Tumor antigen-specific interleukin-10-producing T-cell response in patients with head and neck squamous cell carcinoma

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Abstract. Interleukin-10 (IL-10) is a highly pleiotropic cytokine that regulates immunological homeostasis through anti-inflammatory and/or immunostimulatory functions. Moreover, IL-10 is well known to exert diverse roles in tumor immunology and immunotherapy. The present study investigated the presence of circulating tumor antigen-specific IL-10-producing T cells in patients with head and neck squamous cell carcinoma (HNSCC), and determined factors that may influence the immunodynamics of IL-10-producing T cells. *In vitro*, peripheral blood mononuclear cells (PBMCs) stimulated with the tumor antigens p53 and MAGE-A4 were evaluated for interferon (IFN)-γ/IL-10 production using the IFN-γ/IL-10 double-color enzyme-linked immunosorbent spot assay. The proportion of T cells expressing immune checkpoint molecules in PBMCs was analyzed using flow cytometry. Of the 18 patients with HNSCC, 2 (11.1%) and 9 (50.0%) exhibited p53-specific IFN-γ and IL-10 production, respectively. Meanwhile, MAGE-A4-specific IFN-γ and IL-10 production was detected in 4 (28.6%) and 7 (50.0%) of 14 patients. In the p53-specific responses, IL-10-producing T cells were observed in significantly more patients than IFN-γ producing T cells (P=0.0275). In both $CD4^+$ and $CD8^+$ T cells, the proportion of T cells expressing lymphocyte activation gene-3 (Lag-3) was significantly lower in patients with p53-specific IL-10 production than in those without. In certain patients, Lag-3 blockade enhanced tumor antigen-specific IL-10. Taken together, the present study successfully demonstrated that tumor antigen-specific IL-10-producing T cells exist in the peripheral blood of patients with HNSCC and that Lag-3^+ T cells may serve an important role in modulating IL-10-producing T cells. These findings provide novel insights into the roles of IL-10 and Lag-3 in mediating antitumor immune responses.

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Abbreviations: IL-10, interleukin-10; PBMC, peripheral blood mononuclear cell; IFN-γ, interferon-γ; ELISPOT, enzyme-linked immunosorbent spot; Lag-3, lymphocyte activation gene-3; HNSCC, head and neck squamous cell carcinoma; Treg, regulatory T-cell; Th, T helper; Tr1, T regulatory type 1; SFC, spot-forming-cells; PD-1, programmed cell death-1; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; Tim-3, T-cell immunoglobulin and mucin-domain containing-3; TNF-α, tumor necrosis factor-α

Key words: IL-10, p53, MAGE-A4, tumor antigen, HNSCC, Lag-3

Introduction

Interleukin-10 (IL-10) is a pleiotropic cytokine that regulates immunological homeostasis through anti-inflammatory and/or immunostimulatory functions (1-3). IL-10 has been implicated in immunopathogenesis during tumor development and progression. Accumulating evidence indicates that IL-10 plays a key role in establishing and maintaining a protumor microenvironment as a potent immunosuppressive cytokine. In particular, major immunosuppressive cells, such as regulatory T cells (Tregs), myeloid-derived suppressor cells, and tumor-associated macrophages, in the tumor microenvironment utilize IL-10 as one of the multiple immunosuppressive mechanisms (4,5). Conversely, IL-10 can also exhibit immunostimulatory properties, including the induction of proliferation and cytotoxic activity of $CD8^+$ T cells. Emmerich *et al* demonstrated that treatment with IL-10 could activate tumor-resident $CD8^+$ T cells and suppress well-established large tumors in mouse tumor models (6). Guo *et al* also revealed that the IL-10-Fc fusion protein expands and enhances the cytotoxic function of terminally exhausted $CD8^+$ tumor-infiltrating lymphocytes that do not respond to immune checkpoint inhibitors (7). Thus, IL-10 possesses diverse roles in tumor immunology and immunotherapy (8,9). In addition, the amount and timing of IL-10 exposure may critically impact its function in antitumor immunity.

IL-10 is produced by various cell types, including $CD4^+$ T cells. Among $CD4^+$ T cells, T helper (Th)1, Th2, Th17, and T regulatory type 1 (Tr1) cells are notable producers

of IL-10 (10,11). Simultaneously, IL-10 can directly and/or indirectly suppress Th responses following specific antigen stimulation and potentially induce the formation of a negative feedback loop to regulate immune responses. To date, several reports have shown that tumor antigen-specific regulatory T cells that produce IL-10 exist in the peripheral circulation, as well as in the tumor microenvironment (12-14). Conversely, tumor antigen-specific Th effector cells producing IL-10 in patients with cancer have received considerably less attention. Regarding IL-10 expression in Th effector cells, IL-10 secretion from Th2 cells is stable, whereas that from Th1 and Th17 cells was found to be unstable and conditional (10). Thus, the role of tumor antigen-specific IL-10-producing T cells in antitumor responses is more complex than that of $CD8^+$ cytotoxic T lymphocytes.

In the present study, we identified circulating tumor antigen-specific IL-10-producing T cells in patients with head and neck squamous cell carcinoma (HNSCC) and explored factors influencing the immunodynamics of IL-10-producing T cells.

Materials and methods

Patients and blood collection. During March 2019 to April 2021, blood samples were obtained at Gunma University Hospital from 18 patients with HNSCC who did not receive any anticancer drugs, radiotherapy, or surgery prior to blood collection. Patients with autoimmune diseases, severe infections, or receiving steroid treatment were excluded in this study. The median age of patients was 63.5 years (range: 48-77 years). Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation, followed by cryopreservation. This study was approved by the Ethics Committee of Gunma University Hospital (approval no. HS2017-152). Written informed consent was obtained from all patients.

Immunohistochemical expression of tumor antigens, p53 and MAGE-A4 in HNSCC. During March 2019 to April 2021, HNSCC samples were obtained by biopsy or surgical resection at Gunma University Hospital from the same patients who provided blood samples. The use of HNSCC samples was also approved by the Ethics Committee and patient consent was obtained. Immunohistochemical analysis of p53 and MAGE-A4 expression in tumor specimens was performed as described previously (15). Briefly, formalin-fixed paraffin-embedded specimens sectioned at 3 μm were deparaffinized. Antigen retrieval was achieved by boiling samples at 98˚C for 30 min with 20% zinc sulfate solution and citrate buffer (pH 6.0) for p53 and MAGE-A4 staining, respectively. After blocking, slides were incubated for 2 h with primary antibodies (anti-p53 antibody, NCL-L-p53-DO7, NOVOCASTRA; anti-MAGE-A4 antibody, clone 57 B, MERCK), followed by overnight incubation at 4˚C. Subsequently, slides were incubated with a secondary antibody (Histofine Simple Stain MAX-PO (MULTI), Nichirei), and the reaction products were detected with 3,3'-diaminobenzidine (DAB, DOJINDO, Kumamoto, Japan). Sections were counterstained with Mayer's hematoxylin.

The sections were evaluated by two independent, blinded researchers (H. Tada and K.C.). For p53, specific staining in >10% of tumor cells was defined as positive expression. For MAGE-A4, each specimen was considered positive if specific staining was present.

In vitro sensitization and interferon (IFN)-γ/IL-10 double-color enzyme-linked immunosorbent spot (ELISPOT) assay. Thawed PBMCs were cultured with recombinant tumor antigen protein (10 μg/ml of p53 or MAGE-A4) in a final volume of 0.5 ml AIM-V medium, supplemented with 10 IU/ml IL-2 and 5 ng/ml IL-7 in a 48-well tissue culture plate. After 4 days, AIM-V medium (0.5 ml) containing 10 IU/ml IL-2 was added to each well. After three days of culture, PBMCs were harvested as effector cells and examined for IFN-γ/IL-10 production using the ELISPOT assay. For blocking assay, mouse control IgG1κ (P3.6.2.8.1; eBioscience) or anti-lymphocyte activation gene 3 (Lag-3) antibodies (17B4; AdipoGen, Liestal, Switzerland) (10 μg/ml each) were added throughout the culture period.

ELISPOT assays were performed using the Human IFN-γ/IL-10 double-color ELISPOT kit (Cellular Technology Ltd., Cleveland, OH, USA) according to manufacturer protocol. Briefly, a 96-well plate was precoated with IFN-γ/IL-10 capture antibody and incubated at 4˚C overnight. Harvested effector cells ($1\text{-}5\text{x}10^4$ cells/well) were plated into a precoated plate and co-cultured with PBMCs ($1\text{x}10^5$ cells/well) in the presence of p53 or MAGE-A4 protein (10 μg/ml each). The plates were incubated at 37˚C for 24 h. After incubation, the plates were washed and developed with anti-human IFN-γ (FITC) and FITC-horseradish peroxidase and IL-10 (Biotin) and streptavidin-alkaline phosphatase, respectively. The number of spot-forming cells (SFC) in each well was counted using a CTL-ImmunoSpot Analyzer (Cellular Technology Ltd.). The mean number of spots in control wells (no protein) was subtracted from the mean number of spots in the experimental wells, and the results were expressed as SFC per $5\text{x}10^4$ cells, as described previously (15). A T-cell response to a given tumor antigen was considered to be positive if at least 10 cells per $5\text{x}10^4$ responder cells secreted IFN-γ or IL-10.

Flow cytometric analysis. Flow cytometry was performed using a FACSVerse flow cytometer (BD Biosciences) to analyze the proportion of T cells expressing immune checkpoint molecules in PBMCs, as reported previously (16). Briefly, cryopreserved PBMCs were thawed, blocked using BD Fc Block (BD Bioscience, San Jose, CA, USA), and stained with antibodies specific for CD3, CD4, CD8, programmed cell death-1 (PD-1), cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), and Lag-3. As a negative control, cells were stained with a mouse IgG isotype control (BD Biosciences). The data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). The gating strategy is illustrated in Fig. S1.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 8.0; GraphPad Software, San Diego, CA, USA). Unpaired two-tailed t-test was performed to determine the presence of a significant difference between the number of SFC in protein-stimulated

Table I. Patient characteristics and T-cell response to p53 and MAGE-A4 proteins.

Patient no.	Age, years	Sex	Primary site	T	N	M	Stage	p53 staining	T-cell response (p53) IFN-γ	T-cell response (p53) IL-10	MAGE-A4 staining	T-cell response (MAGE-A4) IFN-γ	T-cell response (MAGE-A4) IL-10
1	72	M	Larynx	4a	0	0	IVA	+	-	+	+	+	-
2	61	M	Oro	2	2b	0	IVA	-	-	+	+	-	+
3	59	M	Hypo	4b	3b	0	IVB	-	-	+	+	ND	ND
4	72	M	Oro	4a	3b	1	IVC	+	-	+	+	-	-
5	62	M	Hypo	1	0	0	I	-	-	-	+	-	-
6	77	M	Hypo	4a	1	0	IVA	+	-	+	+	-	-
7	76	M	Larynx	4a	2c	0	IVA	-	-	-	+	+	-
8	48	M	Hypo	4b	1	0	IVB	+	-	-	+	+	+
9	69	M	Hypo	4a	3b	0	IVB	-	-	-	-	-	-
10	73	M	Larynx	3	0	0	III	+	-	-	-	-	-
11	62	M	Oro	4a	3b	0	IVB	-	-	+	+	-	+
12	57	M	Larynx	2	0	0	II	-	+	+	-	ND	ND
13	63	M	Oro	4a	2b	0	IVA	+	-	-	+	ND	ND
14	56	M	Hypo	3	0	0	III	+	-	-	+	ND	ND
15	74	M	Hypo	2	2c	0	IVA	-	-	+	-	-	+
16	74	M	Oro	4a	2c	0	IVA	NA	+	-	NA	+	+
17	64	M	Larynx	3	0	0	III	+	-	+	+	-	+
18	58	M	Larynx	3	0	0	III	-	-	-	+	-	+

M, male; F, female; Oro, oropharynx; Hypo, hypopharynx; IFN-γ, interferon-γ; NA, not available; ND, not done.

and unstimulated wells, as described previously (17). Fisher's exact test of independence was used to determine differences in categorical variables. Kaplan-Meier curves were plotted and compared using log-rank tests to compare survival curves between patients with and without p53-specific IL-10 production. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients' characteristics and p53 and MAGE-A4 expression. Table I summarizes the characteristics of the included patients. The primary tumor sites included the larynx (n=6), oropharynx (n=5), and hypopharynx (n=7). Immunohistochemical analyses were performed on 17 available tumor specimens. Fig. 1A and B show representative immunohistochemical staining results for p53 and MAGE-A4. p53 and MAGE-A4 were detected in 8 (47.1%) and 13 (76.5%) patients, respectively (Table I).

Tumor antigen-specific IFN-γ/IL-10 production. In vitro, PBMCs stimulated with p53 or MAGE-A4 protein were evaluated using IFN-γ/IL-10 double-color ELISPOT assays (Fig. 2). Of the 18 patients with HNSCC, 2 (11.1%) and 9 (50.0%) patients showed p53-specific IFN-γ and IL-10 production, respectively. Meanwhile, MAGE-A4 specific IFN-γ and IL-10 production were detected in 4 (28.6%) and 7 (50.0%) of 14 patients (Table I). Three patients (pt-8, 12, 16) exhibited both IFN-γ and IL-10 production in response to the same tumor antigen. In the p53-specific responses, IL-10-producing T cells were observed in significantly more patients than IFN-γ producing T cells (P=0.0275, Table II). There was no significant correlation between clinical factors and tumor antigen-specific IL-10 production (Table SI). To evaluate the prognostic significance of p53-specific IL-10 production, Kaplan-Meier survival analyses were performed for patients with and without p53-specific IL-10 production (Fig. S2). Although patients with p53-specific IL-10 production appeared to have a better prognosis for overall survival, the difference was non-significant (overall survival, P=0.2518; relapse-free survival, P=0.5868).

Comparison of proportions of T-cells expressing immune checkpoint molecules. To further elucidate the immunological context underlying tumor antigen-specific IL-10 production, we focused on p53-specific IL-10 production and determined the proportion of T cells expressing immune checkpoint molecules in the peripheral blood. In both $CD4^+$ and $CD8^+$ T cells, the proportion of T cells expressing Lag-3 was significantly lower in patients who exhibited p53-specific IL-10 production than in those who did not, as shown in Fig. 3.

Enhancement of tumor antigen-specific IL-10 production by blockade of Lag-3. Finally, we investigated whether anti-Lag-3 antibodies enhanced tumor antigen-specific IL-10 production. In some patients, Lag-3 blockade significantly enhanced tumor antigen-specific IL-10 production (Fig. 4A and B). Meanwhile, Lag-3 blockade failed to enhance p53 and MAGE-A4-specific IFN-γ production in all patients (Fig. S3).

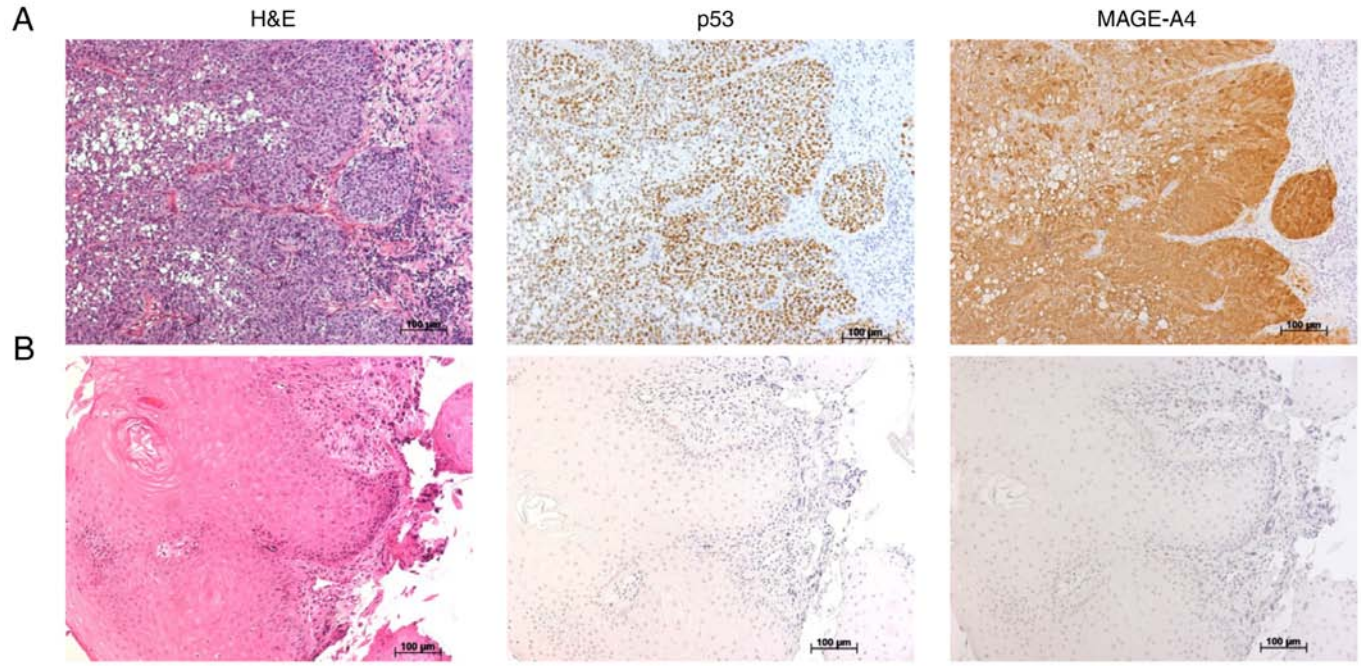

Figure 1. Immunohistochemical analysis of two tumor antigens, p53 and MAGE-A4 in HNSCC. (A) A p53 positive and MAGE-A4 positive case (pt-4) and (B) a p53 negative and MAGE-A4 negative case (pt-9) (x100 magnification). HNSCC, head and neck squamous cell carcinoma; H&E, hematoxylin and eosin.

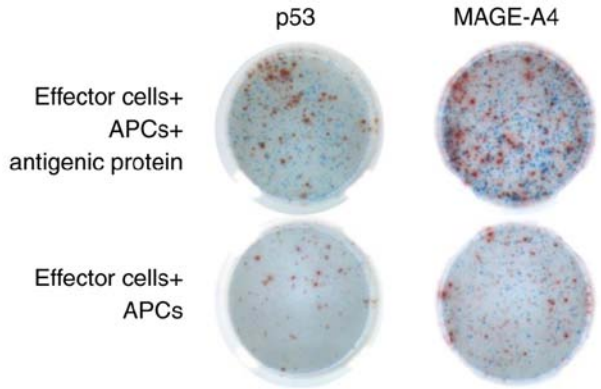

Figure 2. IFN-γ/IL-10 production in response to p53 and MAGE-A4 protein in a patient with head and neck squamous cell carcinoma. Representative well imaging of IFN-γ/IL-10 double-color ELISPOT assay detecting p53 and MAGE-A4-specific T-cell responses (p53, pt-12; MAGE-A4, pt-16). Images of ELISPOT wells show the IFN-γ-producing cells (red spots) and IL-10-producing cells (blue spots). APCs, antigen-presenting cells; IFN-γ, interferon-γ; ELISPOT, enzyme-linked immunosorbent spot.

Discussion

In the current study, we demonstrated that tumor antigen-specific IL-10-producing T cells circulate in the peripheral blood of patients with HNSCC, and their detection rate was significantly higher than that of IFN-γ-producing T cells. In patients with HNSCC, similar to other types of cancers, various immunosuppressive mechanisms are activated, and the functions of relevant effector cells are suppressed not only within the tumor sites but also at systemic sites, including the peripheral blood, bone marrow, and lymph nodes (18-20). Our results may reflect the systemic immunosuppressive status of patients with HNSCC. In some patients whose tumors did not express p53 or MAGE-A4, we detected the presence of tumor antigen-specific T-cells. Consistently, Heusinkveld *et al* (21) and Hoffmann *et al* (22,23) reported similar findings regarding p53. The authors discussed the possibility that p53-negative tumors represent immune escape variants and/or harbor p53 mutations that do not result in overexpression. There was no significant association between tumor antigen-specific IL-10-producing T cells and clinical factors, including prognosis, mirroring the high heterogeneity of IL-10-producing T cells and the dual function of IL-10 in tumor promotion and suppression. To date, high serum IL-10 levels have been associated with poor prognosis in several malignancies, including gastric cancer (24), malignant myeloma (25), and lung cancer (26). In contrast, studies have shown that serum IL-10 levels do not correlate with prognosis (27-29). More recently, a relationship between IL-10 and the clinical benefits of immune checkpoint inhibitors has been reported (30,31). The level of serum IL-10 and percentage of $CD4^+$ $IL\text{-}10^+$ PBMCs were associated with prognosis and treatment response in patients treated with immune checkpoint inhibitors, respectively. However, IL-10 is produced not only by tumor antigen-specific T cells but also by several immune cells, including dendritic cells, macrophages, B cells, and neutrophils (32). To clarify the clinical significance of tumor antigen-specific IL-10 production in patients with cancer, it is necessary to consider the types of tumor antigens and $CD4^+$ T cell subsets that produce IL-10. Furthermore, it is also essential to elucidate the relationship between IL-10-producing T cells within the tumor microenvironment and tumor characteristics such as PD-L1 expression, tumor mutation burden, and microsatellite instability.

Notably, the proportion of $Lag\text{-}3^+$ T-cells was significantly lower in patients with p53-specific IL-10-producing T cells. Lag-3 is an immune inhibitory checkpoint expressed on exhausted $CD4^+$ and $CD8^+$ T cells in the context of persistent tumor antigen stimulation, as well as on immune regulatory cells,

Table II. Tumor antigen-specific T-cell responses in patients with HNSCC.

Tumor antigen	Cytokine	T-cell responses		P-value
		Positive	Negative	
p53	IFN-γ	2	16	0.0275
	IL-10	9	9	
MAGE-A4	IFN-γ	4	10	0.4401
	IL-10	7	7	

HNSCC, head and neck squamous cell carcinoma.

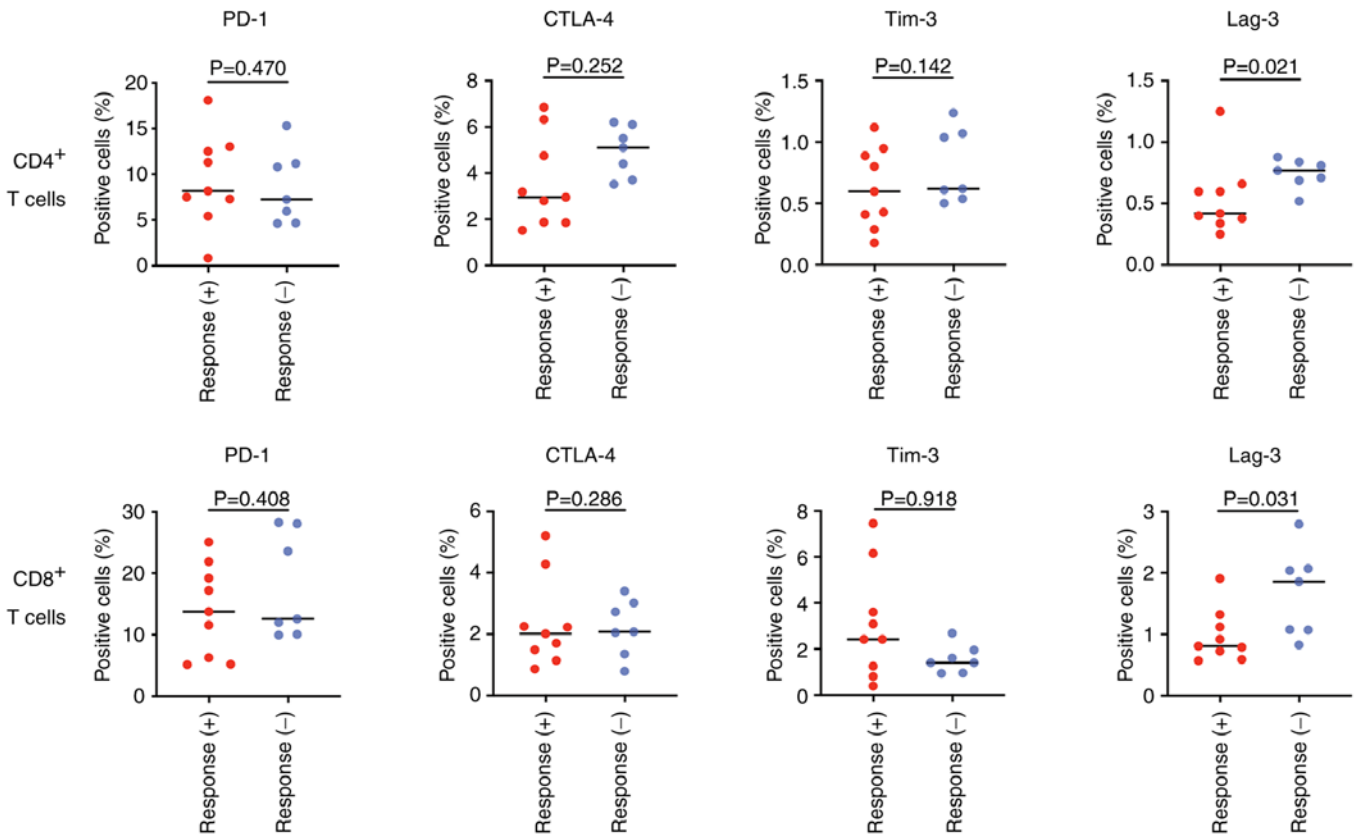

Figure 3. Comparison of the proportion of the immune checkpoint molecules, PD-1, CTLA-4, Tim-3 and Lag-3 expressing T cells, for each $CD4^+$ and $CD8^+$ T cells. Response (+), positive for p53-specific IL-10 production; Response (-), negative for p53-specific IL-10 production. PD-1, programmed cell death-1; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; Tim-3, T-cell immunoglobulin and mucin-domain containing-3; Lag-3, lymphocyte activation gene-3.

including Tregs and Tr1 cells (33,34). Therefore, in addition to the exhausted status of $CD4^+$ and $CD8^+$ T cells, $Lag\text{-}3^+$ regulatory cells may be involved in tumor antigen-specific IL-10-producing T-cell responses. Particularly, $CD4^+$ Tr1 cells are induced in the periphery upon antigen stimulation, producing high amounts of IL-10 and exhibiting robust immunosuppressive effects (35). As expected, the blockade of Lag-3 could reinvigorate tumor antigen-specific IL-10 production in some patients, suggesting the existence of a mechanism through which IL-10 production from T cells may be suppressed by IL-10 from Lag-3+ regulatory cells. Conversely, tumor antigen-specific IFN-γ production was not induced. Matsuzaki *et al* have reported that $CD8^+Lag\text{-}3^+PD\text{-}1^+$ T cells were more impaired in IFN-γ/tumor necrosis factor (TNF)-α production than $Lag\text{-}3^+PD\text{-}1^-$ or $Lag\text{-}3^-PD\text{-}1^-$ T cell subsets in NY-ESO-1-specific $CD8^+$ T cells derived from patients with ovarian cancer; therefore, dual blockade of PD-1 and Lag-3 efficiently augmented cytokine production of tumor antigen-specific $CD8^+$ T cells (36). Thus, Lag-3 blockade alone may be insufficient to induce and activate tumor antigen-specific IFN-γ production.

The present study has several limitations other than the small number of cases. As naïve $CD4^+$ Th cells differentiate into different subsets of Th cells depending on the cytokine milieu, the Th cell balance continuously changes depending on the immune status and/or composition of the tumor microenvironment. Moreover, Bonertz *et al* demonstrated that the repertoires of tumor antigens recognized by Tregs and effector/memory T cells differ in patients with colorectal cancer (14). Thus, tumor

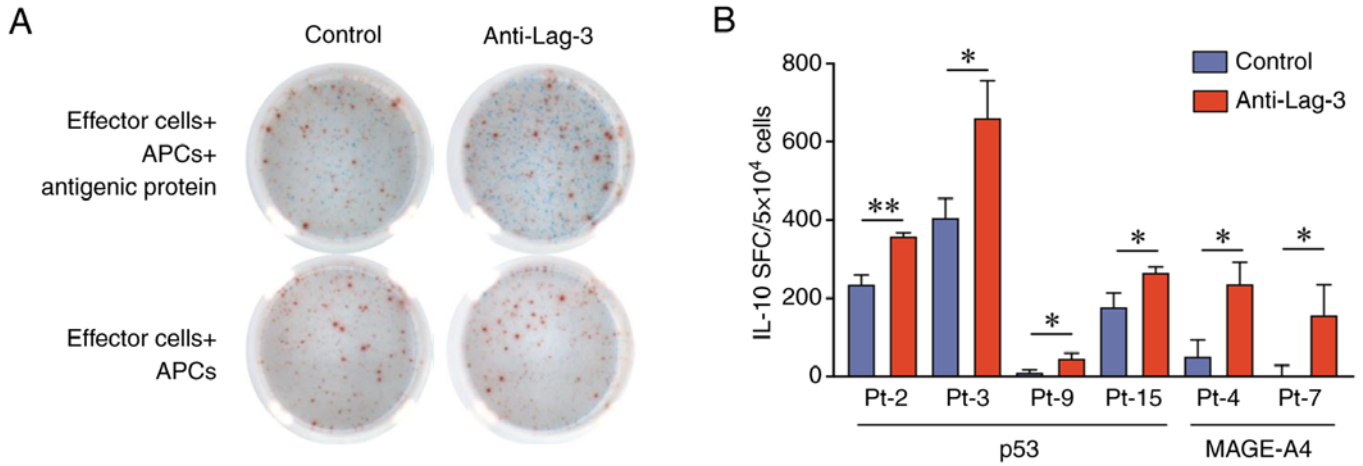

Figure 4. Enhancement of IL-10 production in response to p53 and MAGE-A4 by Lag-3 blockade. (A) Representative well imaging of IFN-γ/IL-10 double-color ELISPOT assay detecting MAGE-A4-sepecific T-cell response in pt-4. Images of ELISPOT wells show the IFN-γ-producing cells (red spots) and IL-10-producing cells (blue spots). The number of IL-10-producing cells was increased by Lag-3 blockade, whereas the number of IFN-γ-producing cells was unchanged. (B) Quantification of results from the ELISPOT assay enhancing p53-specific IL-10 production by Lag-3 blockade. Student's two-tailed t-test was performed to determine whether Lag-3 blockade significantly enhanced tumor antigen-specific IL-10 production. *P<0.05, **P<0.01. APCs, antigen-presenting cells; SFC, spot-forming cells; IFN-γ, interferon-γ; ELISPOT, enzyme-linked immunosorbent spot; Lag-3, lymphocyte activation gene-3.

antigen-specific T cells that produce IL-10 may exhibit distinct behaviors depending on whether they are effector or regulatory T cells. To identify the type of Th cells or Tr1 producing IL-10 in response to tumor antigens, further analyses, such as single-cell proteomic analysis, are required.

To the best of our knowledge, this is the first report to indicate that tumor antigen-specific IL-10-producing T cells are present in the peripheral blood of patients with HNSCC. Nevertheless, it remains unclear whether these tumor antigen-specific IL-10-producing T cells function as effectors or regulatory cells. Lag-3^{+} T cells play an important role in modulating IL-10-producing T cells. These findings provide new insights into the roles of IL-10 and Lag-3 in mediating antitumor immune responses.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KC conceived and designed the study. MH, KM, HTak and HTad acquired the data. KC and ST confirm the authenticity of all the raw data. KC, HTak, YT, MM, TO, ST and KC performed data analysis and interpretation. KC wrote the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Gunma University Hospital (approval no. HS2017-152). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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