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Alterations in composition of immune cells and impairment of anti-tumor immune response in aged oral cancer-bearing mice

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ABSTRACT

Objectives: Aging has been suggested to be associated with immune dysregulation. An understanding of alterations in the host immunity with advancing age is, therefore, important for designing immune therapy for elderly cancer patients. In this context, not much is known about age-associated alterations in the immune system in oral cancer.

Methods: To evaluate age-associated alterations in the immune system, which might affect anti-tumor immune responses in oral cancer, we performed a comparative analysis of the proportion of different immune cells, the proliferative capacity of T cell compartment, and the response against immune therapies targeting immune check point molecules between young and aged oral cancer-bearing mice.

Results: The proportion of immune regulatory cells, such as regulatory T cells and myeloid derived suppressor cells, was significantly increased in aged mice compared to that in young mice. Moreover, the expression of PD-1 and CTLA-4 on both $CD4^+$ and $CD8^+$ T cells was elevated in aged mice compared to that in young mice, and the proliferative abilities of $CD4^+$ and $CD8^+$ T cells derived from aged mice were significantly reduced following stimulation of T-cell receptors. Moreover, tumor growth was significantly enhanced in aged mice compared to that in young mice. However, immunotherapies targeting PD-1, CTLA-4, and PD-L1 resulted in faster tumor regression in aged mice than in young mice.

Conclusions: Together, our results indicate that age-associated alterations in the immune system are directly associated with the impairment of anti-tumor immunity in aged mice bearing oral cancer, and might facilitate the progression of the tumor.

Introduction

Accumulating evidence has revealed that age-associated immune dysregulation affects the ability to resist diseases, including cancer, and inflammatory and autoimmune diseases [1–3]. Notably, most of the cancers occur in the elderly, aged 65 years or above [4]. Oral cancer is one of the most common malignancies occurring worldwide. Although the major risk factors of oral cancer have been shown to be the use of tobacco [5] and alcohol [6], and human papillomavirus infection [7], the prevalence of oral cancer is also dependent on aging [8]. Despite the fact that the outcome of treatment of oral cancer has remarkably improved owing to recent advances in diagnostic techniques and therapies, highly metastatic and refractory cancer remain challenging to treat [8]. Therefore, new therapeutic strategies are required to improve the outcome in patients with more advanced oral cancer. Immunotherapy is a new therapeutic strategy currently being evaluated for the treatment of recurrent and metastatic oral cancer.

It has been suggested that aging is accompanied by numerical alterations in the cellular components of innate and adaptive immunity [9–15], and these alterations might facilitate the incidence and development of cancers in the elderly [16,17]. Therefore, an understanding of age-associated and tumor-associated decline in immunity could be equally important in optimizing immunotherapeutic interventions in the elderly cancer patients [16,17]. However, little attention has been paid in determining the influence of age-associated alterations in elderly oral cancer patients, and investigations on the response to immunotherapies against oral cancer have not been carried out in aged, tumor-bearing, hosts.

Recently cancer immunotherapies, typically based on targeting the immune check-point molecules, including CTLA-4, PD-1, and PD-L1,

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have been clinically successful and have been demonstrated to improve clinical outcomes in patients with recurrent or metastatic cancer [18–20]. However, the response rate to these agents remains low, and one of the most critical issues is to identify predictive markers for patients who derive greater benefits from the use of these agents.

In the present study, we investigated age-associated alterations in the immune system in oral cancer-bearing mice with the objective of understanding as to how advancing age influences the tumor immunity and the response to immunotherapeutic agents targeting immune check-point inhibitors. Our data reveals that these agents are more effective in aged mice than in young mice, suggesting that immunotherapies targeting immune check-point molecules might be the desirable strategies in elderly oral cancer patients. We also discuss the possible involvement of immunosenescence in the impairment of antitumor immunity and tumor progression in oral cancer.

Materials and methods

Mice and cell lines

Young (8-week-old) and aged (17-month-old) female C3H/HeN mice were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) and were housed under specific pathogen-free conditions according to institutional guidelines at the University of Toyama (Toyama, Japan). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University.

The oral squamous cell carcinoma cell line, NR-S1K, was kindly provided by Dr. M. Azuma from the Department of Molecular Immunology, Graduate School, Tokyo Medical and Dental University. This cell line was derived from the NR-S1 cell line, which, in turn, was established from C3H/HeN mice [21]. Cells were maintained in RPMI1640 medium, supplemented with 10% fetal bovine serum.

Tumor model

NR-S1K cells (1×10^6) were administered subcutaneously into the right masseter of C3H/HeN mice and the tumor area (length × width) was measured every three days using a caliper, as described previously [22]. When the tumor surface area was approximately 150 mm^2, mice were sacrificed, and cells from tumors, peripheral blood, spleen, and lymph nodes were analyzed by flow cytometry.

As a therapeutic model, administration of blocking antibodies against immune checkpoint molecules (CTLA-4, PD-1, and PD-L1) was initiated at day 7 of tumor inoculation, and was continued in each group of mice, once every week. Control mice received saline. Tumor sizes (length × width) were determined at 5-day intervals.

Antibodies and reagents

The following antibodies were obtained from eBioscience (San Diego, CA): FITC-conjugated antibody against mouse PD-1; PE-conjugated antibody against mouse CD152; PerCP-Cy5.5-conjugated antibody against mouse CD4; APC-conjugated antibody against mouse CD62L; APC-eFluor 780-conjugated antibody against mouse Ly-6G (Gr-1); purified CD16/32 mAb and functional-grade antibodies against CD3 and CD28. FITC-conjugated antibody against mouse F4/80, APC-conjugated antibody against mouse Foxp3, and PerCP-Cy5.5-conjugated antibody against mouse CD11b were obtained from TONBO Biosciences (San Diego, CA). PE-Cy7-conjugated antibody against mouse CD8 and FITC-conjugated antibody against mouse CD44 were obtained from Invitrogen (Carlsbad, CA), whereas PE-conjugated antibody against mouse CD206 was obtained from Biolegend (San Diego, CA). For *in vivo* experiment, antibodies against mouse CTLA-4 (clone: 9D9), mouse PD-1 (clone: RPM1-14), and mouse PD-L1 (clone: 10F.9G2) were purchased from BioXcell (West Lebanon, NH). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen.

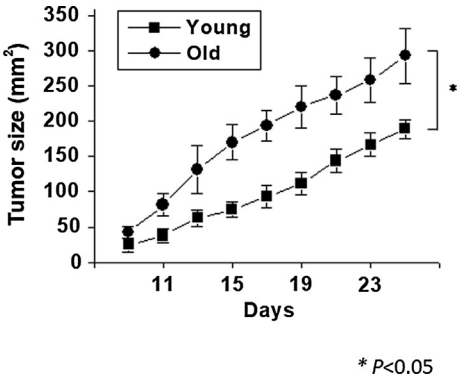


Fig. 1. Growth curve of subcutaneous tumor in young and aged mice. The sizes of tumors in mice were measured at the indicated time points (n = 4/group). The mean time required for the tumor surface areas to increase to 150 mm^2 was shorter in aged mice (7 ± 0.5 days) compared to that in young mice (10 ± 1.5 days). * $p < 0.05$.

Flow cytometry

Samples were blocked with purified FcR blocking mAb (eBioscience), washed, and suspended in phosphate-buffered saline (PBS), supplemented with 2% fetal bovine serum, 0.05% NaN_3, and a saturating concentration of fluorochrome-conjugated mAbs, as described previously [22,23]. Cells were analyzed using FACS Canto II (Becton Dickinson, San Jose, CA).

Cell purification

Splenic $CD3^+$ T cells were isolated from naïve C3H/HeN mice using a Pan T-cell Isolation Kit (Miltenyi Biotec, Auburn, CA). Flow cytometry of purified cells routinely indicated > 90% purity.

Tumor dissociation

Tumors were harvested and digested at 37 °C for 60 min with 0.02 mg/mL DNase I (Roche, Switzerland) and 1 mg/mL collagenase type IV (Sigma–Aldrich, St Louis, MO). Single cell suspension was generated by filtration through a 70-μm cell strainer [23].

T cell proliferation assay

In vitro T-cell proliferation assays were performed, as described previously [22,24]. Splenic $CD3^+$ T cells were labeled with CFSE and cocultured for 72 h in the absence or presence of 0.1 μg/mL mAbs against CD3 and CD28. The CFSE dilution was measured by flow cytometry.

Statistical analysis

Groups were compared by Student's *t*-test or ANOVA, and a value of $p < 0.05$ was considered to be statistically significant.

Results

Subcutaneously injected tumors grow faster in aged mice than in young mice

Differences in the tumor growth rates between young and aged mice have been demonstrated in previous studies [25–30]. To compare the growth rates of oral cancer between young and aged mice, NR-S1K oral squamous carcinoma cells were injected subcutaneously into the right masseter of young and aged C3H/HeN mice and tumor surface areas were monitored over time. Subcutaneous tumors with a surface area of 150 mm^2 usually developed in about three weeks in young mice,

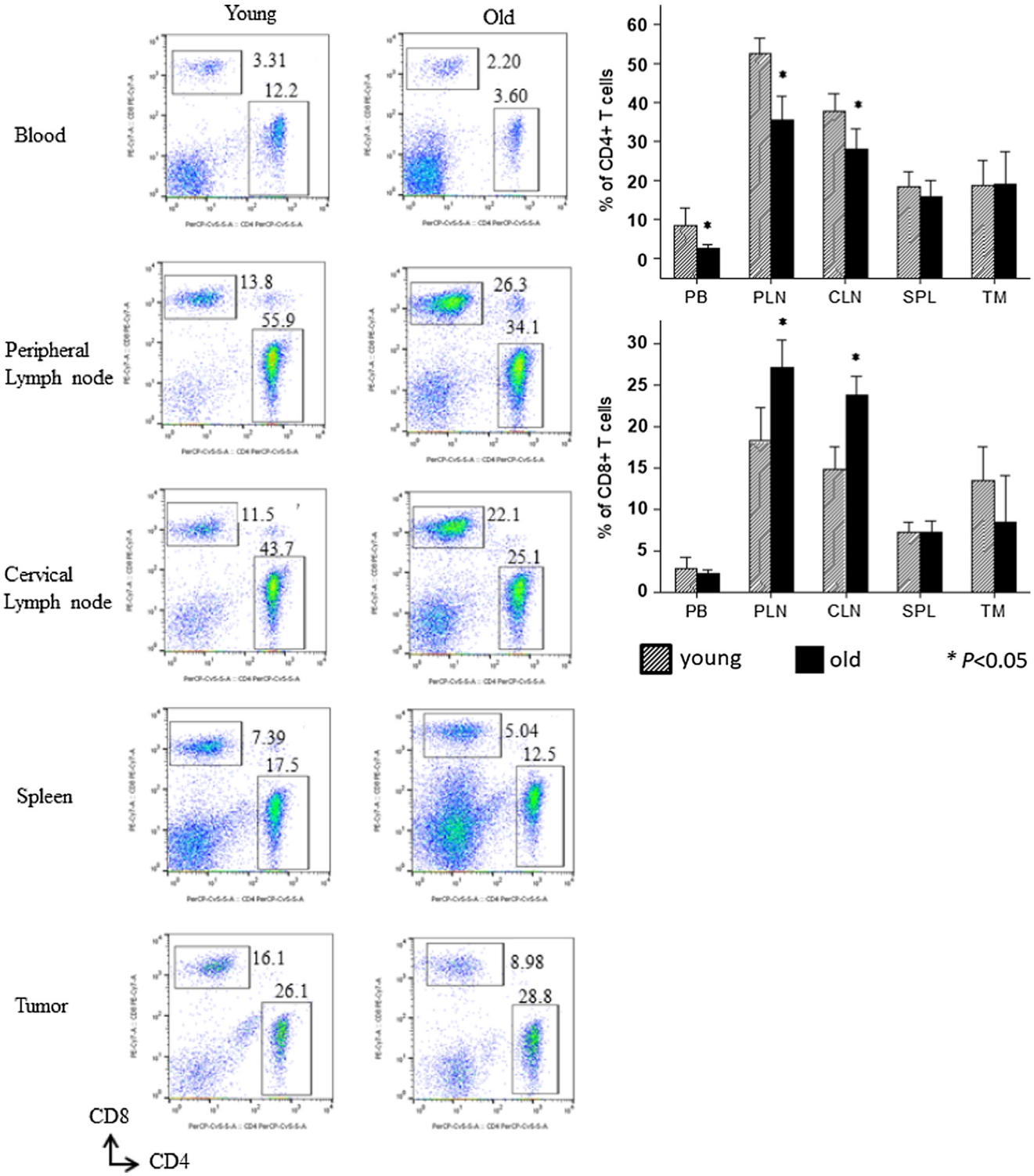
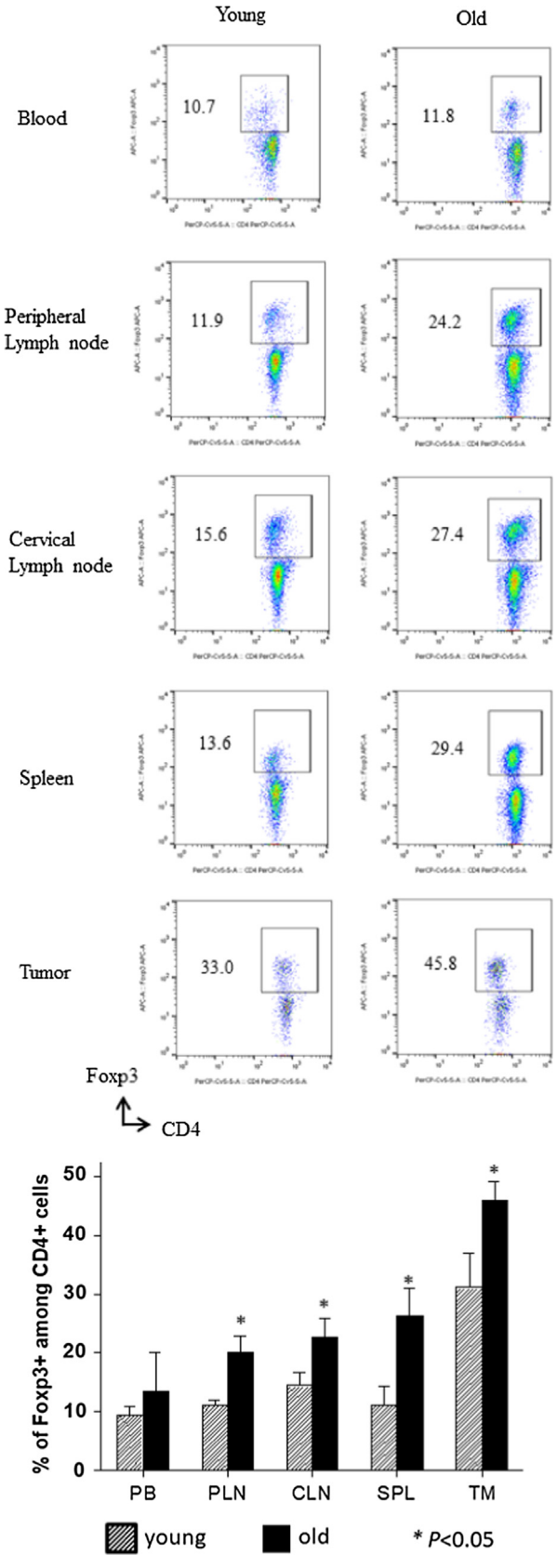


Fig. 2. Age-associated alterations in immune cell populations in oral cancer-bearing mice. Young and old mice were challenged with NR-S1K oral squamous carcinoma cells. At the time points when subcutaneous tumor had surface areas of 150 mm^2, peripheral blood, cervical lymph nodes, peripheral lymph nodes, spleen, and tumors were harvested and the percentages of different immune cell types in each organs in NR-S1K tumor-bearing young or old mice were determined by flow cytometry. Representative scatter plots and summary of these results are shown (n = 4/group); $p < 0.05$, young vs. old. (A) $CD4^+$ and $CD8^+$ T cells, (B) $CD4^+Foxp3^+$ regulatory T cells (Tregs), (C) $CD11b^+Gr\text{-}1^+$ myeloid derived suppressor cells (MDSCs), and (D) $F4/80^+CD206^+$ tumor-associated macrophages (TAMs).

whereas in aged mice, about two weeks were required for the same (Fig. 1). These data revealed that the growth of grafted oral cancer was faster in aged mice compared to that in young mice.

Total proportion of regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) is elevated in aged mice

To evaluate the age-associated immunological alterations in oral cancer, we first compared immune cell populations between young and



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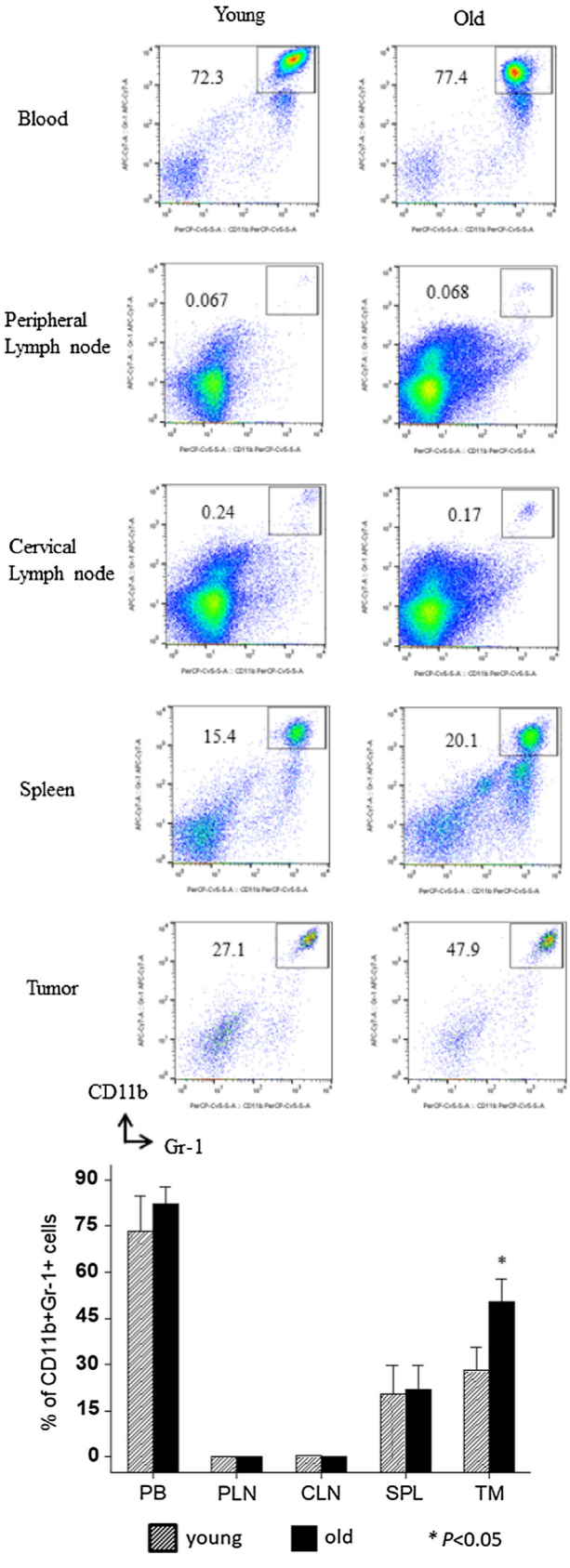

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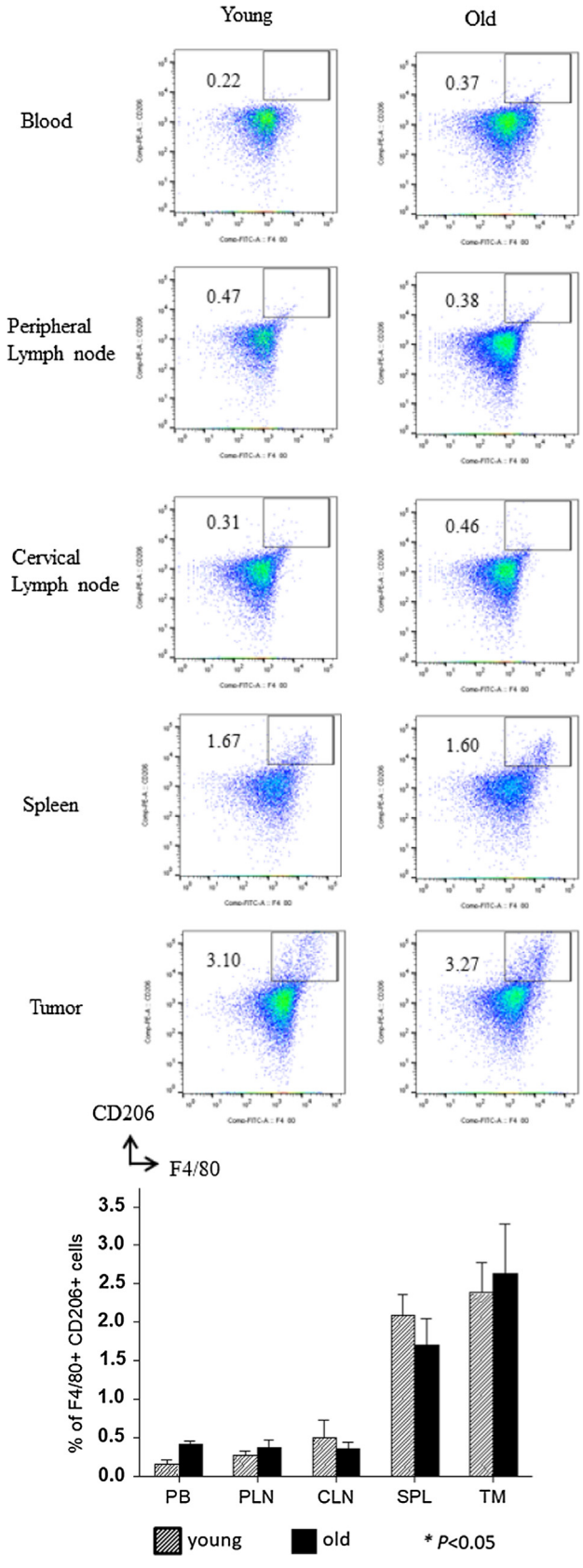
aged mice. At time points when surface area of subcutaneous tumors was 150 mm^2, peripheral blood, cervical lymph nodes, peripheral lymph nodes, spleen, and tumors were harvested and the percentages of different immune cell types in each organs were determined. The proportion of $CD4^+$ T cells was significantly reduced in peripheral blood, peripheral lymph nodes, and cervical lymph nodes of aged mice



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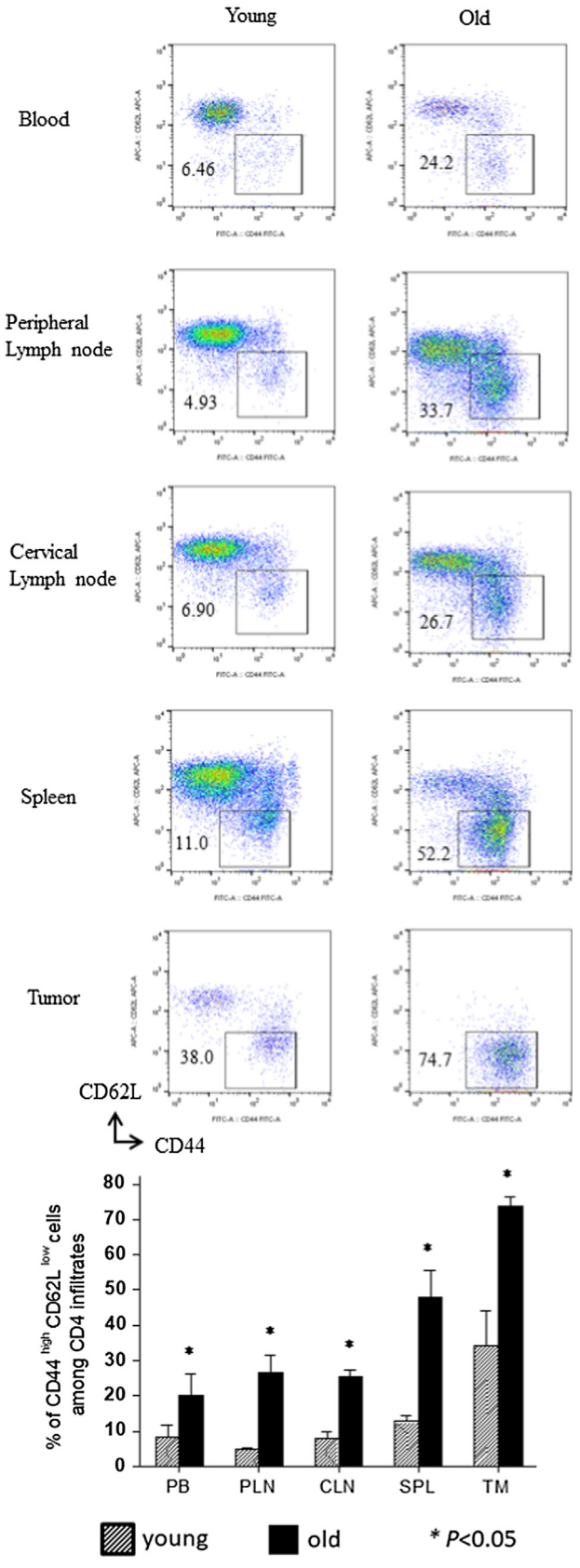
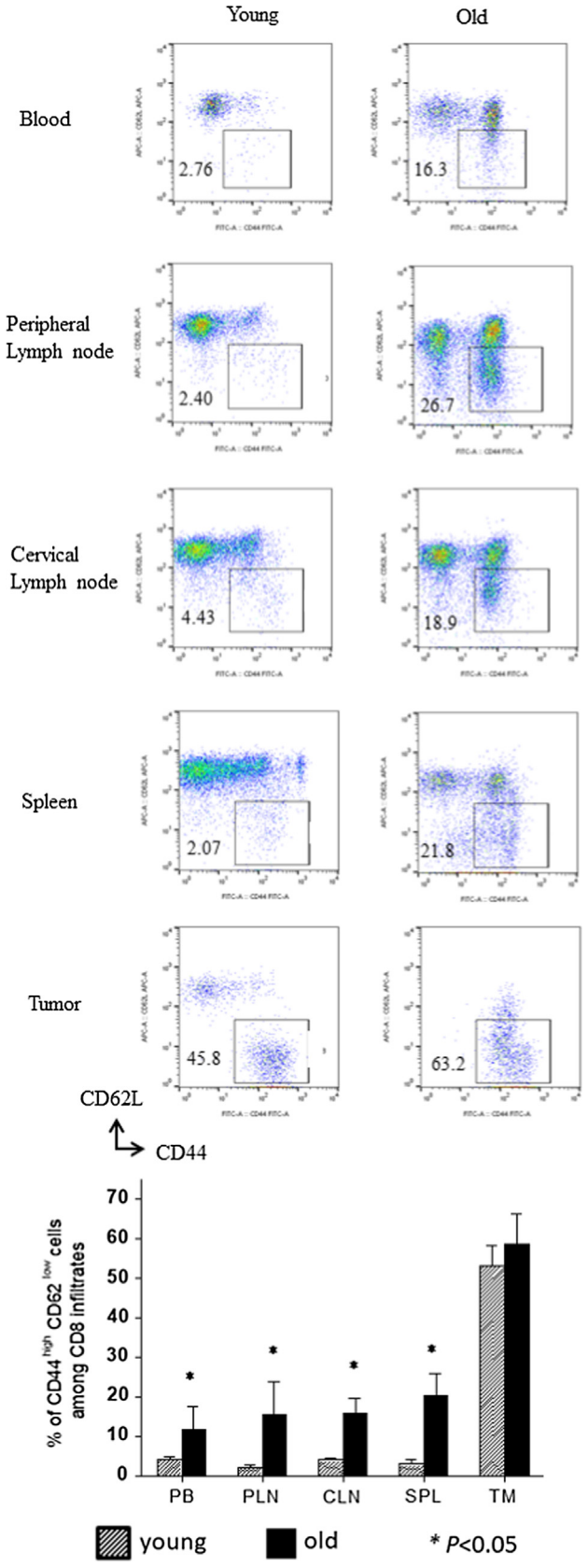


Fig. 3. Phenotypic profiles of T cells from young and old oral cancer-bearing mice. Composition of CD44 $_{high}$ and CD62L $_{low}$ in CD4^{+} T cells (A) and CD8^{+} T cells (B) from peripheral blood, cervical lymph nodes, peripheral lymph nodes, spleen, and tumors of young and old NR-S1K cancer-bearing mice was determined by flow cytometry. Representative scatter plots and summary of these results are shown (n = 4/group).



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compared to that in young mice, and the proportion of $CD8^+$ T cells was significantly reduced in peripheral lymph nodes and cervical lymph nodes of aged mice compared to that in young mice (Fig. 2A).

Several studies with mice have shown that aging affects the proportion of immune regulatory cells, such as $CD4^+Foxp3^+$ regulatory T cell (Tregs) and $CD11b^+Gr\text{-}1^+$ myeloid derived suppressor cells (MDSCs) [31–36]. Thus, we investigated whether aging affected the proportion of these immune regulatory cells in oral cancer-bearing mice. The proportion of Tregs was significantly increased in cervical lymph nodes, peripheral lymph nodes, spleen, and tumors, but not in peripheral blood of aged mice compared to that in young mice (Fig. 2B). Moreover, the proportion of MDSCs was significantly increased in tumors but not in other organs of aged mice compared to that in young mice (Fig. 2C). In contrast, the proportion of tumor-associated macrophages (TAMs) was not altered between young and aged mice (Fig. 2D). These observations suggest that advancing age results in the accumulation of immune regulatory cells, which may facilitate immune dysregulation in oral cancer-bearing hosts.

Composition of T cells in aged mice is altered with an increase in memory T cells

One of the most notable alterations in the T cell compartment during aging is the reduction of naïve T cells, which results in the elevation of memory T cells [37]. While the proportions of both $CD4^+$ and $CD8^+$ T cells were maintained during aging, in the present study, we examined the proportions of $CD44_{high}$ and $CD62L_{low}$ phenotypes of $CD4^+$ and $CD8^+$ T cells, which have been shown to be the phenotypes of memory T cells [38,39]. As shown in Fig. 3A, the proportions of $CD44_{high}$ and $CD62L_{low}$ CD4 T cells were significantly increased in peripheral blood, cervical lymph nodes, peripheral lymph nodes, spleen, and tumors of aged mice compared to those in young mice. The proportions of $CD44_{high}$ and $CD62L_{low}$ CD8 T cells were significantly increased in peripheral blood, cervical lymph nodes, peripheral lymph nodes, spleen, but not in tumors of aged mice compared to that in young mice (Fig. 3B).

Expression of immune checkpoint molecules is increased on T cells from aged mice

The expression of immune checkpoint molecules, including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) on T cells has been shown to affect the decline of anti-tumor immunity and contributes to tumor progression in various types of cancers [40–44]. However, the expression status of these molecules on T cells in the tumor microenvironment of oral cancer, especially with advancing age, has not yet been studied. We compared the expression of these immune checkpoint molecules on T cells between young and aged mice. As shown in Fig. 4A, $CD4^+$ T cells in peripheral blood, peripheral lymph node, cervical lymph node, spleen and tumors of aged mice had increased levels of both CTLA-4 and PD-1 compared to that in young mice. $CD8^+$ T cells in cervical lymph node, spleen, and tumor, but not in peripheral blood and peripheral lymph node, of aged mice exhibited increased levels of CTLA-4 compared to that in young mice. Also, $CD8^+$ T cells in peripheral lymph node, cervical lymph node, and tumor, but not in peripheral blood and spleen, of aged mice had increased levels of PD-1 compared to that in young mice (Fig. 4B). These results suggest that advancing age can enhance the expression of immune check point molecules on T cells, which may facilitate immune dysregulation in oral cancer-bearing hosts.

Proliferation capacity of T cells is markedly reduced in aged mice

Data from previous animal and human studies have revealed that age-associated decline in immunity is mainly due to reduced T cell reactivity, including reduced capacity of T cells to proliferate and survive following stimulation of T-cell receptors (TCRs) [9–11]. We investigated as to how advancing age affects the function of T cell

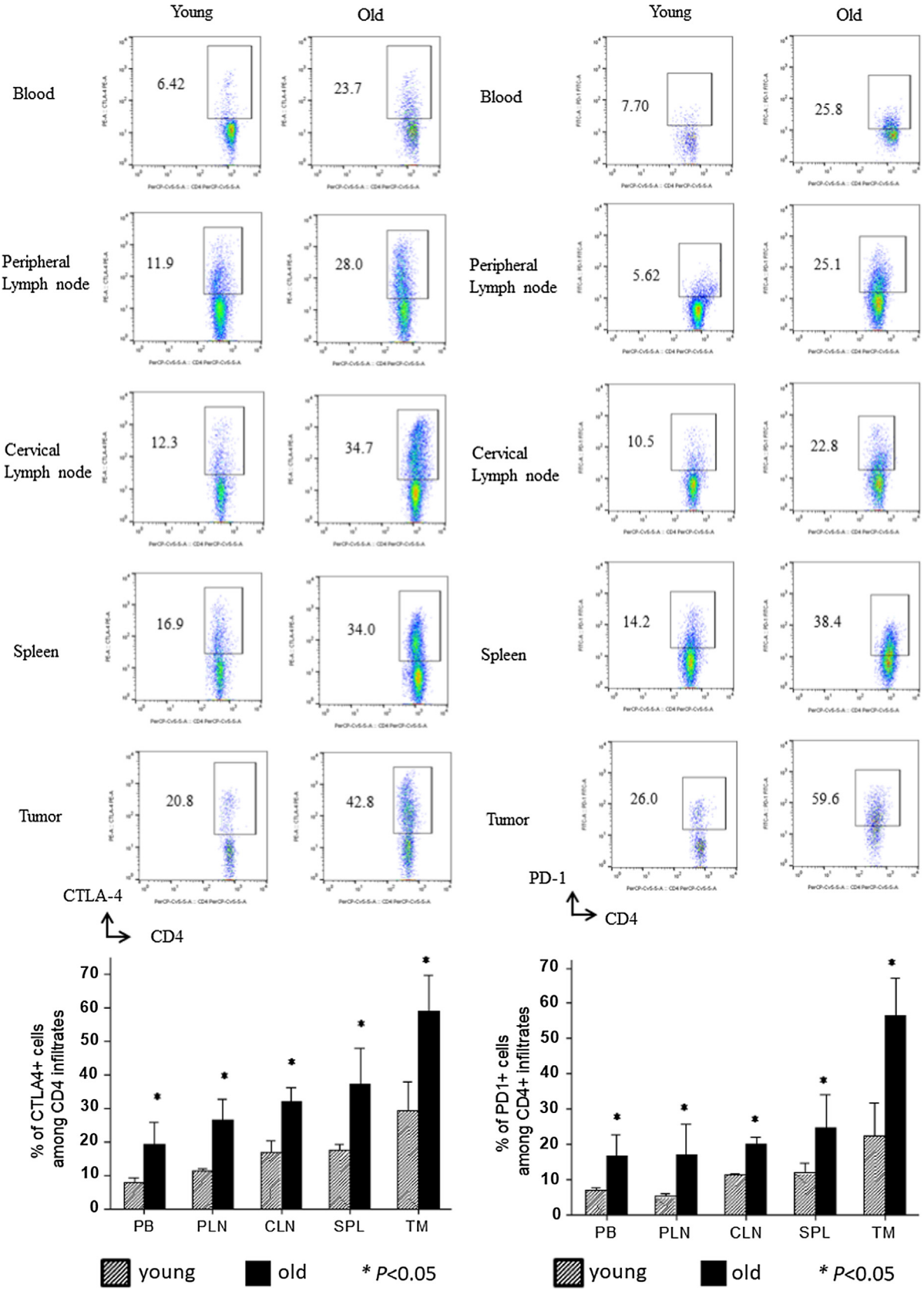
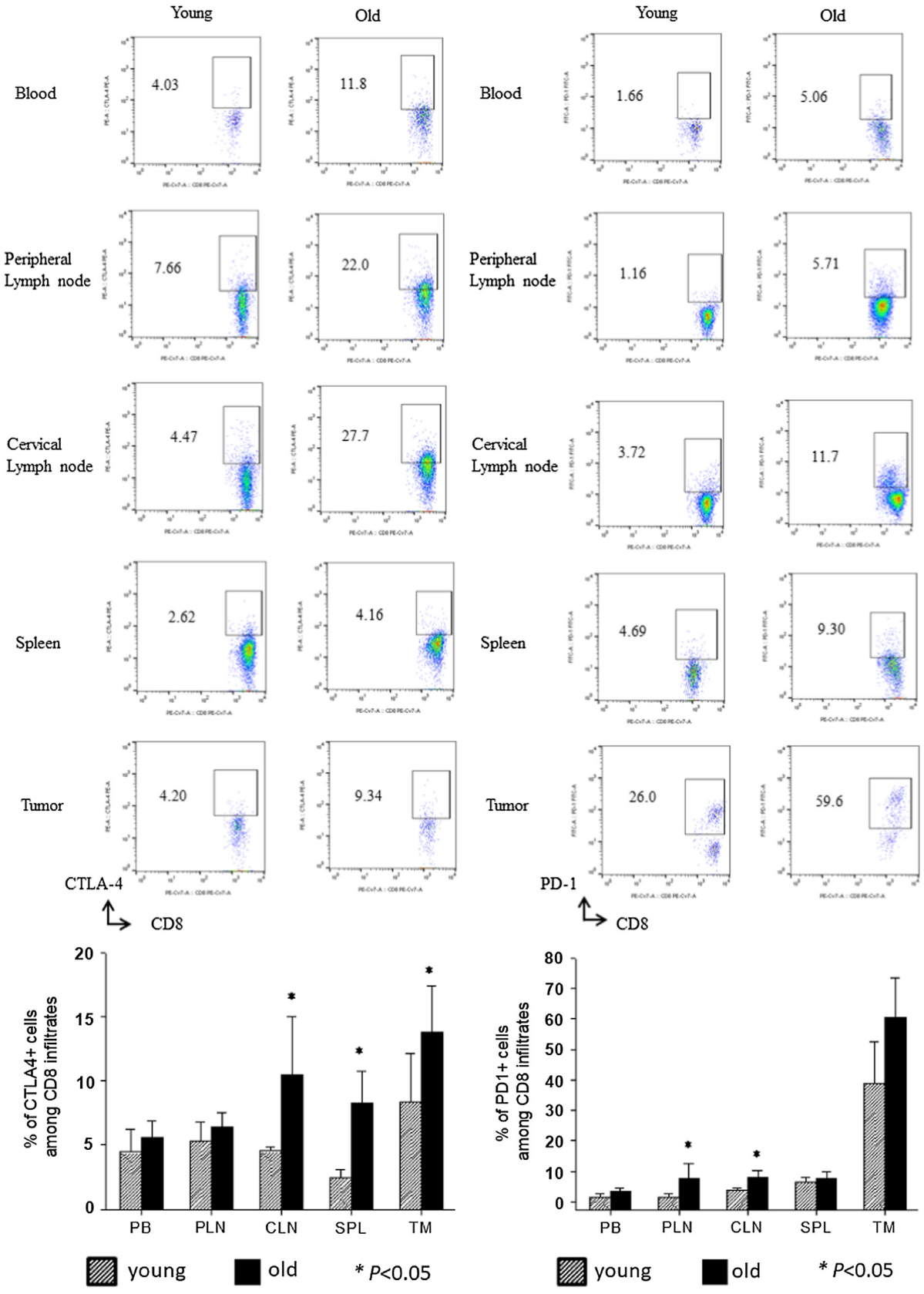


Fig. 4. Expression of immune checkpoint molecules in T cells from young and old cancer-bearing mice. Surface expression of CTLA-4 and PD-1 on $CD4^+$ T (A) and $CD8^+$ T (B) cells in peripheral blood, cervical lymph nodes, peripheral lymph nodes, spleen, and tumors of young and old NR-S1K cancer-bearing mice was analyzed by flow cytometry. Representative scatter plots and summary of these results are shown (n = 4/group).



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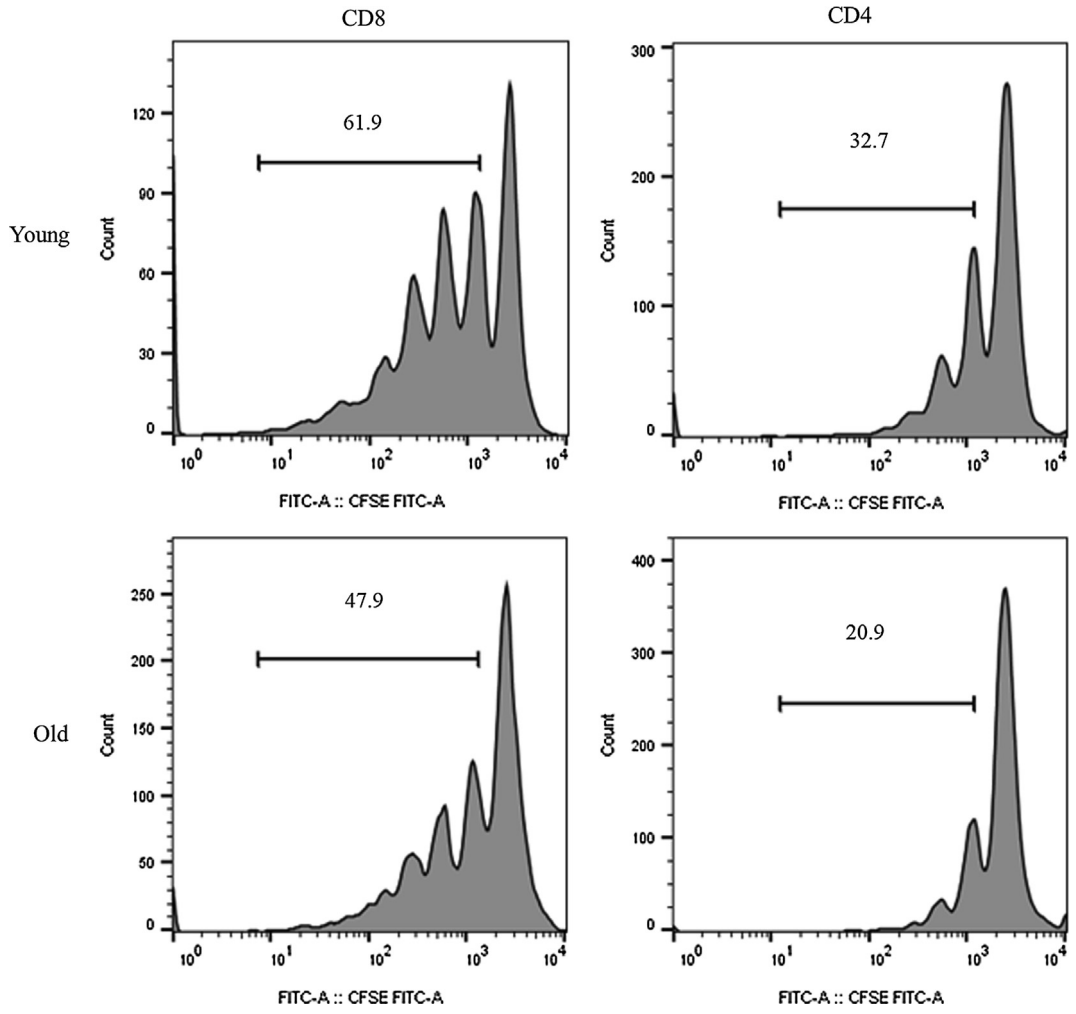

Fig. 5. Proliferation capacity of T cells from young and old oral cancer-bearing mice. Splenic Pan T cells from young and old cancer-bearing mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured with or without 0.1 μg/mL mAbs against CD3 and CD28 for 72 h. T cell proliferation was measured by CFSE dilution. Experiments were performed three times and similar results were obtained. Representative histograms from one experiment are shown.

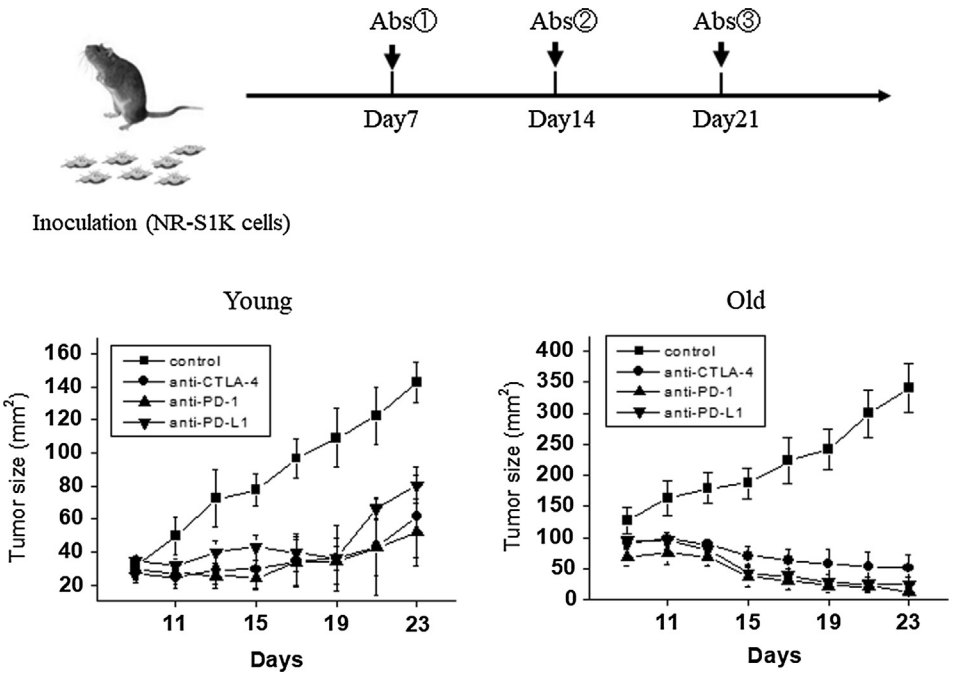

Fig. 6. Inhibitory effects of immune checkpoint inhibitors (ICIs) on tumor growth in young and old oral cancer-bearing mice. Young and old mice were inoculated with NR-S1K oral squamous carcinoma cells. From day 7 of tumor inoculation, administration of blocking antibodies against immune checkpoint molecules (CTLA-4, PD-1, and PD-L1) was initiated and continued in each group of mice once every week. Control mice received saline. Tumor sizes in mice were measured at the indicated time points ($n = 4$/group); $p < 0.05$, ICIs vs. saline.

compartment in oral cancer-bearing mice. As shown in Fig. 5, the proliferation of both $CD4^+$ and $CD8^+$ T cells from aged mice was significantly disrupted following TCR stimulation compared to that in young mice.

Immunotherapies targeting PD-1, CTLA-4, and PD-L1 are highly effective in treating aged tumor-bearing mice

Anti-CTLA-4 antibody improves T cell activation and T cell priming, and anti-PD-1 and PD-L1 antibodies can prevent negative signals to $PD\text{-}1^+$ anti-cancer T cells [45,46]. The three classes of immune checkpoint inhibitors of CTLA-4, PD-1, and PD-L1 have shown clinically remarkable antitumor effects in an increasing number of elderly cancer patients [18–20]. However, comparative effects of these immune checkpoint inhibitors in young and aged hosts have not been much investigated. To test the effects of age on treatments with these immune check point inhibitors, we compared the efficacy of these antibodies between young and aged tumor-bearing mice. As shown in Fig. 6, the efficacy of all three agents was more in aged tumor-bearing mice compared to that in young tumor-bearing mice.

Discussion

The present study is the first to demonstrate age-associated alterations in the immune system in oral cancer-bearing mice. We performed a comparative analysis of the composition of immune cells, the proliferation of T cells, and the response against immune therapies targeting immune check-point molecules between young and aged oral cancer-bearing mice. There are numerous studies demonstrating successful outcomes of antitumor immune therapy in animal models; however, the clinical efficacy of immune therapies in cancer patients has not been satisfactory [16,17]. These immune therapies are mainly applied to elderly persons, many of whom have dysregulated immune system, which contributes to evasion of antitumor immune therapies. Although most preclinical studies on antitumor immune therapy have been carried out on young animals, in the studies which addressed the age-associated alteration in immune response and compared the difference in the reaction against immune therapies between the young and the old, the responses to immune therapies were reduced in old animals compared to that in young animals [47–49].

Both animal and human studies have revealed age-associated alterations in the cellular components of innate and adaptive immunity, viz. impairment of proliferation of T cells [9], reduced survival of T cells following TCR stimulation [10], impairment of effector function of T cells [11], impairment of cytotoxic activity of T cells [12], impairment of the response to exogenous antigens on B cells [13], decreased number or impaired function of APCs, such as dendritic cells and macrophages [14,15], qualitative alteration of natural killer and natural killer T cells [50], decreased expression of costimulatory molecules [51], decreased expression and impaired function of TLRs [52], accumulation of immune regulatory cells, such as Tregs cells and MDSCs [31–36]. These age-associated alterations in the immune system might contribute to the poor effect of immunotherapies in elderly cancer patients. Consistently, in the present study, impaired proliferative capacity of T cells following TCR stimulation and increased number of immune regulatory cells, including Tregs and MDSCs in aged mice, was observed compared to that in young mice. We compared the effects of cancer immunotherapies targeting CTLA-4, PD-1, and PD-L1 in young and old oral cancer-bearing mice. Surprisingly, all the three agents were more effective in treating old, rather than young, tumor-bearing mice. Therefore, targeting immune check point molecules would be desirable in old oral cancer-bearing hosts, because age-associated phenotypic alterations in immune cells, specifically with increased expression of immune check-point molecules including PD-1, CTLA-4, and PD-L1, may facilitate the effect of these antibodies.

It has been suggested that the number of PD-1 expressing lymphocytes is higher in good responders of PD-1 blockade among melanoma patients [53]. In addition, higher numbers of PD-L1 expressing tumor cells and macrophages have been demonstrated to be positively correlated with the response to PD-1 blockade in melanoma patients [53].

In the present study, we observed that the expression of PD-1 and CTLA-4 in T lymphocytes was more abundant in aged mice compared to that in young mice. Moreover, the proportion of PD-L1 expressing MDSCs was also increased in aged mice compared to that in young mice. Increased number of PD-1 expressing T cells has been suggested to be due to the elevation of dysfunctional T cells, such as anergic and exhausted T cells, or memory T cells, and the response of PD-1 blockade has been suggested to be associated with reinvigoration of these dysfunctional T cells [54,55]. Therefore, the good response of immune check-point inhibitors in aged mice, in the present study, could be associated with reinvigoration of these dysfunctional T cells. One of the most critical issues is whether tumor growth can be accelerated in aged hosts. In the present study, the tumor growth was observed to be faster in aged mice compared to that in young mice. However, it is widely accepted that tumor growth is delayed in aged hosts compared to that in young hosts. In previous studies, it has been demonstrated that the tumor growth is slower in aged mice than in young mice in the B16/F10 mouse melanoma model [25], the EHS mouse sarcoma model [26], and the SP1mouse fibrosarcoma model [27]. In humans, bronchogenic [28], breast [29], and colon cancers [30] have been shown to grow slowly in elderly patients compared to that in relatively younger patients. Faster tumor growth in young mice has been suggested to be associated with faster cell proliferation and decreased tumor cell apoptosis [56], increased angiogenesis [26,27], and immune system in young hosts [57,58]. On the other hand, it was demonstrated that the growth of TRAMP-C2 mouse prostate tumor cells was similar in aged and young mice, and the tumor growth in aged mice was associated with high levels of MMP2 and MMP9 activity and angiogenesis [59]. Together, the effects of aging on tumor growth are likely to be tumor-cell dependent, although further study would be necessary to show how aging influences the acceleration of tumor growth in oral cancer.

Conclusions

It is very likely that advancing age is associated with the magnitude of immune dysregulation in tumors. Therefore, an understanding of how advancing age affects immune responses in cancer-bearing hosts could be important in providing new opportunities for the development of cancer immune therapy. We found profound alterations in the function of T cell compartment in aged oral cancers, which might directly relate to the impairment of anti-tumor immunity in elderly oral cancer-bearing hosts. Therefore, a strategy to effectively and specifically target these age-associated alterations in the T cell compartment could be a desirable strategy in elderly oral cancer patients.

Declaration of Competing Interest

All authors of this manuscript have no conflict of interest.

Acknowledgements

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