

Persistent restoration to the immunosupportive tumor microenvironment in glioblastoma by bevacizumab

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Although vascular endothelial growth factor (VEGF) promotes the immunosuppressive microenvironment, the efficacy of bevacizumab (Bev) on tumor immunity has not been fully investigated. The present study used 47 glioblastoma tissues obtained at 3 different settings: tumors of initial resection (naïve Bev group), tumors resected following Bev therapy (effective Bev group), and recurrent tumors after Bev therapy (refractory Bev group). The paired samples of the initial and post-Bev recurrent tumors from 9 patients were included. The expression of programmed cell death-1 (PD-1)/PD ligand-1 (PD-L1), CD3, CD8, Foxp3, and CD163 was analyzed by immunohistochemistry. The PD-L1+ tumor cells significantly decreased in the effective or refractory Bev group compared with the naïve Bev group ($P < .01$ for each). The PD-1+ cells significantly decreased in the effective or refractory Bev group compared with the naïve Bev group ($P < .01$ for each). The amount of CD3+ and CD8+ T cell infiltration increased in the refractory Bev group compared with the naïve Bev group (CD3, $P < .01$; CD8, $P = .06$). Both Foxp3+ regulatory T cells and CD163+ tumor-associated macrophages significantly decreased in the effective or refractory Bev group compared with the naïve Bev group (Foxp3, $P < .01$ for each; CD163, $P < .01$ for each). These findings were largely confirmed by comparing paired initial and post-Bev recurrent tumors. Bevacizumab restores the immunosupportive tumor microenvironment in glioblastomas, and this effect persists during long-term Bev therapy.

KEYWORDS

bevacizumab, PD-1, PD-L1, TAM, Treg

1 | INTRODUCTION

Despite the lack of improvement in overall survival, Bev has been extensively used for the treatment of newly diagnosed as well as recurrent high-grade gliomas because of the benefits in progression-free

survival and patients' performance status.¹⁻³ However, tumors refractory to Bev are often highly invasive, with no effective therapy at present. Thus, elucidation of the mechanism of action and resistance is imperative to overcome resistance to Bev and develop a more effective therapy.

Abbreviations: Bev, bevacizumab; DC, dendritic cell; HIF-1 α , hypoxia-inducible factor-1 α ; HPF, high-power field; IL, interleukin; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; TAM, tumor-associated macrophage; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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We have previously reported, using tumor specimens resected following Bev therapy (under the Bev efficacy), that Bev induces the normalization of vascular structure, reduction of microvessel density, and improves tumor oxygenation.⁴ In addition, by comparing tumors resected before treatment (initial tumor), following Bev therapy (under the Bev efficacy), and at the time of recurrence, tumor hypoxia was regained while the microvessel density was still paradoxically lowered in recurrent tumors after Bev therapy, suggesting that the re-activation of tumor angiogenesis might not be involved in the first stage of refractoriness to Bev.⁵

Bevacizumab is known to enhance the effects of immunotherapy, because VEGF-A suppresses antitumor immunity by inhibiting the maturation of DCs and stimulating the proliferation of Tregs.^{6,7} Indeed, the combination of atezolizumab, the anti-PD-L1 antibody, and Bev induced a strong and synergistic antitumor effect on tumors with high levels of VEGF-A, by the induction of CTLs.^{8,9}

This study aims to investigate the status and change of immunosuppressive cells and immune checkpoint molecules regarding Bev usage. The analyses were carried out by immunohistochemistry using and comparing tumor specimens resected at 3 different settings: tumors before any treatment, tumors resected following Bev therapy (under the Bev efficacy), and recurrent tumors after Bev therapy. To the best of our knowledge, this is the first study to show the status of key molecules/cells in the tumor immune

microenvironment regarding Bev therapy using actual human tumor specimens.

2 | MATERIALS AND METHODS

2.1 | Patients and tissues

The present study used 47 glioblastoma tissues from 31 patients obtained at 3 different settings: 15 tumors resected following Bev therapy (under the Bev efficacy, defined as the “effective Bev group”), 20 tumors of initial glioblastoma (defined as the “naïve Bev group”), and 12 post-Bev recurrent tumors (defined as the “refractory Bev group”) (Table S1). The 15 tumors resected following Bev therapy (cases 1-15) were the tumors resected after 1-3 times of the preoperative Bev administration at a dose of 10 mg/kg for a safer surgical resection or as a consequence of the clinical course, and included 3 tumors enrolled in the phase II study of neoadjuvant Bev (UMIN000025579). Tumor resection was carried out 21-36 days after the last Bev treatment. Fourteen of the 15 patients (except case 1) were newly diagnosed cases. One patient (case 1) was treated with Bev at tumor recurrence, and resection was carried out under the Bev efficacy.

Six of the 15 patients in the effective Bev group had recurrent disease. Among these 6 patients, 2 underwent surgical resection

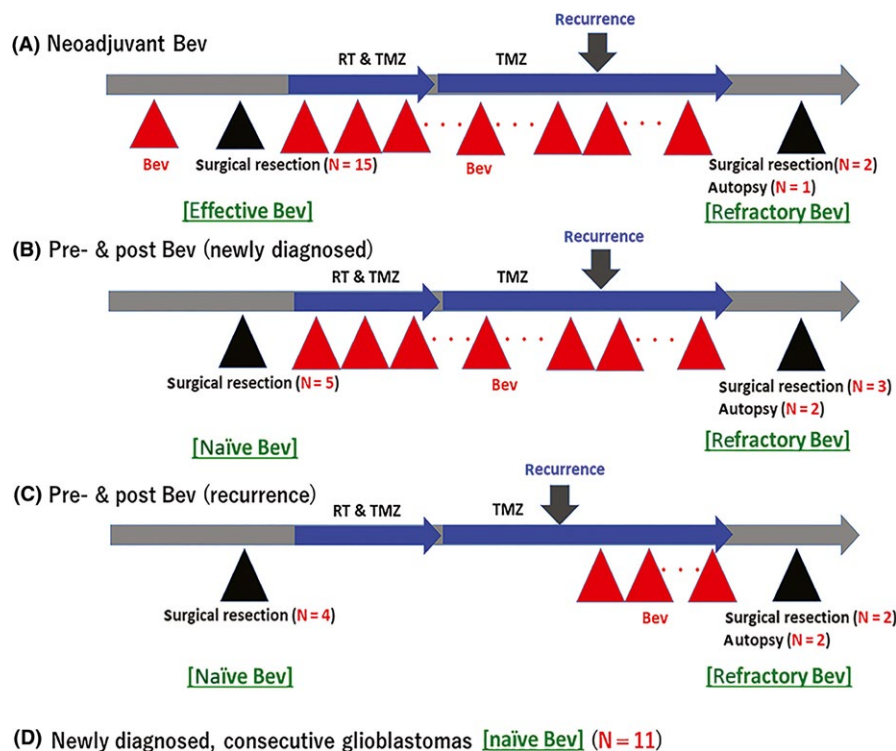


FIGURE 1 Patients and tissues. The present study used 47 glioblastoma tissues obtained from 3 different settings: 15 tumors resected under neoadjuvant bevacizumab (Bev) therapy (A; defined as the “effective Bev group”), 20 initial glioblastomas before treatment (B-D; defined as the “naïve Bev group”), and 12 recurrent tumors of post Bev therapy (A-C; defined as the “refractory Bev group”). The 15 patients in the effective Bev group (cases 1-15) were treated 1-3 times with preoperative Bev at a dose of 10 mg/kg, and resection was carried out 21-36 days after the last Bev treatment (A). In the refractory Bev group, 3 tumors were recurrent from 3 patients after neoadjuvant Bev therapy for newly diagnosed glioblastomas (A). In the naïve and refractory Bev groups, paired samples of pre- and post-Bev therapy were collected from 9 patients (B,C). RT, radiotherapy; TMZ, temozolomide

for the recurrent tumor (cases 2 and 5), and autopsy tissue was obtained from 1 patient (case 3) (Figure 1A). Therefore, 3 tumors of the refractory Bev group were recurrent ones after neoadjuvant Bev therapy. Nine tumors in each of the naïve and refractory Bev group were paired samples of pre- and post-Bev therapy obtained from 9 patients (Figure 1B,C). Five of the 9 patients (case 17, 18, 20, 21, and 22) were treated with the Stupp regimen (radiotherapy plus concomitant and adjuvant temozolomide)¹⁰ with Bev (typically, 10 mg/kg every 2 weeks)^{1,2} following initial resection, and the other 4 patients (case 16, 19, 23, and 24) were treated with Bev (10 mg/kg every 2 weeks) at tumor recurrence following the Stupp regimen. All 9 recurrent tumors were refractory to Bev despite the initial response to Bev; 5 (case 16, 17, 18, 21, and 24) were resected recurrent tumors, and the other 4 (case 19, 20, 22, and 23) were postmortem (Figure 1B,C). The interval between resection/death and the last Bev treatment was 4-208 days (median, 33 days).

In the naïve Bev group, the other 11 tumors included 10 consecutive newly diagnosed glioblastomas (all primary glioblastomas) and 1 secondary glioblastoma before neoadjuvant Bev therapy in case 1 (recurrence of anaplastic astrocytoma) (Figure 1D).

Tumor resection was carried out at any of the 3 collaborative institutes (Keio University Hospital, Tokyo; Kagawa University Hospital, Kagawa; and Jikei University Kashiwa Hospital, Chiba) in Japan between January 2014 and December 2017. Written informed consent was obtained from all the 31 patients, and the analyses were carried out at Keio University School of Medicine. This translational research was approved by the Institutional Review Board of all three institutes.

2.2 | Immunohistochemical analyses

Histopathological analyses were undertaken on 4- μ m sections of formalin-fixed, paraffin-embedded tissue of 47 tumors. All of the available tissue blocks were examined for each of the following analyses.

Standard immunohistochemistry was carried out. Sections were deparaffinized and rehydrated. The antigen retrieval was carried out in citrate buffer (pH 6 for PD-1, CD3, Foxp3, and CD163), or in Tris buffer (pH 9 for PD-L1 and CD8) using microwave irradiation or autoclave (PD-L1). The sections were blocked for 60 minutes in 2.5% horse serum (ImmPRESS Detection Systems; Vectorlabs, Burlingame, CA, USA). The sections were incubated overnight at 4°C with anti-PD-1 Ab (1:50, NAT105; Abcam), anti-PD-L1 Ab (1:500, 28-8; Abcam, Cambridge, MA, USA), anti-CD3 Ab (1:100, ab5690; Abcam), anti-CD8 Ab (1:100, 144B; Abcam), anti-Foxp3 Ab (1:100, ab54501; Abcam), and anti-CD163 Ab (1:100, ab87099; Abcam), and then incubated with anti-mouse, or rabbit Ig secondary Ab (ImmPRESS Detection Systems; Vectorlabs) for 60 minutes at room temperature. The products were visualized with peroxidase-diaminobenzidine reaction. All expressions were assessed by consensus of 4 authors with blinded clinical information (RT, TT, KO, and HS).

For the assessment of PD-1, CD3, CD8, Foxp3, and CD163, the stained sections were screened at low-power field ($\times 40$), and 5 hot spots were selected. The number of positive cells in these areas were counted at HPF $\times 400$, 0.47 mm².

The expression of PD-L1 was scored as a percentage of tumor cells expressing PD-L1, as described previously (3+, $\geq 50\%$; 2+, $\geq 5\%$ and $< 50\%$; 1+, $\geq 1\%$ and $< 5\%$; and 0, $< 1\%$).¹¹

2.3 | Statistical analyses

One-way ANOVA with post-hoc test was used to compare counts of PD-1-, CD3-, CD8-, Foxp3-, or CD163-positive (+) cells, and the ratio of Foxp3+ cells to CD8+ cells. Paired t-test was used for comparison between paired initial and post-Bev recurrent tumors, or paired neoadjuvant Bev and recurrent tumors. Mann-Whitney *U* test was used to compare the scores of PD-L1 expressions. All statistical analyses were carried out with IBM SPSS statistics. *P* value $< .05$ was considered to be statistically significant.

3 | RESULTS

Clinical characteristics and the results of immunohistochemical analyses of 47 tumors are summarized in Tables S1 and S2.

3.1 | Expression of PD-L1 and PD-1

The expression of PD-L1 was observed on the cell membrane and in the cytoplasm of tumor cells (Figure 2A). Endothelial cells were also occasionally stained. In the naïve Bev group, the PD-L1 score was 2 or 3 in 19 of 20 cases. In contrast, in the effective Bev group, 12 of 14 cases displayed score 0, and 11 of 12 cases displayed score 0 or 1 in the refractory Bev group (Figure 2B,C). A significant difference was observed between the effective/refractory Bev group and the naïve Bev group (effective vs naïve, $P < .01$; refractory vs naïve, $P < .01$). The comparison of paired initial and post-Bev recurrent tumors revealed a significant decrease of PD-L1 expression in post-Bev recurrent tumors ($P < .01$) (Figure 2D). In the comparison between paired neoadjuvant Bev and recurrent tumors, the PD-L1 staining score slightly increased in the recurrent tumors in all three cases.

The number of PD-1+ cells was significantly decreased in the effective or refractory Bev group compared with the naïve Bev group (naïve Bev group, 7.60/5HPF; refractory Bev group, 2.67/5HPF; effective Bev group, 2.93/5HPF; naïve vs refractory or effective Bev group, $P < .01$) (Figure 2E,F). There was also a trend towards decreased expression of PD-1+ cells in the post-Bev recurrent tumors in a comparison between paired initial and post-Bev recurrent tumors (initial tumors, 6.78/5HPF; post-Bev recurrent tumors, 2.89/5HPF, $P = .056$) (Figure 2F). There was no significant difference in the number of PD-1+ cells between paired neoadjuvant Bev and recurrent tumors (initial tumors, 1/5HPF; post-Bev recurrent tumors, 2/5HPF, $P = .29$).

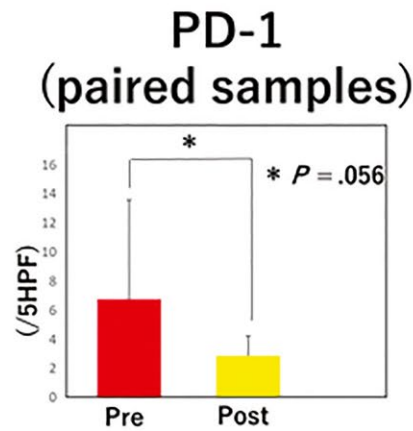
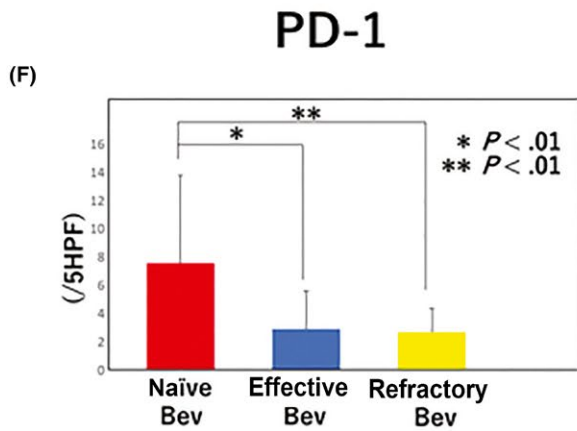
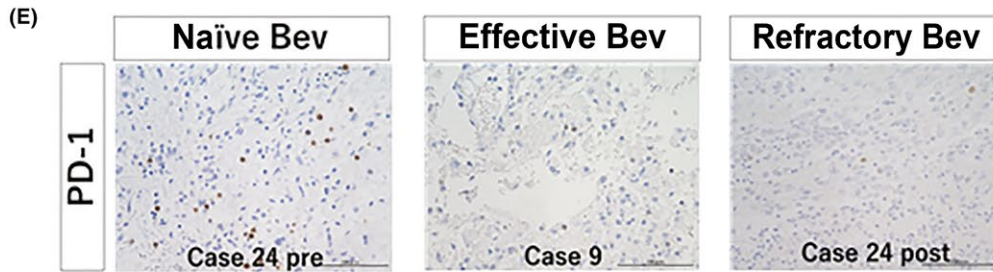
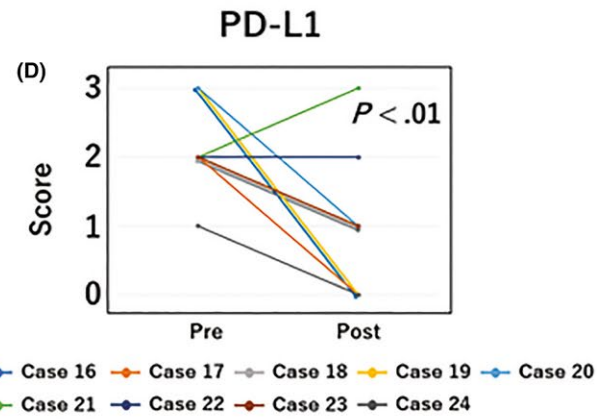
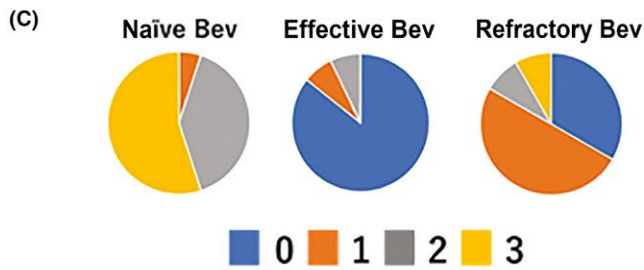
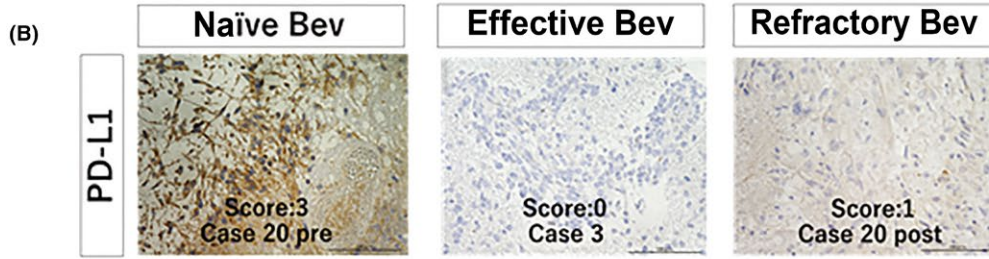
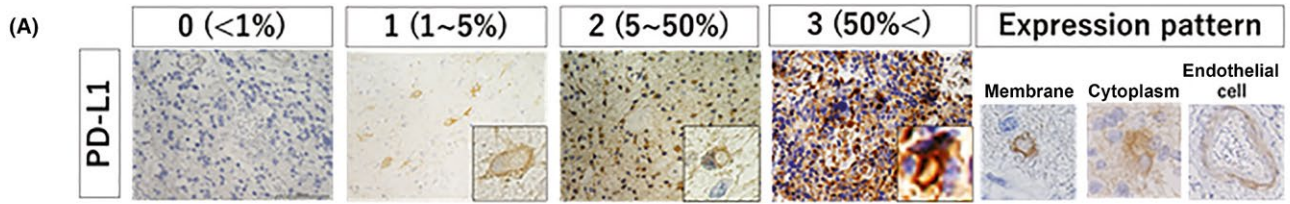


FIGURE 2 Programmed cell death-1 (PD-L1) and PD ligand-1 (PD-1) expression among glioblastoma patients with naïve, effective, and refractory bevacizumab (Bev) therapy. A, PD-L1 expression was scored as a percentage of positive cells to the total tumor cells, as previously described (tumor cells scored as percentage of PD-L1-expressing tumor cells: 3 points, $\geq 50\%$; 2 points, $\geq 5\%$ and $< 50\%$; 1 point, $\geq 1\%$ and $< 5\%$, and 0 point, $< 1\%$; original magnification, $\times 400$; scale bar = 100 μm). B, Representative photomicrographs of PD-L1 immunohistochemistry in tumors in the naïve, effective, and refractory Bev groups. PD-L1 expression was observed on a few tumor cells in the effective Bev (case 3, score 0) and refractory Bev groups (case 20 post, score 1). In contrast, PD-L1 expression was observed on most tumor cells in the naïve Bev group (case 20 pre, score 3; original magnification, $\times 400$; scale bar = 100 μm). C, Comparison of PD-L1 scores among naïve, effective, and refractory Bev groups. In the naïve Bev group, 19 of 20 cases scored 2 or 3. In contrast, in the effective Bev group, 12 of 14 cases scored 0, and 11 of 12 cases scored 0 or 1 in the refractory Bev group. A significant difference was observed between the effective/refractory Bev group and the naïve Bev group (effective vs naïve, $P < .01$; refractory vs naïve, $P < .01$). D, Comparison of PD-L1 scores between naïve and refractory Bev groups using paired samples from 9 patients with glioblastoma. There was a significant decrease of PD-L1 expression in post-Bev recurrent tumors ($P < .01$). Pre, tumors of initial resection (naïve Bev); Post, recurrent tumors following Bev therapy (refractory Bev). E, PD-1+ cells were few in tumor of the effective Bev (case 9, 3/5 high-power fields [HPF]) and refractory Bev groups (case 24 post, 3/5HPF). In contrast, PD-1 expression was frequently observed in tumors of the naïve Bev group (case 24 pre, 12/5HPF) (original magnification, $\times 400$; scale bar = 100 μm). F, Number of PD-1+ cells was significantly decreased in the effective or refractory Bev group compared with the naïve Bev group (naïve vs refractory or effective Bev group, $P < .01$) Comparison between paired initial and post-Bev recurrent tumors revealed that the number of PD-1+ cells was decreased in post-Bev recurrent tumors ($P = .056$)

3.2 | Expression of CD3 and CD8

The refractory Bev group showed a trend toward an increased number of CD8+ T cells compared with the naïve Bev group (naïve Bev group, 14.9/5HPF; refractory Bev group, 20.8/5HPF; effective Bev group, 18.6/5HPF; naïve vs refractory Bev group, $P = .06$; naïve vs effective Bev group, $P = .12$) (Figure 3A,B). In a comparison between the paired initial and post-Bev recurrent tumors, although the number of CD8+ cells was numerically increased in the recurrent tumors, the difference was not statistically significant (initial tumors, 16.6/5HPF; post-Bev recurrent tumors, 21.3/5HPF, $P = .2$) (Figure 3C). Comparison between the paired neoadjuvant Bev and recurrent tumors showed that the number of CD8+ cells increased in recurrent tumors in all 3 cases (neoadjuvant Bev tumors, 11/5HPF; recurrent tumors, 19/5HPF, $P = .06$) (Table S2).

The number of CD3+ T cells increased in the refractory Bev group compared to the naïve Bev group (naïve Bev group, 16.4/5HPF; refractory Bev group, 30.1/5HPF; effective Bev group, 24.6/5HPF; naïve vs refractory Bev group, $P < .01$) (Figure 3A,B). This trend was confirmed by comparison between paired initial and post-Bev recurrent tumors (naïve Bev group, 21.1/5HPF; refractory Bev group, 31.1/5HPF, $P < .01$) (Figure 3C). There was no significant difference in the number of CD3+ cells between paired neoadjuvant Bev and recurrent tumors (neoadjuvant Bev tumors, 16.7/5HPF; recurrent tumors, 27.0/5HPF, $P = .21$) (Table S2).

3.3 | Expression of Foxp3

The number of Foxp3+ T cells, or Tregs, decreased in the effective or refractory Bev group compared with the naïve Bev group (naïve Bev group, 5.3/5HPF; refractory Bev group, 0.9/5HPF; effective Bev group, 1.4/5HPF; naïve vs refractory or effective Bev group, $P < .01$) (Figure 4A,B). The comparison between paired initial and post-Bev recurrent tumors revealed that the number of Foxp3+ T cells decreased in post-Bev recurrent tumors (naïve Bev group, 4.78/5HPF; refractory Bev group, 0.89/5HPF, $P < .05$) (Figure 4C). The number of Tregs was similar between paired neoadjuvant Bev and recurrent tumors (neoadjuvant Bev tumors, 0.33/5HPF; recurrent tumors, 1/5HPF, $P = .32$). The Foxp3/CD8 ratio was decreased in the

effective and refractory Bev group compared with the naïve Bev group, suggesting improvement of the tumor microenvironment by Bev treatment (naïve Bev group, 0.43; refractory Bev group, 0.05; effective Bev group, 0.07; naïve vs refractory or effective Bev group, $P < .01$) (Figure 4D).

3.4 | Expression of CD163

The number of CD163 positive (+) cells decreased in the effective or refractory Bev group compared with the naïve Bev group (naïve Bev group, 54.8/5HPF; refractory Bev group, 12.4/5HPF; effective Bev group, 18.4/5HPF; naïve vs refractory or effective Bev group, $P < .01$) (Figure 4A,B). The comparison between paired initial and post-Bev recurrent tumors revealed a significant decrease in the number of CD163+ in post-Bev recurrent tumors (naïve Bev group, 46.6/5HPF; refractory Bev group, 14.4/5HPF, $P < .01$) (Figure 4C). The number of CD163+ cells was similar between paired neoadjuvant Bev and recurrent tumors (neoadjuvant Bev tumors, 8/5HPF; recurrent tumors, 6/5HPF, $P = .22$).

4 | DISCUSSION

The present study showed that, using human glioblastoma specimens resected at 3 different settings in an evaluation of Bev therapy, Bev downregulates the expression of PD-1 and PD-L1 immune checkpoint molecules, and decreases the number of immunosuppressive Tregs and TAMs. Moreover, the infiltration of cytotoxic T cells was numerically increased by Bev therapy. These results were largely confirmed by the comparison of paired pre-Bev initial and post-Bev recurrent tumors obtained from 9 patients. Importantly, those changes in the key molecules/cells in tumor immunity began immediately after the initial Bev treatment and persisted during long-term Bev therapy, even in recurrent tumors refractory to Bev. Vascular endothelial growth factor (VEGF) promotes the immunosuppressive tumor microenvironment by inhibiting the maturation of DCs, disrupting T-cell infiltration into tumors and stimulating the proliferation of Tregs.^{6,7} Some previous studies suggested that the inhibition of the VEGF pathway provided a favorable tumor microenvironment in

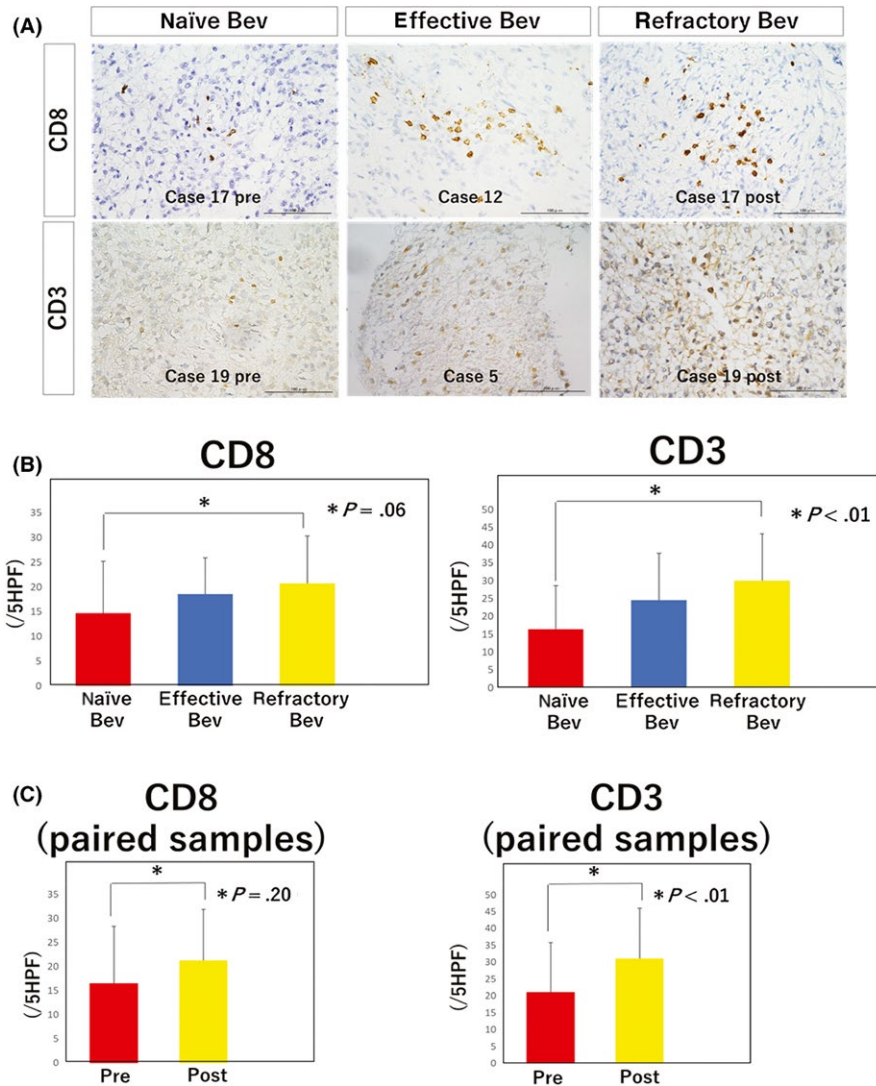


FIGURE 3 Expression of CD8 and CD3 among glioblastoma patients with naïve, effective, and refractory bevacizumab (Bev) therapy. Representative photomicrographs of CD8 (A), and CD3 (B) immunohistochemistry in tumors in the naïve, effective, and refractory Bev groups (original magnification, $\times 400$; scale bar = 100 μm). A, Top row: CD8 immunohistochemistry. Left, naïve Bev group (case 17 pre, 10/5 high-power fields [HPF]); center, effective Bev group (case 12, 29/5HPF); right, refractory Bev group (case 17 post, 28/5HPF). Bottom row: CD3 immunohistochemistry. Left, naïve Bev group (case 19 pre, 10/5HPF); center, effective Bev group (case 5, 28/5HPF); right, refractory Bev group (case 19 post, 26/5HPF). B, A trend was noted toward increased CD8+ T cell infiltration in the tumors of the refractory Bev group compared with the naïve Bev group (naïve vs effective Bev group, $P = .26$; naïve vs refractory Bev group, $P = .06$). There was a significant difference in the number of CD3+ T cells between the naïve and refractory Bev groups (naïve vs effective Bev group, $P = .07$; naïve vs refractory Bev group, $P < .01$). C, The trend of CD8+ T cells was not confirmed by comparison between the paired initial and post-Bev recurrent tumors ($P = .2$). The trend of CD3+ T cells was confirmed ($P < .01$)

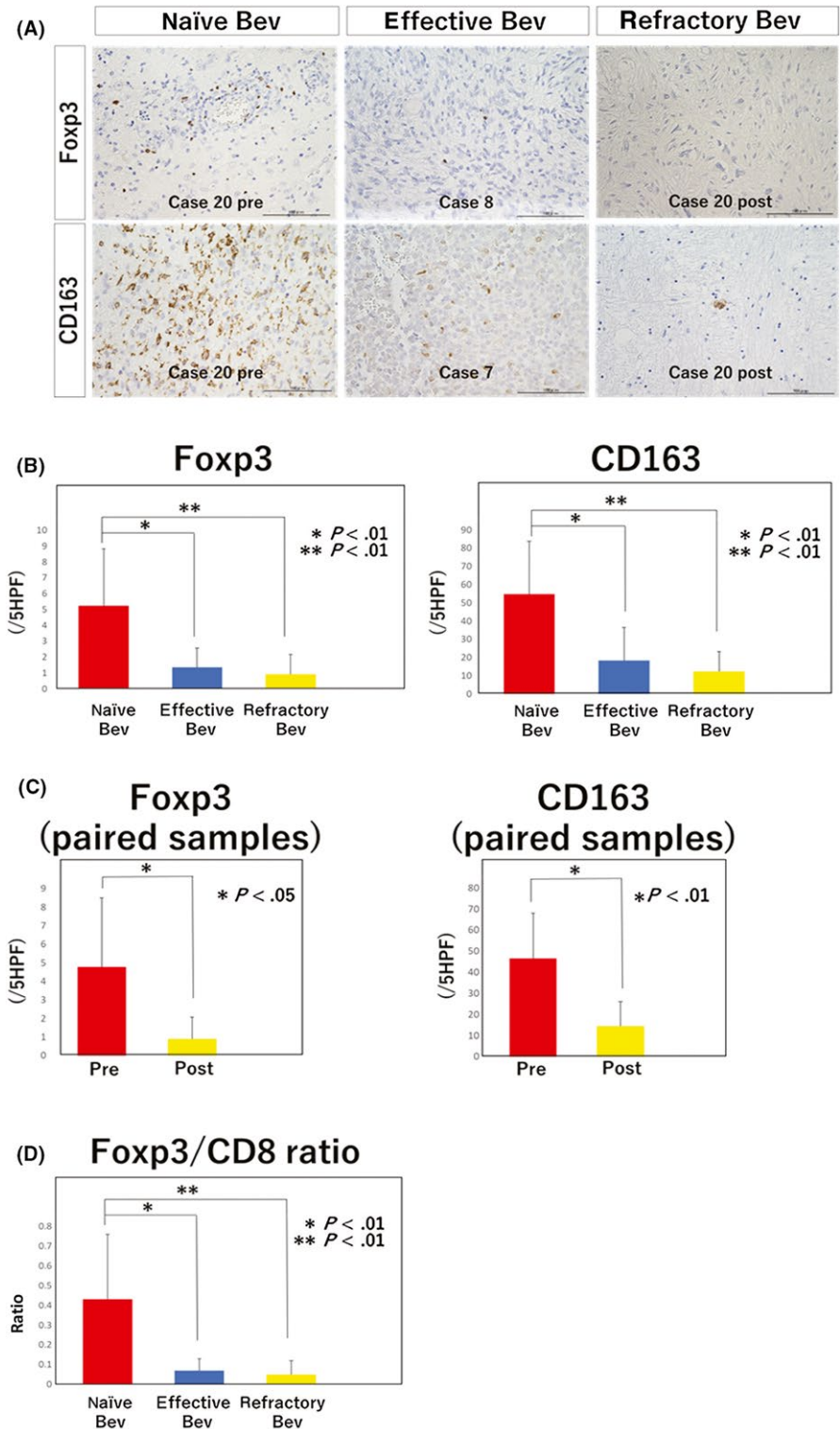
the antitumor immune response.¹²⁻¹⁴ However, this is the first study to show the overall picture of the key molecules/cells in tumor immunity in relation to antiangiogenic therapy based on the evidence using human specimens. Furthermore, it is the first to reveal that the phenomenon sustains during long-term Bev therapy.

4.1 | Programmed cell death ligand-1/PD-1/CD3/CD8/Tregs

Programmed cell death ligand-1 is frequently expressed in glioblastomas reportedly in 61%-88% of tumor cells.¹⁵ Previous studies showed that

high PD-L1 expression correlated with worse OS in patients with glioblastoma.¹⁶ The expression of PD-L1 is induced by various pro-inflammatory molecules such as γ -interferon and VEGF-A in the tumor microenvironment.^{17,18} However, to date, no study has reported the change in PD-L1 expression under antiangiogenic therapy. For the first time, the present study showed that PD-L1+ tumor cells decreased in the effective and refractory Bev groups compared with the naïve Bev group. Importantly, the downregulation of PD-L1 was persistent even in tumors refractory to Bev, suggesting that continuous Bev treatment could regulate the expression of PD-L1 over an extended duration. This phenomenon could be attributable to the inhibition of VEGF-dependent upregulation of PD-L1.

FIGURE 4 Foxp3 and CD163 expression among glioblastoma patients with naïve, effective, and refractory bevacizumab (Bev) therapy. Representative photomicrographs of Foxp3 (A) and CD163 (B) immunohistochemistry in tumors in the naïve, effective, and refractory Bev groups (original magnification, $\times 400$; scale bar = 100 μm). A, Top row: Foxp3+ Tregs were frequently observed in tumor of the naïve Bev group (case 20 pre, 14/5 high-power fields [HPF]), whereas those were few in tumors of the effective Bev (case 8, 2/5HPF) and refractory Bev groups (case 20 post, 0/5HPF). Bottom row: CD163+ TAMs were abundant in tumors of the naïve Bev group (case 20 pre, 52/5HPF), whereas those were less frequent or few in tumors of the effective Bev (case 7, 24/5HPF) and refractory Bev groups (case 20 post, 6/5HPF). B, Number of Foxp3+ T cells was significantly decreased in the effective and refractory Bev groups compared with the naïve Bev group (naïve vs refractory or effective Bev group, $P < .01$). Number of CD163+ cells significantly decreased in the effective or refractory Bev groups compared with the naïve Bev group (naïve vs refractory or effective Bev group, $P < .01$). C, Comparison between paired initial and post-Bev recurrent tumors revealed that the number of Foxp3+ T cells or CD163+ cells decreased in post-Bev recurrent tumors (Foxp3, $P < .05$; CD163, $P < .01$). D, Foxp3/CD8 ratio was significantly decreased in the effective and refractory Bev groups compared with the naïve Bev group (naïve vs refractory or effective Bev group, $P < .01$).



In addition, inhibition of immunosuppressive cells, including TAMs, under or after Bev might also have contributed to the decrease of the percentage of tumor cells expressing PD-L1, because soluble inflammatory factors such as γ -interferon, tumor necrosis factor- α , IL-10 and IL-6 derived from TAMs were known to induce PD-L1 of cancer cells.¹⁹

Some previous studies reported the induction of CD8+ T cells under VEGFR inhibition. Manning et al¹³ showed that treatment

with anti-VEGFR2 Ab led to both increased tumor-specific CD8+ TILs and regression of mouse breast tumors. Du Four et al¹² assessed PBMCs of patients with recurrent glioblastoma treated with a VEGFR inhibitor (axitinib), and reported that axitinib elevated the number of naïve CD8+ T cells and central memory CD4+ and CD8+ T cells. Wallin et al reported the combination of atezolizumab, the anti-PD-L1 Ab, and Bev increased intratumoral CD8+ T cells in metastatic

renal cell carcinoma through vascular normalization and endothelial cell activation.^{8,9}

In the present study, tumor-infiltrating CD3 or CD8+ T cells numerically increased after Bev therapy, especially in tumors of the refractory Bev group, compared with the naïve Bev group.

Regulatory T cells produce transforming growth factor- β , IL-10, and granzyme B, which trigger the apoptosis of cytotoxic T cells.²⁰ Therefore, immune suppression by Tregs is linked to a failure of cancer immunotherapy.²¹ Regulatory T cells were also shown to be induced during chemotherapy for various cancers.^{22,23} For instance, the population of Tregs increases rapidly, known as the rebound phenomenon, during long-term temozolomide therapy for glioblastoma.²⁴ Some studies investigated the status of Tregs under antiangiogenic therapy. Flow cytometry analyses revealed that the Treg proportion decreased in the peripheral blood of patients with metastatic colorectal cancer and kidney cancer treated with antiangiogenic therapy, including Bev, sorafenib, and sunitinib.²⁵⁻²⁷ In the present study, the number of Foxp3+ Tregs in the effective and refractory Bev groups was significantly decreased compared with the naïve Bev group. This is the first study to show that Tregs were downregulated in tumors under antiangiogenic therapy, and continuous Bev treatment might regulate Tregs persistently over a long duration, even in recurrent tumors following continuous Bev therapy.

Glioblastomas contain a greater number of PD-1+ TILs than lower-grade gliomas,²⁸ and the PD-1 expression on TILs is significantly increased in recurrent tumors compared with initial tumors.²⁹ Foxp3+ Tregs expressing PD-1 were also shown to be enriched in human glioblastoma tissue.³⁰ Expression of PD-1 on CD8+ T cells and Foxp3+ Tregs is associated with VEGF-A exposure, and blocking of the VEGF/VEGFR pathway downregulated the frequency of PD-1 expression on these cells.³¹⁻³³ In the present study, the total number of PD-1+ cells, including CD8+ T cells and Tregs, was significantly decreased in the effective or refractory Bev group compared with the naïve Bev group. Although it was difficult to tell which cells PD-1 was expressed on after Bev therapy, the decrease of the overall PD-1 expression suggested that the inhibition of the VEGF pathway provides a favorable microenvironment in the immune function.

Du Four et al reported that Tregs and PD-1 expression on CD4+ and CD8+ T cells in PBMCs significantly increased in patients with glioblastoma after resistance to a VEGFR inhibitor.¹² The cause for the discrepancy in Tregs and the PD-1 status in the recurrent stage between their study and ours remains unclear; however, it could be attributed to the difference of Treg status between PBMCs and tumors or the difference in the target of inhibition, VEGF compared with VEGFR.

4.2 | Tumor-associated macrophages

Bone marrow-derived cells, including CD163+ TAMs, have been associated with tumor progression, angiogenesis, invasion, metastasis, and treatment evasion.^{34,35} Typically, CD163+ TAMs behave as M2 macrophages to suppress the host immune response.^{36,37}

Tumor-associated macrophages promote angiogenesis and invasion by various cytokines or growth factors, including basic fibroblast growth factor, MMP9, and VEGF-A.³⁵ In addition, a previous study involving autopsy specimens reported that CD163+ TAMs increased in the infiltrative edge of glioblastomas following antiangiogenic therapy comprising Bev and cediranib.³⁸ The present study revealed that the number of CD163+ TAMs in tumors of the effective and refractory Bev groups was significantly decreased compared with that in the naïve Bev group. We did not observe any difference in CD163+ cell density between the tumor bulk and infiltrative edge.

4.3 | Effect of continuous VEGF blockade vs recovery of hypoxia on infiltration of immune-suppressive cells

Hypoxia affects the tumor immune microenvironment by upregulation of VEGF-A via HIF-1 α .³⁹ In addition, hypoxia is directly associated with immunosuppressive cells and molecules.³⁹ The conversion of naïve T cells into Tregs is controlled by the transcription factor Foxp3, which is upregulated in hypoxia through HIF-1.⁴⁰ The hypoxic condition also contributes to phenotypic control of macrophages into a specific M2 phenotype (TAM).⁴¹ Hypoxia-inducible factor-1 α was also able to activate the expression of PD-L1 by binding of HIF to a specific hypoxic response element in the promoter of PD-L1 in cancer cells.^{42,43} We have previously reported that Bev improves tumor oxygenation in glioblastomas,⁴ and that tumor hypoxia is regained in the post-Bev refractory tumors, to a similar or even higher level compared with the paired pre-Bev initial tumors.⁵ In the present study, however, immunosuppressive cells and molecules in the tumors of refractory Bev group were still suppressed compared with the naïve Bev group despite recovery of hypoxia. The expression of immune checkpoint molecules and immunosuppressive cells were strongly associated with VEGF-A.^{17,18,31-33,35} Continuous trapping of a certain amount of VEGF-A by long-term Bev therapy might have contributed to the persistent suppression of immunosuppressive cells/molecules in tumors of the refractory Bev group. These results suggest that VEGF-A might be the key regulator of the tumor immune microenvironment in glioblastomas.

4.4 | Limitations of the study

Some could argue that the tumors in the refractory Bev group were the recurrent tumors following not only Bev but also radiotherapy and temozolomide, and those therapies might also have affected the immunohistochemical results. However, the expression of PD-1 and PD-L1 was known to increase in recurrent glioblastomas,^{16,29} and the results contrary to those were very likely due to the effect of Bev. Another limitation of the present study was the paucity of the number of paired tissues from the same patients because salvage surgery following Bev failure is extremely rare. Ideally, studies using a larger number of paired samples are warranted to confirm the findings of this study.

5 | CONCLUSION

This study showed that Bev downregulates the expression of PD-1 and PD-L1 immune checkpoint molecules, and suppresses the infiltration of immunosuppressing cells, in line with the previous finding that cytotoxic T cells increase by Bev therapy. Importantly, the condition was sustained during long-term Bev therapy, and persisted even in recurrent tumors refractory to Bev.

Due to higher infiltration of CD3+ and CD8+ T cells in tumors of the refractory Bev group compared with the effective Bev group, as well as significant decrease of the ratio of Foxp3+ to CD8+ cells in the effective and refractory Bev groups compared with the naïve Bev group, long-term therapy with Bev could exert more potent effects on assistance for tumor immunity. These findings suggest that the immune microenvironment under Bev therapy is persistently favorable and might provide the rationale for the continued use of Bev beyond progression.⁴⁴ Because Bev could enhance the efficacy of immunotherapy, novel strategies with the combination of Bev and certain cancer immunotherapies, such as peptide-pulsed or DC-based vaccines, appear to be reasonable treatment.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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