SK7), CD4 (RPA-T4), CD8 (RPA-T8), CD45RO (UCHL1), CD25 (M-A251), CD62L (DREG-56), CD127 (eBioRDR5), CD38 (HIT2), HLA-DR (L243), and CD14 (MφP9). All antibodies were directly conjugated to FITC, phycoerythrin (PE), PE–cyanine 7 (PE-Cy7), allophycocyanin (APC), or APC-eFluor 780. For compensation, PBMCs were also stained with IgG isotype-matched controls of all fluorescent dyes: FITC-CD4, PE-CD4, PE-Cy7-CD3, APC-CD4, and APC-eFluor 780-CD62L. Staining was carried out at 4°C for 30 min, protected from light, in PBS with 1% heat-inactivated FCS (Life Technologies, Carlsbad, CA, USA) and 0.1% sodium azide (Sigma-Aldrich, St. Louis, MO, USA). All antibodies were purchased from BD Biosciences and eBioscience (San Diego, CA, USA). After washing, samples were immediately analyzed by flow cytometry using an Attune Acoustic Focusing Cytometer (Life Technologies). Acquired data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis. The Mann–Whitney U-test or Wilcoxon signed-rank test were used to test for differences in the medians between the two groups. The Friedman test followed by Dunn's multiple comparisons test was used to test for differences in the medians between the three groups. Two-sided P-values <0.05 were considered to be significant. All statistical analyses were carried out using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Differences in proportion and activation status of peripheral $CD8^+$ T cells between healthy donors and HNSCC patients. The proportion and activation status of peripheral $CD8^+$ T cells in 60 HNSCC patients were compared with those from 20 HDs. The gating strategy is shown in Figure 1(a). Lymphocytes expressing both CD3 and CD8 were gated as $CD8^+$ T cells, then divided into four populations by expression of CD45RO and CD62L: naïve T cells were defined as CD45RO⁻
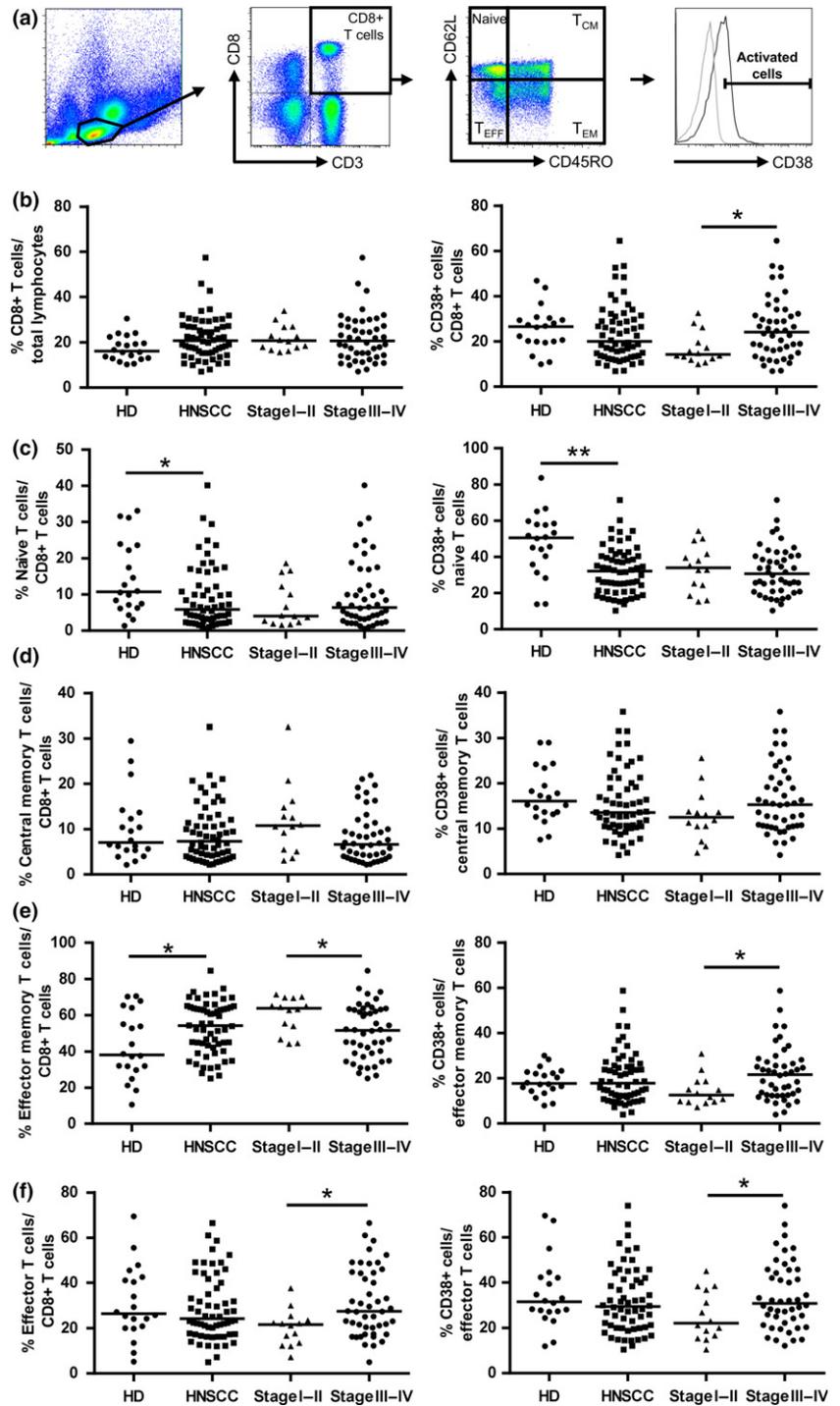
Fig. 1. Proportion and activation status of peripheral CD8$^+$ T cells including those subpopulations in patient with head and neck squamous cell carcinoma (HNSCC) and healthy donors (HDs). Peripheral blood mononuclear cells isolated from patients ($n = 60$) and HDs ($n = 20$) were stained for expression of surface markers, then analyzed by flow cytometry. The gating strategy is shown in (a). Lymphocytes expressing both CD3 and CD8 were gated as CD8$^+$ T cells, then divided into four populations according to their expression of CD45RO and CD62L. Activation status in each gate was evaluated using the surface expression of CD38. Graphs on the left side of (b–f) show the proportion status, and those on the right side of (b–f) show activation status in each population. *$P < 0.05$; **$P < 0.01$. T$_{CM}$, central memory T cells; T$_{EFF}$, effector T cells; T$_{EM}$, effector memory T cells.

CD62L$^+$, T$_{CM}$ as CD45RO$^+$ CD62L$^+$, T$_{EM}$ as CD45RO$^+$ CD62L$^-$, and T$_{EFF}$ as CD45RO$^-$ CD62L$^-$. Activation status in each gate was evaluated using surface expression levels of CD38.

There was no difference in the proportion of whole CD8$^+$ T cells between HDs and HNSCC patients (Fig. 1b). The proportion of naïve T cells was significantly lower in HNSCC patients than in HDs (Fig. 1c); in contrast, the proportion of T$_{EM}$ cells was significantly higher in HNSCC patients (Fig. 1e). These results indicated that the proportion shift from naïve to T$_{EM}$ cells occurred in patients with HNSCC. In addition, the proportion of T$_{EM}$ cells was significantly lower in

patients with stage III–IV tumors than those with stage I–II (Fig. 1e); conversely, the proportion of T$_{EFF}$ cells was higher in patients with stage III–IV tumors than those with stage I–II (Fig. 1f). These results indicated that the proportion shift from T$_{EM}$ cells to T$_{EFF}$ cells occurred with the progression of the tumors.

There was no difference in whole CD8$^+$ T cells expressing CD38 between HDs and HNSCC patients (Fig. 1b); however, the levels of naïve T cells expressing CD38 were significantly lower in HNSCC patients (Fig. 1c). There were no differences between HDs and HNSCC patients in other subpopulations; however, T$_{EM}$ cells and T$_{EFF}$ cells expressing CD38 were

significantly higher in stage III–IV tumors than in stage I–II tumors (Fig. 1e,f).

Differences in proportion and activation status of peripheral regulatory immune cells between HDs and HNSCC patients. Proportion and activation status of peripheral Tregs and MDSCs in 60 HNSCC patients were compared with 20 HDs. The gating strategy of Tregs is shown in Figure 2(a). Lymphocytes expressing both CD3 and CD4 were gated as $CD4^+$ T cells; then Tregs were identified by expression of CD25 and lack of CD127. Activation status in each gate was evaluated using the expression level of CD38. There was no difference in the proportion of whole $CD4^+$ T cells between HNSCC patients and HDs (Fig. 2b); however, the proportion of Tregs was significantly higher in HNSCC patients than in HDs (Fig. 2c). There were no differences in $CD4^+$ T cells and Tregs expressing CD38 between HDs and HNSCC patients (Fig. 2b,c).

The gating strategy of MDSCs is shown in Figure 2(d). The MDSCs were identified by surface expressions of CD14 and lack of HLA-DR. The proportion of MDSCs was significantly higher in HNSCC patients than in HDs (Fig. 2e).

Dynamic changes of T cells and MDSCs after TPF therapy. The changes of status described previously after TPF therapy were also investigated. Peripheral blood samples of 16 patients treated with TPF therapy were collected before cycle 1 day 1 (day 0), 5 days after the start of treatment (day 6), and at the end of cycle 1 (day 21) and compared with each other. The proportion of $CD8^+$ T cells was decreased significantly at day 6 (Fig. 3), whereas that of $CD4^+$ T cells increased (Fig. 3f). Among the subpopulations of $CD8^+$ T cells, naïve T cells increased most at day 6 and returned to the baseline level at day 21 (Fig. 3b). Meanwhile, the proportion of T_{CM} cells decreased significantly at day 21 compared with baseline (Fig. 3c), whereas T_{EFF} cells increased (Fig. 3e). The expression levels of activation marker CD38 increased significantly at day 21 compared with baseline levels in $CD8^+$ T cells, especially in T_{EM} cells and T_{EFF} cells (Fig. 3a,d,e).

The proportion of Tregs decreased significantly at day 6 (Fig. 3g). The expression levels of CD38 on Tregs increased significantly at day 21 compared with baseline (Fig. 3g). The proportion of MDSCs decreased transiently at day 6 and recovered then to baseline level at day 21 (Fig. 3h).

Discussion

Immune cell subsets in peripheral blood from patients with malignancies show complex and distinct pattern from HDs.
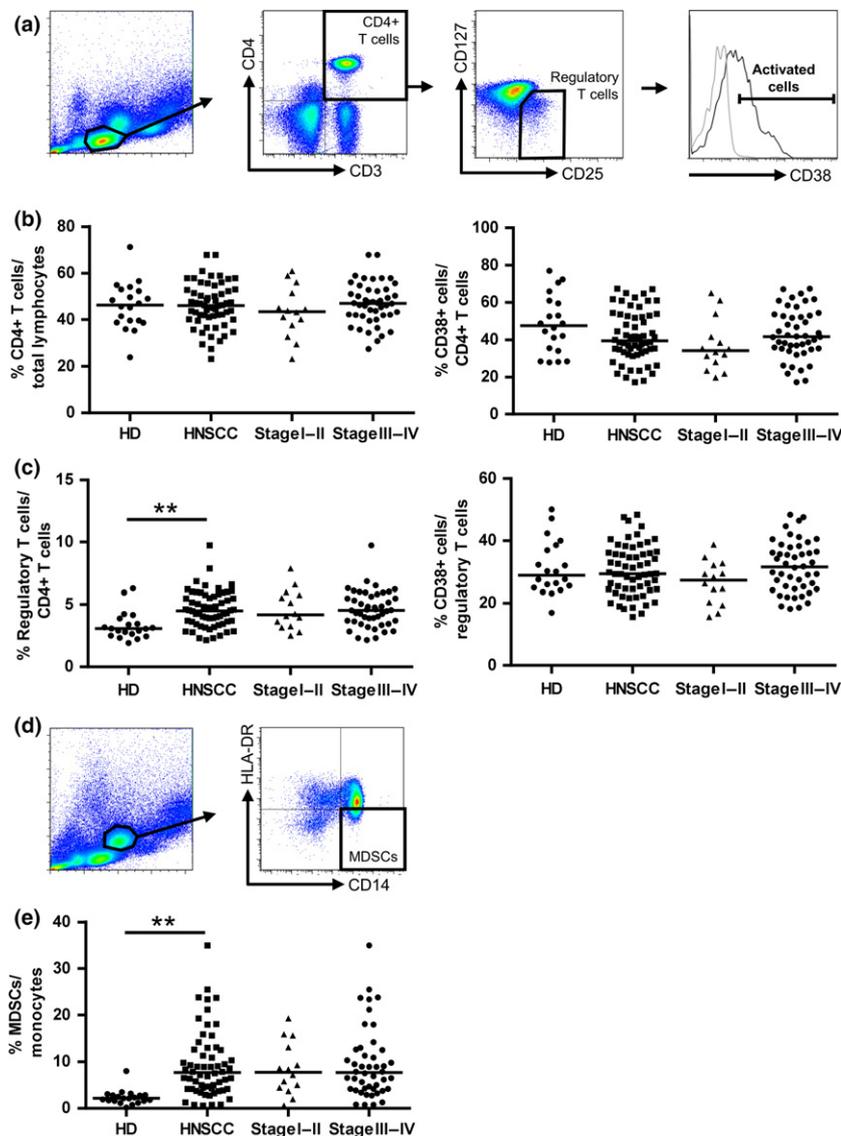

Fig. 2. Proportion and activation status of peripheral $CD4^+$ T cells and regulatory T cells (Tregs), and proportion status of myeloid-derived suppressor cells (MDSCs) in 60 patients with head and neck squamous cell carcinoma (HNSCC) and 20 healthy donors (HDs). The gating strategy of $CD4^+$ T cells and Tregs is shown in (a). Lymphocytes expressing both CD3 and CD4 were gated as $CD4^+$ T cells, then Tregs were identified according to their expression of CD25 and the lack of CD127. Activation status in each gate was evaluated according to their expression of CD38. Graphs on the left side of (b,c) show proportion status; those on the right side of (b,c) show activation status in each gate. The gating strategy of MDSCs is shown in (d). MDSCs were identified according to their surface expression of CD14 and lack of HLA-DR. The graph (e) shows proportion status of MDSCs. *$P < 0.05$; **$P < 0.01$.
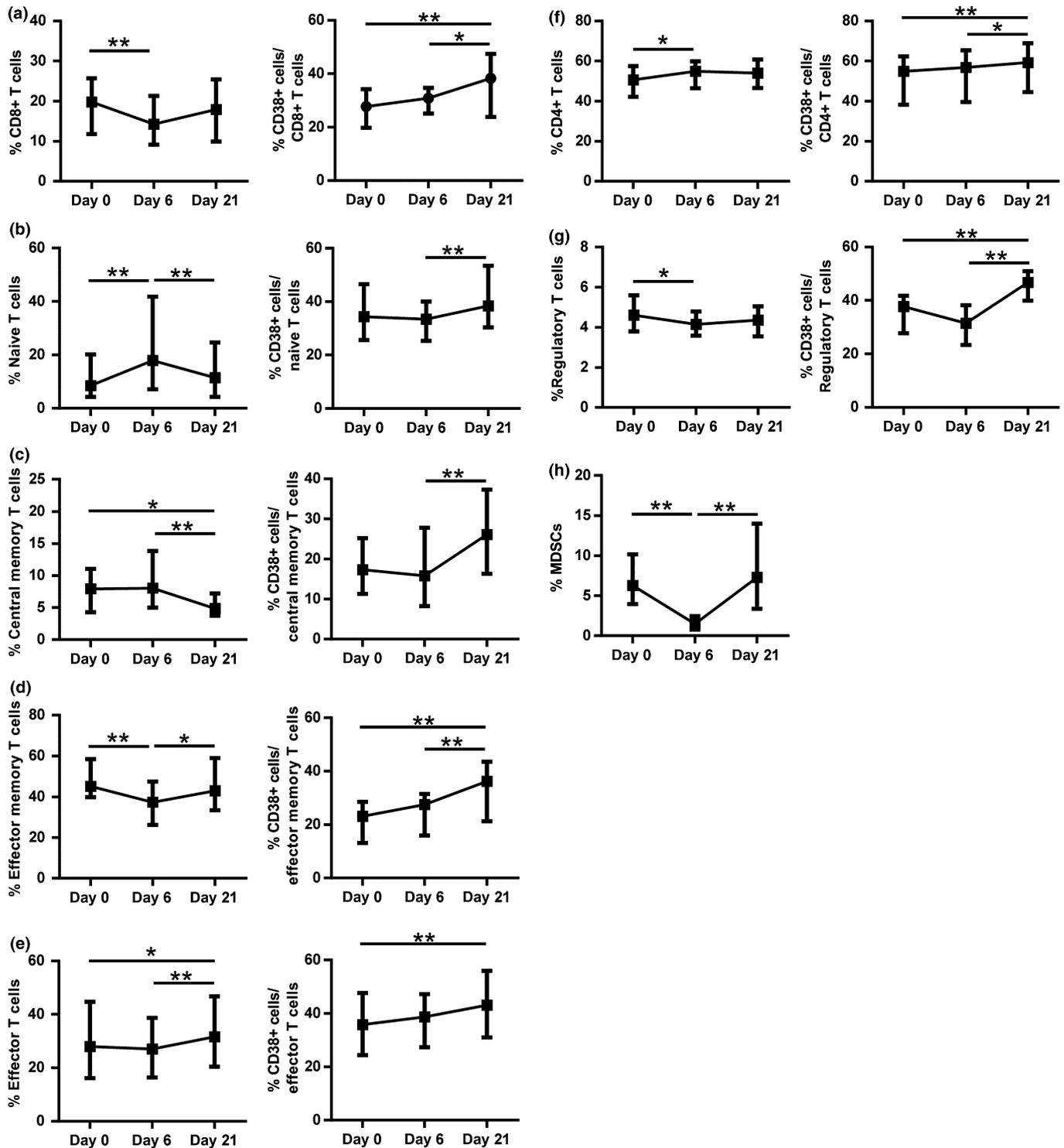
Fig. 3. Changes in the proportion and activation status of T cells and myeloid-derived suppressor cells (MDSCs) following addition of docetaxel to the cisplatin and 5-fluorouracil induction regimen therapy. Peripheral blood samples of 16 patients treated with chemotherapy were collected before cycle 1 day 1 (day 0), 5 days after the start of treatment (day 6), and at the end of cycle 1 (day 21), and compared with each other. Graphs on the left side show the changes of proportion status, and those on the right side show the changes of activation status in each gate. *$P < 0.05$; **$P < 0.01$.

Our previous observations indicated that the percentage of CD8^{+} T cells in the peripheral circulation of patients with HNSCC was not different from those of HDs;[5] therefore, in the present study, we focused on the subsets of CD8^{+} T cells

and intensively investigated the proportion of each subset as well as their activation status.

Our results showed that the proportion of naïve T cells was lower and that of T$_{EM}$ cells was higher in HNSCC patients

than in HDs. With the progression of tumors, the amount of tumor antigens released from primary tumors increased, and tumor-specific $CD8^+$ T cells are primed by antigen-presenting cells engulfing these tumor antigens.[28] The longevity and intermittent turnover of antigen-specific memory $CD8^+$ T cells is MHC-independent and maintained through the action of interleukin (IL)-15, which is produced by a variety of non-T cells including monocytes, macrophages, and dendritic cells.[29] Indeed, the presence of IL-15 in the sera and tumor tissue has been reported in several carcinomas including HNSCC.[30,31] Interestingly, patients with advanced stage carcinoma showed a marked decrease in T_{EM} compared with those with at an early stage, suggesting that dysfunction and phenotypic changes in these IL-15-producing cells might progress in patients with advanced stage carcinoma. Turksma et al.[14] reported similar findings, with a significant shift from naïve to T_{EM} in HNSCC patients. However, in their study, $CD3^+$ T cells not $CD8^+$ T cells were characterized on the basis of the expression of CD45RA, CD45RO, and CD27, indicating that the bulk populations of T cells were analyzed. In the present study, we could clearly show a dynamic change in $CD8^+$ T cell subsets in HNSCC patients.

With respect to effector T_{EFF} cells, Kuss et al.[6] revealed that the frequency of effector $CD8^+$ $CD45RO^-$ $CD27^-$ cells was increased in HNSCC patients regardless of the disease status, and these expanded effector T cells were zeta-negative and sensitive to apoptosis. On the contrary, our data showed that the proportion of T_{EFF} was not different between HNSCC patients and HDs, rather it was different according to the disease status. One possibility could be due to the markers used for characterization of $CD8^+$ T-cell subsets. Next, we investigated the activation status of each $CD8^+$ T-cell subset, and found that the proportion of activated $CD8^+$ T cells in HNSCC patients was not significantly different compared with that in HDs. Nevertheless, the proportion of activated $CD8^+$ T cells, especially activated T_{EM} and T_{EFF}, was dramatically increased in patients with advanced stage carcinoma, suggesting that $CD8^+$ T cells stimulated with a certain type of tumor antigen might proliferate and be maintained with the progression of tumors. Regardless of such suggested antitumor immune responses, as expected, the proportion of Tregs and $CD14^+$ $HLA-DR^-$ MDSCs was elevated in the peripheral blood of patients with HNSCC, and they did not correlate with tumor stages. Head and neck squamous cell carcinoma is known to produce various factors including angiogenic factors and pro-inflammatory cytokines,[32] and these molecules could induce and expand MDSCs. Further analysis is required to clarify the immunosuppressive function of MDSCs in HNSCC. Although the proportion of Tregs was elevated in HNSCC patients, that of activated Tregs was not. Regulatory T cells consist of two distinct subsets, naturally occurring Tregs and inducible Tregs. $CD4^+$ $CD25^+$ $CD127^-$ T cells examined in this study represent naturally occurring Tregs, whereas inducible Tregs are induced in the periphery by adequate antigenic stimulation in the presence of immunoregulatory cytokines such as IL-10 and transforming growth factor-β with disease process.[33,34] Therefore, the immunosuppressive behavior of inducible Tregs might be involved in the status of naturally occurring Tregs.

Although various changes regarding the proportion and/or activation status of T-cell subsets in peripheral blood caused by chemotherapy have been reported in several malignant tumors, including thoracic malignancies,[20] ovarian cancers,[11] and pancreatic cancers,[35] it still remains unclear how TPF induction chemotherapy against HNSCC modulates the immune cell profile. During chemotherapy, the peripheral immune cell profile regulated by complex homeostatic mechanisms could be influenced by a variety of factors, including the type of chemotherapeutic agents, clinical pharmacokinetics and pharmacodynamics of chemotherapeutic agents, and their differences in sensitivity to chemotherapeutic agents. However, our data showed a certain trend; namely, a decrease in T_{CM} cells and an increase in T_{EFF} cells were observed among $CD8^+$ T-cell subsets after TPF therapy. These findings suggest that the release of tumor antigens by dying tumor cells might cause the differentiation of T_{CM} cells into T_{EFF} cells. Moreover, an increase in the proportion of activated $CD8^+$ T cells might support the suggestion that TPF chemotherapy not only directs killer T cells but also activates tumor antigen-specific $CD8^+$ T cells.

Two immune regulatory cells are transiently decreased by TPF therapy but recover promptly to baseline levels. Compared with Tregs, MDSCs change more rapidly and dramatically in response to TPF chemotherapy. This difference might be because all three chemotherapeutic agents used in TPF therapy have selective cytotoxic activity against MDSCs,[23–25] whereas only docetaxel has been reported to have cytotoxic activity against Tregs.[36] Myeloid-derived suppressor cells are a mixture of myeloid progenitor cells at varying stages of differentiation; therefore, compared with Tregs, they may contain populations with high chemosensitivity. One of the acknowledged limitations of the present study includes the relatively small number of samples, and further analysis in a larger cohort, as well as for correlation with treatment efficacy and prognostic outcome, is necessary.

In conclusion, despite the immunosuppressive status in patients with HNSCC, the shift in proportion from naïve $CD8^+$ T cells to T_{EM} suggested the induction and maintenance of tumor-specific immune responses mediated by $CD8^+$ T cells. Moreover, chemotherapy can trigger not only a transient reduction of immune regulatory cells but also further activation of $CD8^+$ T cells. Further elucidation regarding immune cell profiles in HNSCC patients receiving chemotherapy may provide new insights into the development of chemo-immunotherapy against HNSCC.

Disclosure Statement

The authors declare have no conflict of interest.

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