

Myeloid Cells Induce Infiltration and Activation of B Cells and CD4⁺ T Follicular Helper Cells to Sensitize Brain Metastases to Combination Immunotherapy

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Myeloid Cells Induce Infiltration and Activation of B Cells and CD4⁺ T Follicular Helper Cells to Sensitize Brain Metastases to Combination Immunotherapy

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46 **Running title**47 B and T_{FH} cells sensitize brain metastases to immunotherapy

48

49 **Significance**

50 B cell and CD4⁺ T follicular helper cell activation via BAFF/APRIL from abundant
51 myeloid cells in the intracranial tumor microenvironment enables a combinatorial
52 effect of CTLA-4 and PD-1 blockade in brain metastases.

53

54 **Disclosure of Conflict of Interest**

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Abstract

Brain metastasis (BM) is a poor prognostic factor in cancer patients. Despite showing efficacy in many extracranial tumors, immunotherapy with anti-PD-1 monoclonal antibody (mAb) or anti-CTLA-4 mAb appears to be less effective against intracranial tumors. Promisingly, recent clinical studies have reported that combination therapy with anti-PD-1 and anti-CTLA-4 mAbs has a potent antitumor effect on BM, highlighting the need to elucidate the detailed mechanisms controlling the intracranial tumor microenvironment (TME) to develop effective immunotherapeutic strategies. Here, we analyzed the tumor-infiltrating lymphocytes in murine models of BM that responded to anti-CTLA-4 mAb to anti-PD-1 mAb. Activated CD4⁺ T follicular helper (T_{FH}) cells with high CTLA-4 expression characteristically infiltrated the intracranial TME, which were activated by the combination anti-CTLA-4 and anti-PD-1 treatment. Loss of T_{FH} cells suppressed the additive effect of CTLA-4 blockade on anti-PD-1 mAb. B cell-activating factor belonging to the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) produced by abundant myeloid cells, particularly CD80^{hi}CD206^{lo} pro-inflammatory M1-like macrophages, in the intracranial TME, induced B cell and T_{FH} cell infiltration and activation. Furthermore, the intracranial TME of patients with non-small cell lung cancer featured T_{FH} and B cell infiltration as tertiary lymphoid structures. Together, these findings provide insights into the immune cell crosstalk in the intracranial TME that facilitates an additive anti-tumor effect of CTLA-4 blockade with anti-PD-1 treatment, supporting the potential of a combination immunotherapeutic strategy for BM.

Introduction

Brain metastasis (BM) is reported to occur in 10%-30% of patients with solid tumors (1), most commonly originating from lung cancer, breast cancer, or melanoma (1,2). BM indicates disease progression from the primary tumor and is associated with a poor prognosis. Despite the use of cytotoxic chemotherapy and targeted therapies, including platinum doublets, epidermal growth factor receptor tyrosine kinase inhibitors, v-raf murine sarcoma viral oncogene homolog B1 inhibitors, and human epidermal growth factor receptor 2 inhibitors, their effectiveness against BM remains limited (3). The median survival for patients with BM from melanoma or non-small cell lung cancer (NSCLC) is reported to be approximately 4-8 months (4,5). A key factor contributing to this poor prognosis is the blood-brain barrier (BBB), which restricts drug penetration into BM (3,6). This highlights the need for the development of novel therapies to overcome this challenge.

In the last decade, immune checkpoint inhibitors (ICIs), such as anti-PD-1/PD-L1 or CTLA-4 monoclonal antibodies (mAbs), have been approved based on evidence of their clinical activity and have been used for various cancer types (7-9). In analyses of patients with BM, the response rate to anti-PD-1 mAb monotherapy was approximately 20% in melanoma and 30% in NSCLC, respectively (10). In addition, anti-CTLA-4 mAb monotherapy was effective in approximately 16% of melanoma patients with BM (11). These results appear to be slightly inferior to those observed in extracranial tumors in phase III trials (12,13). Furthermore, anti-PD-1 mAbs without anti-CTLA-4 mAbs seem to be less effective against

intracranial tumors than against extracranial ones in patients with melanoma or NSCLC (14,15). In contrast, combination therapy with anti-PD-1 and anti-CTLA-4 mAbs is more effective in patients with melanoma, particularly those with BM, than anti-PD-1 mAb monotherapy, with comparable response rates and progression-free survival (PFS) for both intracranial and extracranial tumors (14,16). Similarly, in NSCLC, combination therapy with an anti-CTLA-4 mAb showed comparable efficacy in patients with and without BM (17-19), which contrasts with treatments lacking an anti-CTLA-4 mAb (15). Taken together, combination therapy with anti-PD-1 and anti-CTLA-4 mAbs appears to robustly activate antitumor immunity in BM compared to anti-PD-1 mAb alone.

ICIs exert antitumor effects by activating effector T cells through the inhibition of immune checkpoint molecules (20). Therefore, effector T cells in the tumor microenvironment (TME) and their interactions with the surrounding cells play crucial roles in ICI efficacy (20). There are almost no lymphocytes in healthy brain parenchyma, but disruption of the BBB due to cancer progression induces immune-cell infiltration (21,22), resulting in an extremely heterogeneous intracranial TME consisting of a wide variety of cells, including immune cells, glial cells, and neurons (21). However, the relationship between the BM-specific TME and variations in immunotherapy outcomes is not yet fully understood.

In this study, we observed that the combination of PD-1 and CTLA-4 blockade demonstrated remarkable efficacy against intracranial tumors in murine models compared to monotherapy with either antibody alone. Analysis of tumor-infiltrating

lymphocytes (TILs) in these models revealed that CD4⁺ T follicular helper (T_{FH}) cells with high CTLA-4 expression, rather than regulatory T (T_{reg}) cells, exhibited higher infiltration into intracranial tumors compared to subcutaneous tumors. Furthermore, PD-1 and CTLA-4 double blockade significantly activated T_{FH} cells in the intracranial TME, and knocking out T_{FH} cells almost eliminated the additive effect of CTLA-4 blockade. We further investigated the mechanisms underlying the increased infiltration of T_{FH} cells into the intracranial TME and identified a key role for intracranial B cell-activating factor belonging to the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) produced by abundant myeloid cells, particularly CD80^{hi}CD206^{lo} pro-inflammatory M1-like macrophages. These findings suggest that PD-1 and CTLA-4 double blockade is highly effective against BM, specifically due to the characteristics of the BM-related TME, thereby supporting the combination strategy for BM.

Materials and Methods

Mice

The C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). CD4-cre mice and Bcl6^{fl} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CD4-cre/Bcl6^{fl/fl} mice were generated by crossbreeding CD4-cre mice and Bcl6^{fl} mice. All mice were maintained under pathogen-free conditions in the animal facilities. All animal experiments were approved by the Animal Care and Use Committee of Okayama University in accordance with the Animal Welfare and

Management Act of Japan.

Cell lines

LL/2 (murine lung cancer), B16F10 (murine melanoma) and A20 (murine lymphoma) cell lines were purchased from ATCC (cat #CRL-1642, RRID: CVCL_4358; cat #CRL-6475, RRID: CVCL_0159; and cat #TIB-208, RRID: CVCL_1940, respectively). LL/2 and B16F10 cells were cultured in DMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biosera, Cholet, France) and 2% Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), while A20 cells were cultured in RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FBS and 2% Penicillin-Streptomycin at 37°C in 5% CO₂, respectively. All cells were confirmed to be Mycoplasma-negative using the Mycoplasma PCR Detection Kit (Applied Biological Materials, British Columbia, Canada), following the manufacturer's instructions, before being used in experiments. Cell lines were used in experiments within 20 passages post-thawing.

Virus production and transfection

Luciferase-transduced cells were generated using the lentiviral vectors purchased from Addgene (Watertown, MA, USA). The following lentiviral vectors were gifts: pMDLg/pRRE, from Didier Trono (Addgene plasmid #12251; <http://n2t.net/addgene:12251>; RRID: Addgene_12251) (23); pRSV-Rev, from Didier

Trono (Addgene plasmid #12253; <http://n2t.net/addgene:12253>; RRID: Addgene_12253) (23); pMD2.G, from Didier Trono (Addgene plasmid #12259; <http://n2t.net/addgene:12259>; RRID: Addgene_12259); and pHAGE PGK-GFP-IRES-LUC-w, from Darrell Kotton (Addgene plasmid #46793; <http://n2t.net/addgene:46793>; RRID: Addgene_46793) (24). The viral vectors were transfected into packaging cells using Opti-MEM and Lipofectamine 3000 (Thermo Fisher Scientific). After 48 hours, the supernatant was collected and used to transfect LL/2 or B16F10 cells.

Reagents

Rat anti-mouse PD-1 mAb (RMP1-14) was purchased from BioLegend (San Diego, CA, USA; RRID: AB_2800575) and anti-mouse CTLA-4 (9D9) was purchased from Bio X Cell (West Lebanon, NH, USA; RRID: AB_10949609). PLX3397 was obtained from Selleck Chemicals (Houston, TX, USA). TACI-Fc was generated as previously described (25). Briefly, the TACI-Fc expression vector was designed to express the human IgG1 Fc-Avi Tag-HisTag-fused protein (TACI-Fc) under the control of the CMV promoter (26-28). Cells transfected with the TACI-Fc expression vector were diluted, and the recombinant TACI-Fc protein was purified from the culture supernatants using a HisTrap HP column (Cytiva, Marlborough, MA, USA) according to the manufacturer's protocol. The purified protein was passed through a PD-10 column (Cytiva) equilibrated with phosphate-buffered saline (PBS), and the purity of the protein was checked using SDS-PAGE and western blotting. The protein

concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Intracranial and subcutaneous tumor models

Six- to 10-week-old female mice were used as dual-transplant murine models (**Fig. 1A**). Briefly, LL/2 cells (2×10^5) or B16F10 cells (1×10^5) suspended in 100 μ L PBS were injected subcutaneously into the back as an extracranial tumor on day 0. Simultaneously, the same amount of tumor cells suspended in 3 μ L PBS were injected into the right cerebral hemisphere (2 mm to the right and 2 mm in front of bregma, 3 mm deep) with a Hamilton microliter syringe (Hamilton Company, NV, USA) as an intracranial tumor. Mice were randomized into groups and intraperitoneally (i.p.) injected with anti-PD-1 mAb (200 μ g/body), anti-CTLA-4 mAb (200 μ g/body) or anti-PD-1 and anti-CTLA-4 mAbs on day 6. Antibodies were administered on days 6, 9, and 12. PLX3397 was dissolved in glycerol (Katayama Chemical, Osaka, Japan) and diluted with 1% CMC-Na (FUJIFILM Wako Pure Chemical Corporation) to a final concentration of 1 mg/mL. PLX3397 (0.2 mg/body) or a solution of glycerol diluted in 1% CMC-Na (control) was administered orally daily from day 0 to 13. TACI-Fc (40 μ g/body) or control PBS was i.p. administered on days 0, 5, and 10. Tumor volumes were measured manually on days 6, 9, 12, and 14 for subcutaneous tumors or via bioluminescence signals on days 6 and 14 for intracranial tumors. All *in vivo* experiments were performed at least twice.

Bioluminescent imaging

For imaging of intracranial tumors, luciferase-transduced tumor-bearing mice were intraperitoneally administered a dose of 150 mg/kg D-luciferin (Summit Pharmaceuticals International, Tokyo, Japan) under anesthesia. Bioluminescent images were obtained using the IVIS Imaging System (Xenogen, Alameda, CA, USA). Radiance was averaged within the region of interest during the plateau state using the Living Image 3.0 (Xenogen).

Evaluation of competition among anti-PD-1 mAb clones

To evaluate the competition among anti-PD-1 mAb clones, CD4⁺ T cells were isolated from mouse spleens using a CytoFLEX SRT (Beckman Coulter, Brea, CA, USA). Plates used for culture were pre-coated with 2 µg/mL anti-CD3e (BioLegend; RRID: AB_2616673) in PBS or with only PBS overnight at 4°C and rinsed with PBS just before adding cells. The sorted cells were cultured with or without 1 µg/mL CD28 mAbs (BioLegend; RRID: AB_11147170), anti-PD-1 mAb (RMP1-14, 10 µg/mL) or anti-PD-L1 mAb (10F.9G2; Selleck Chemicals, 10 µg/mL). After 24 hours, the cells were stained with PE-Cy7-conjugated anti-PD-1 mAb (RMP1-30; BioLegend; RRID: AB_572017) or with an anti-PD-1 mAb (RMP1-14) followed by secondary staining using PE-conjugated anti-rat IgG2a (BioLegend; RRID: AB_2564487) to assess PD-1 expression. After repeated PBS washes, additional molecules were stained, then analyzed by flow cytometry.

Flow cytometry analysis

Tumor tissues were collected on day 14 and dissociated using gentleMACS™ (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were washed with FACS buffer (PBS with 2% FBS), followed by FcR blocking with anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA, USA; RRID: AB_394656). The cells were stained with surface antibodies. Intracellular staining was performed with the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) and specific antibodies.

For intracellular cytokine and CD40L staining, cells were cultured prior to staining. Plates used for culture were pre-coated with 5 µg/mL anti-CD3e (BioLegend) in PBS overnight at 4°C and rinsed with PBS just before adding cells. Cells were suspended in RPMI-1640 containing 2 µg/mL anti-CD28 (BioLegend) and 1 µL/mL Protein Transport Inhibitor GolgiPlug™ (BD Biosciences), then cultured on anti-CD3e coated plates for 6 h at 37°C. After incubation, the cells were collected and stained. To stain TILs from mice treated with anti-PD-1 rat IgG2a mAb (RMP1-14), we used the same mAb, followed by secondary staining with PE-conjugated anti-rat IgG2a.

When we evaluated major histocompatibility complex class II (MHC-II) expression, cells were treated with 100 ng/mL recombinant murine IFNγ (Pepro Tech, Rocky Hill, NJ, USA) for 24 hours.

The antibodies used for staining were diluted according to the manufacturer's instructions (**Supplementary Table S1**). Given the dynamic nature of CTLA-4, which

continuously cycles between the cell surface and intracellular compartments upon T cell activation (29), intracellular staining was used for accurate quantification of CTLA-4 expression (30). Accordingly, we employed intracellular staining to evaluate CTLA-4 expression, following the manufacturer's instructions as previously described (30). Cells were analyzed on BD LSR Fortessa™ X-20 (BD Biosciences). FACSDiva and FLOWJO v10 software (BD Biosciences) were used for data analysis. The gating strategies are shown in **Supplementary Fig. S1-3**.

Immunohistochemistry (IHC) in mice

Tumor tissues from mice were fixed in 10% neutral-buffered formalin for 8 h immediately after collection, followed by paraffin embedding. Paraffin-embedded sections were cut in 5 µm slices, deparaffinized, and rehydrated. Antigen retrieval was performed in pH 6.0 Citrate buffer (LSI Medience Corporation, Tokyo, Japan) at 121°C for 10 min. After blocking endogenous peroxidase activity with 3% H₂O₂, the tissues were treated with 3% Bovine Serum Albumin (BSA; Nacalai Tesque, Kyoto, Japan) in PBS to block nonspecific reactions in a humid box at room temperature (RT). Anti-mouse CD11b/ITGAM antibody (E4K8C; Cell Signaling Technology, Beverly, MA, USA) or anti-mouse CD79a antibody (EPR26537-114; Abcam, Cambridge, UK) diluted in 3% BSA was added to tissues and incubated overnight in a humid box at 4°C. The tissues were washed, then incubated with SignalStain® Boost IHC Detection Reagent (HRP, Rabbit; Cell Signaling Technology) for 30 min in a humid box at RT. After washing, 3,3'-diaminobenzidine (DAB) staining was

performed with SignalStain[®] DAB Substrate Kit (Cell Signaling Technology). Counterstaining was performed with hematoxylin, and the tissues were mounted. All images were obtained using an all-in-one fluorescence microscope BZ-X700 (Keyence, Osaka, Japan) at the Central Research Laboratory of the Okayama University Medical School. Positive cell detection was performed using QuPath software (v0.5.1; <https://qupath.github.io/>).

Patients and IHC

BM specimens from 19 patients with advanced NSCLC who underwent BM resection between 2008 and 2022 were used for IHC analysis (**Supplementary Table S2**). All patients provided written informed consent to participate in this study. The study protocol was approved by the Institutional Review Board and Ethics Committee of the Okayama University Hospital. This study was conducted in accordance with the principles of the Declaration of Helsinki. Paraffin embedded sections of surgical specimen were similarly cut in 5 μ m slices, deparaffinized and rehydrated. For the detection of B cells, antigen retrieval was followed by permeabilization with 0.5% tween20 (Merck, Darmstadt, Germany) dissolved in PBS. After blocking endogenous peroxidase activity and nonspecific reaction, tissues were incubated overnight with anti-human CD79a antibody (HM57; Abcam) diluted in 3% BSA at 4°C. DAB staining was performed using the SignalStain[®] Boost IHC Detection Reagent (HRP, Mouse; Cell Signaling Technology) and DAB Substrate Kit.

For multi-color IHC, EDTA buffer pH 8.0 was used at 121°C for 20 min in

antigen retrieval method. After blocking nonspecific reaction, tissues were incubated overnight with anti-human CD4 antibody (4B12; Thermo Fisher Scientific) and anti-human CXCR5 antibody (EPR23463-30; Abcam) diluted in 3% BSA at 4°C and washed. Donkey anti-mouse IgG H&L Alexa Fluor 488 (Abcam) and anti-rabbit IgG H&L Alexa Fluor 555 (Abcam), diluted in 3% BSA, were used as secondary antibodies and reacted with the tissues at RT. The samples were washed and sealed using a TrueVIEW Autofluorescence Quenching Kit with DAPI (Vector Laboratories, Burlingame, CA, USA). All images were captured using an all-in-one fluorescence microscope BZ-X700 (Keyence) at the Central Research Laboratory of the Okayama University Medical School.

Statistical analysis

GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. The relationships of continuous variables across groups were compared using two-tailed t-tests or one-way analysis of variance (ANOVA). The relationships between tumor volume curves were compared using two-way ANOVA. For multiple comparisons, Bonferroni correction was applied. Statistical significance was defined as $p < 0.05$. P-values were indicated as follows. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; NS, not significant.

Data availability

All raw data generated in this study are available upon request from the corresponding

author.

Results

Adding anti-CTLA-4 mAb to anti-PD-1 mAb increases the antitumor effect against intracranial tumors

We encountered clinical cases of NSCLC or melanoma in which BM lesions responded dramatically to combination therapy with anti-PD-1 and anti-CTLA-4 mAbs (**Supplementary Fig. S4A and B**). Thus, we first attempted to demonstrate the effect of adding an anti-CTLA-4 mAb to an anti-PD-1 mAb on intracranial tumors in murine models. Although there have been several reports on murine models in which tumors were implanted both intracranially and extracranially to explore ICI efficacy against BM, the number of tumor cells and the timing of implantation differed between the two locations in these models (31,32). In this study, to assess the differences in ICI efficacy between tumor locations, we created a dual-transplant murine model in which equal numbers of luciferase-introduced LL/2 (mouse lung cancer cell line) cells were simultaneously transplanted intracranially and subcutaneously (**Fig. 1A**). Subcutaneous tumors were almost eliminated by PD-1 or CTLA-4 blockade alone, and no additive effects were observed in the combination group (**Fig. 1B and C**). In contrast, intracranial tumors showed an apparent reduction in the combination group compared with PD-1 blockade or CTLA-4 blockade alone, whereas no significant differences were observed among the groups just before the administration of anti-PD-1 and/or anti-CTLA-4 mAbs (**Fig. 1D and E**). Similar

trends were observed in a B16F10 melanoma model (**Supplementary Fig. S4C-F**). These results suggest that the additive effect of anti-CTLA-4 mAb in combination with anti-PD-1 mAb can be particularly observed in the BM.

Not T_{reg} cells but T_{FH} cells with high CTLA-4 expression are increased in the intracranial TME

In our models, both PD-1 blockade and CTLA-4 blockade alone almost completely eliminated the subcutaneous tumors. In contrast, both treatments were effective but could not eliminate intracranial tumors. Although many previous studies have shown that intracranial TME (i.e., myeloid cell infiltration) may be related to such insufficient efficacy, few studies have explained the additive effect of CTLA-4 blockade on PD-1 blockade against intracranial tumors (21,22). Thus, to investigate the mechanisms underlying the additive effect, we analyzed TILs from untreated intracranial or subcutaneous tumors on day 14 after implantation using flow cytometry. We evaluated CTLA-4 expression in Foxp3⁺CD4⁺ T_{reg}, Foxp3⁻CD4⁺ conventional T cells (T_{conv} cells), and CD8⁺ T cells from untreated intracranial tumors. As shown in **Figure 2A**, CTLA-4 was most highly expressed in T_{reg} cells, followed by T_{conv} cells, whereas CD8⁺ T cells showed very low expression, which is consistent with previous subcutaneous tumor studies (33,34). Although several studies have shown that tumor-infiltrating T_{reg} cells were a main target of CTLA-4 blockade (35,36), the frequency of T_{reg} cells in intracranial tumors was significantly lower than that in subcutaneous tumors (**Fig. 2B**). In addition, CTLA-4 and ICOS, representative

T_{reg}-cell activation molecules, were expressed significantly lower in intracranial tumor-infiltrating T_{reg} cells than in subcutaneous ones (**Fig. 2C** and **Supplementary Fig. S5A**). These findings indicate that the intracranial TME is not infiltrated by activated T_{reg} cells as compared to the subcutaneous TME in these models, suggesting that tumor-infiltrating T_{reg} cells do not appear to be the main reason for the additive effect against intracranial tumors.

Next, we focused on intracranial tumor-infiltrating T_{conv} cells, which also showed high CTLA-4 expression (**Fig. 2A**) and investigated the relationship between CTLA-4 expression and other molecules. Notably, the high expression of CTLA-4 in tumor-infiltrating T_{conv} cells was accompanied by high expression of PD-1 and ICOS (**Fig. 2D**), which are characteristic of T_{FH} cells (37). We defined PD-1⁺CXCR5⁺ T_{conv} cells as T_{FH} cells (38,39) based on their expression levels in lymph nodes as previously described (38) (**Supplementary Fig. S5B**), and CTLA-4 expression in T_{FH} cells (PD-1⁺CXCR5⁺ T_{conv} cells) was significantly higher than in other T_{conv} cells (non-T_{reg}/non-T_{FH} CD4⁺ T cells) (**Fig. 2E**). Additionally, we used Bcl6, a transcription factor required for T_{FH} cell differentiation, to further define T_{FH} cells (37). Similarly, high CTLA-4 expression was observed in the Bcl6⁺CXCR5⁺ T_{conv} cell fraction (**Supplementary Fig. S5C**). Notably, within this cell population, CD40L-expressing cells, typically associated with T_{FH} cells (40), exhibited particularly high levels of CTLA-4 (**Supplementary Fig. S5D**). In addition, in contrast to T_{reg} cells, the frequency of T_{FH} cells in the intracranial TME was significantly higher than that in the subcutaneous TME, accompanied by high levels of CTLA-4 and ICOS (**Fig. 2F, G**

and **Supplementary Fig. S5E**). Similar trends were observed in the B16F10 melanoma model (**Supplementary Fig. S6A-D**). These findings indicate that activated T_{FH} -cell infiltration with high CTLA-4 expression is a feature of the intracranial TME, which may serve as the main target for CTLA-4 blockade.

Combination of anti-PD-1 and anti-CTLA-4 mAbs activates $CD8^+$ T cells and T_{FH} cells in the intracranial TME

Although $CD8^+$ T cells that directly attack tumor cells generally play crucial roles in ICI efficacy (41), $CD4^+$ T cells have also been reported to exhibit cytotoxicity against MHC-II-expressing tumors in the TME (42). To investigate this, we analyzed MHC-II expression in cancer cells. However, no MHC-II expression was observed in LL/2 and B16F10 cells, even after $IFN\gamma$ treatment (**Supplementary Fig. S7A**), suggesting that $CD8^+$ T cells play central roles in our mouse models. We then focused on the tumor-infiltrating $CD8^+$ T cells under treatment. In the presence of therapeutic anti-PD-1 mAb (Clone: RMP1-14), competition between this therapeutic antibody and another anti-PD-1 mAb (Clone: RMP1-30) was observed (**Supplementary Fig. S7B**). Therefore, we employed a secondary antibody-based method to accurately assess PD-1 expression (**Supplementary Fig. S7B**). As a result, the frequencies of $PD-1^+CD8^+$ T cells, $IFN\gamma^+CD8^+$ T cells, and perforin $^+CD8^+$ T cells in the intracranial TME were increased by PD-1 blockade alone, and these frequencies were further increased by the combination with CTLA-4 blockade (**Fig. 3A-C**). Although $CD8^+$ T cells in the intracranial TME were further activated by adding CTLA-4 blockade,

CTLA-4 expression in CD8⁺ T cells was very low (**Fig. 2A**), suggesting that the effects of CTLA-4 blockade on CD8⁺ T cells in the intracranial TME are likely indirect.

Following high CTLA-4⁺ T_{FH}-cell infiltration into the intracranial TME, we further analyzed tumor-infiltrating T_{FH} cells under treatment. The frequency of T_{FH} cells was increased by PD-1 blockade alone, and this frequency was further increased by the combination with CTLA-4 blockade (**Fig. 3D**). In addition, we analyzed IL-21, a representative cytokine produced by T_{FH} cells (37), based on its levels in lymph nodes (**Supplementary Fig. S7C**). The production of IL-21 was significantly upregulated in the combination treatment group (**Fig. 3E**). Given our previous report of cytotoxic T_{FH}-like cells in MHC-II-expressing tumors (43) and documented cytotoxicity of CTLA-4-deficient T_{FH} cells (44), we analyzed the cytotoxic potential of T_{FH} cells within the intracranial TME. Consistent with the absence of MHC-II expression in the cancer cells used in this study (**Supplementary Fig. S7A**), cytotoxicity in T_{FH} cells within the intracranial TME was scarcely observed and remained comparable after PD-1 and/or CTLA-4 blockade (**Supplementary Fig. S7D**). These results are consistent with those of recent studies showing that T_{FH}-cell derived IL-21 sustains CD8⁺ T cell responses (45-47), suggesting that CD8⁺ T cells could be indirectly activated by CTLA-4 blockade via activated T_{FH} cells in the intracranial TME.

T_{FH}-cell knockout almost abolishes the additive effect of CTLA-4 blockade

To validate the importance of T_{FH} cells in the intracranial TME, we generated CD4-cre/Bcl6^{fl/fl} mice, which are commonly used as a T_{FH} -cell-deficient animal model (37,48). We then implanted tumors intracranially and confirmed a reduction in T_{FH} -cell infiltration in CD4-cre/Bcl6^{fl/fl} mice (**Supplementary Fig. S8**). In intracranial tumors, T_{FH} -cell knockout slightly decreased sensitivity to PD-1 blockade alone, whereas it dramatically decreased sensitivity to the combination, and the additive effect of CTLA-4 blockade was almost abolished (**Fig. 4A and B**).

Analysis of CD8⁺ T cells within intracranial tumors similarly showed that CTLA-4 blockade-mediated additive expansions in PD-1⁺ and IFN γ ⁺CD8⁺ T cells were almost abolished by T_{FH} -cell knockout (**Fig. 4C and D**). This finding supports the notion that the effects of CTLA-4 blockade on CD8⁺ T cells are mediated indirectly through activated T_{FH} cells in the intracranial TME. Overall, CTLA-4⁺ T_{FH} cells activated by CTLA-4 blockade sustained CD8⁺ T cell responses in the intracranial TME, leading to a dramatic response to PD-1 and CTLA-4 double blockade in intracranial tumors.

Abundant myeloid cells maintain robust B cell activity in the intracranial TME

T_{FH} cells are generally involved in germinal center (GC) formation, interaction with B cells, maturation, and antibody production (37). Thus, we analyzed B cells in the TMEs to elucidate the mechanisms of intracranial T_{FH} -cell infiltration. Consistent with the high T_{FH} -cell infiltration, both B cell and plasma cell infiltration in the intracranial TME was significantly higher than that in the subcutaneous TME (**Fig.**

5A). Furthermore, GC B cells, which are crucial for the adaptive immune response through enhanced interactions with T_{FH} cells and are characterized by GL7 and FAS expression (49), were also enriched in intracranial tumors compared to subcutaneous tumors (**Fig. 5B**). Accordingly, the expression levels of B cell surface molecules required for interactions with T_{FH} cells, such as CD40, ICOSL, and MHC-II, were remarkably higher in the intracranial TME than in the subcutaneous TME (**Fig. 5C and D**). Similar trends were observed in the B16F10 melanoma model (**Supplementary Fig. S9A-C**).

High myeloid-cell infiltration is a well-known feature of the intracranial TME (21,22), which was also confirmed by immunohistochemistry (IHC) of mouse tumor specimens (**Supplementary Fig. S9D**). To evaluate whether myeloid cells are related to B cell activity, we used a colony-stimulating factor 1 (CSF-1) inhibitor (PLX3397) (50) to reduce myeloid cell infiltration (**Supplementary Fig. S9E**). Under these conditions, intracranial B and plasma cell infiltration, as evaluated by CD79a immunohistochemistry, was significantly reduced (**Fig. 5E and Supplementary Fig. S9F**). Additionally, intracranial B cell activity was markedly decreased compared to that in the control (**Fig. 5F and G**). These results suggest that abundant myeloid cells regulate B-cell activity in the intracranial TME.

BAFF/APRIL are responsible for the additive effect of CTLA-4 blockade against intracranial tumors

Previous studies have revealed that BAFF, also known as BLyS, TALL-1, and

TNFSF13B, and APRIL, a close homologue of BAFF, play key roles in B cell and plasma cell survival and are mainly produced by myeloid cells (51-53). In addition, our experimental findings suggest that myeloid cells, which are enriched in the intracranial TME, regulate B cell activity in the intracranial TME. Based on these findings, we analyzed BAFF and APRIL expression in intracranial TILs and confirmed that myeloid cells, particularly macrophages, showed high BAFF and APRIL expression, as previously reported (**Fig. 6A** and **Supplementary Fig. S10A**) (51,52). Among macrophages, not CD206^{hi} immunosuppressive macrophages but CD80^{hi} pro-inflammatory macrophages produced more abundant BAFF and APRIL (**Supplementary Fig. S10B** and **C**). Accordingly, CD80^{hi}CD206^{lo} M1-like macrophages mostly produced BAFF and APRIL compared with the other fractions (**Supplementary Fig. S10D** and **E**). To validate the effects of BAFF/APRIL on the intracranial TME, we used a transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI)-Fc, which neutralizes both BAFF and APRIL proteins specifically (53), in a murine model. In mice treated with TACI-Fc, B cell and plasma cell infiltration in the intracranial TME decreased, accompanied by reduced expression of CD40, ICOSL, and MHC-II in B cells (**Fig. 6B-D**). In addition, T_{FH}-cell infiltration in the intracranial TME was decreased by TACI-Fc, accompanied by reduced IL-21 production (**Fig. 6E and F**). Accordingly, the additive effect of the CTLA-4 blockade on the PD-1 blockade in intracranial tumors was almost abolished (**Fig. 6G and H**). Overall, BAFF/APRIL produced by abundant myeloid cells induces B-T_{FH} cell activation in the intracranial TME, contributing to the additive effect of the

CTLA-4 blockade.

T_{FH}⁻ and B-cell infiltration are observed as tertiary lymphoid structures (TLSs) in the intracranial TME of patients with NSCLC

Finally, we stained B and plasma cells (CD79a) and T_{FH} cells (CXCR5 and CD4) in the surgically resected BM samples from 19 patients with NSCLC. We found B and plasma cell clusters in 8 of the 19 samples (42%) (**Supplementary Table S2**). The area of the B and plasma cell cluster was also infiltrated by a large number of T_{FH} cells (**Supplementary Fig. S11A**), which is consistent with the features of TLS. Eleven of the 19 patients (58%) received systemic chemotherapy prior to surgical resection, and 4 of these 11 patients (36%) had TLS in their BM samples. In contrast, 4 of the 8 patients (50%) who did not receive chemotherapy prior to surgical resection had TLS. Among the 5 patients who received chemotherapy within 6 months prior to surgical resection, TLS were observed in 3 patients (60%). Only one patient received ICI therapy, including anti-PD-1 and anti-CTLA-4 mAbs, within 6 months prior to surgical resection. Notably, TLS was detected within the tumor tissue of this patient. Additionally, we assessed the localization of B and plasma cells in the murine intracranial TME with CD79a staining, which revealed clustering of B and plasma cells within or at the margins of intracranial tumors, particularly in mice treated with PD-1 and CTLA-4 blockade (**Supplementary Fig. S11B**), consistent with findings in human clinical samples. Based on our experimental findings, these patients may be candidates for combination therapy.

Discussion

Combination therapies with anti-PD-1 and CTLA-4 mAbs have been proven to be effective in various cancer types such as melanoma (54), NSCLC (55), malignant mesothelioma (56), renal cell carcinoma (57), hepatocellular carcinoma (58), and esophageal cancer (59), but clearly increase severe immune-related adverse events (60,61), prompting clinicians to use this combination for appropriate patients who can obtain actual clinical benefits. Patients with BM are one such candidate because several studies have shown that a combination with CTLA-4 blockade can be particularly effective against BM (14,16-19). However, the detailed mechanisms of the BM-specific TME remain unclear (21,22). Here, we demonstrated the additive effect of CTLA-4 blockade against intracranial tumors using murine models, which is consistent with the results of previous studies. High CTLA-4⁺ T_{FH}-cell infiltration into the intracranial TME contributed to this additive effect. In addition, we found that BAFF/APRIL from abundant myeloid cells in the intracranial TME induced B-T_{FH} cell activation. Our findings provide a novel axis of B-T_{FH} cells via BAFF/APRIL from abundant myeloid cells in the intracranial TME and deep insights into the additive effect of CTLA-4 blockade against BM based on the BM-specific TME, supporting the combination strategy against BM.

Although CD8⁺ T cells that directly attack tumor cells generally play crucial roles in ICI efficacy (41), CD4⁺ T cells, including T_{FH} cells, have been reported to exhibit cytotoxicity against MHC-II-expressing tumors in the TME (42). However,

the tumor cells used in this study lacked MHC-II expression, and no cytotoxicity was observed in T_{FH} cells. Despite the very low expression of CTLA-4 in CD8⁺ T cells, CTLA-4 blockade further activated CD8⁺ T cells in the intracranial TME, suggesting that its effects on CD8⁺ T cells are indirect. Given the high infiltration of CTLA-4⁺ T_{FH} cells in the intracranial TME, we investigated T_{FH} cells as the potential indirect mediator. T_{FH} cells are generally involved in GC formation, interaction with B cells, their maturation, and the production of antibodies (37). Additionally, IL-21 produced by T_{FH} cells induces the proliferation and cytotoxic activity of CD8⁺ effector T cells (45-47). Thus, T_{FH} cells play an important role in antitumor immunity including ICI efficacy (45-47). Furthermore, in our murine experiments, the intracranial TME was more highly infiltrated by CTLA-4⁺ T_{FH} cells than the subcutaneous TME, and PD-1 blockade alone did not sufficiently activate such intracranial T_{FH} cells. Because CTLA-4 generally has suppressive roles in T cell activation (62), CTLA-4 expression in intracranial tumor-infiltrating T_{FH} cells likely suppresses these T_{FH} cells, which can be recovered by CTLA-4 blockade. Accordingly, the addition of anti-CTLA-4 mAb significantly induced T_{FH} cell proliferation and IL-21 production in the intracranial TME, and T_{FH}-cell knockout almost abolished this additive effect in our murine experiments, supporting the hypothesis that CTLA-4 blockade exerts indirect effects on CD8⁺ T cells through activated T_{FH} cells in the intracranial TME. Overall, the high infiltration of CTLA-4⁺ T_{FH} cells in the intracranial TME explains why PD-1 blockade alone was insufficient, whereas the combination of PD-1 and CTLA-4 blockade exhibited pronounced antitumor effects in intracranial tumors.

Myeloid cells, including macrophages, microglia, and monocytes, are major components of immune cells in the intracranial TME (21,22), which appear to be a specific phenotype in the BM and regulate immune cell activity through the upregulation of various cytokines and chemokines (22). Tumor-associated macrophages (TAMs) and microglia are reportedly involved in tumor cell invasion via TGF β , EGF and IL-10 (63) and suppression of lymphocytes due to inhibitory cytokines and the lack of costimulatory molecules (64), thereby promoting tumor growth. Consistent with these previous studies, our murine experiments showed that myeloid cells, including immunosuppressive TAMs, were more abundant in the intracranial TME than in the subcutaneous TME and that treatment with either anti-PD-1 mAb or anti-CTLA-4 mAb alone was less effective against intracranial tumors than against subcutaneous ones. In contrast to these suppressive roles, some TAMs play pro-inflammatory roles and provide antitumor effects (65). Here, we also found a novel antitumor role for TAMs in the intracranial TME in addition to their immunosuppressive roles, namely, BAFF/APRIL-mediated B-T_{FH} cell activation in the intracranial TME. Several studies have shown that BAFF/APRIL are primarily produced by myeloid cells and that BAFF/APRIL promote the survival and proliferation of B and plasma cells by increasing the levels of survival-promoting molecules (51-53). Particularly in our mouse models, intracranial infiltration of GC B cells, which are essential for the adaptive immune response through enhanced interactions with T_{FH} cells, was significantly higher than their infiltration in the subcutaneous TME. Furthermore, BAFF production is reportedly a characteristic of

pro-inflammatory macrophages (66). Consistent with these previous studies, we found that CD80^{hi}CD206^{lo} pro-inflammatory M1-like macrophages produced abundant BAFF/APRIL in the intracranial TME. Together with our data, these pro-inflammatory macrophages likely represent the origin of intracranial BAFF/APRIL production, which in turn leads to B-T_{FH} cell activation in the intracranial TME.

TLS forms in non-lymphoid peripheral tissues with chronic inflammation due to infection or tumors and is composed of abundant T cells, mainly T_{FH} cells, B cells, plasma cells, and follicular dendritic cells (67,68). Tumor-associated TLS generate local adaptive immune responses against tumor antigens via clonal amplification, somatic mutations, and class-switching of immature B cells (67,68). Mature B cells within TLS highly express MHC-I or MHC-II and play a role in inducing tumor-specific T cells, which recognize tumor antigens (69). The presence of TLS in human solid tumor tissues has been examined in primary melanoma, NSCLC, breast cancer and colorectal cancer and is associated with an improved response to immunotherapy and positive prognosis (67). Although it is largely unknown whether TLS form in the TME of BM (70), we found that TLS form in the BM of NSCLC. Similarly, we observed clusters of B and plasma cells within the murine intracranial TME, particularly in mice treated with a combination of PD-1 and CTLA-4 blockade. In our brain metastasis cohort, one patient received a combination of anti-PD-1 and anti-CTLA-4 mAbs within 6 months prior to surgical resection. Notably, we detected TLS within the tumor tissue of this patient. Together with our mouse data, ICI

treatment can induce TLS formation; however, it is difficult to draw definitive conclusions due to the small sample size. While the role of macrophages in TLS formation is not fully understood, BAFF/APRIL reportedly leads to the *in situ* maturation and survival of early infiltrating B cells (71). Taken together with our data, macrophages partially contribute to TLS formation via BAFF/APRIL in the intracranial TME.

Our findings were mainly based on murine experiments, which represent a limitation of this study. Although we found B- and T_{FH}- cell infiltration along with TLS formation in human BM specimens, the sample size was relatively small, and we could not compare them with paired primary tumors. In addition, although the identified intracranial TME could become a predictive biomarker for combination therapies, it is difficult to approach the intracranial TME in a clinical setting, necessitating the use of surrogate markers derived from primary tumors or peripheral blood. Further investigation, including a larger number of clinical samples, is warranted to resolve these limitations.

In summary, we demonstrated the additive effect of CTLA-4 blockade against intracranial tumors in terms of the intracranial TME using murine models. Notably, high CTLA-4⁺ T_{FH}-cell infiltration, induced by the interaction of B-T_{FH} cells via BAFF/APRIL from abundant myeloid cells in the intracranial TME, could contribute to this additive effect. Despite several limitations, our findings provide valuable insights into the additive effect of CTLA-4 blockade against BM based on the BM-specific TME, supporting a combination strategy against BM.

Author contributions

T. Ninomiya performed data curation, formal analysis, and writing – original draft; N. Kemmotsu and F. Mukohara provided clinical samples and performed technical advice; M. Magari and A. Miyamoto performed purifying TACI-Fc and writing – original draft; Y. Ueda, T. Ishino and J. Nagasaki performed formal analysis and technical advice; T. Fujiwara provided resources and performed technical advice; H. Yamamoto performed technical advice; H. Hayashi and K. Tachibana provided clinical data; J. Ishida, Y. Otani, S. Tanaka and S. Toyooka provided clinical samples; I. Okamoto supervised research; Y. Togashi performed conception, data curation, formal analysis, project management, and writing – original draft.

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Figure Legends

Fig. 1. Combination efficacy of PD-1 and CTLA-4 double blockade in subcutaneous and intracranial tumor models

(A) Schematic of the murine model. LL/2 cells (2×10^5) or B16F10 cells (1×10^5) were inoculated subcutaneously and intracranially on day 0. Each treatment was administered on days 6, 9, and 12. We evaluated the intracranial tumor volume using bioluminescence signals obtained from luciferase-transduced cells and tumor-infiltrating lymphocytes by flow cytometry and immunohistochemistry on day 14. **(B)** and **(C)** *In vivo* efficacy of PD-1 and/or CTLA-4 blockade in subcutaneous LL/2 tumors. The mean tumor volume was used to generate tumor growth curves. Tumor growth curves **(B)** and summary on day 14 **(C)** are shown. **(D)** and **(E)** *In vivo* efficacy of PD-1 and/or CTLA-4 blockade against intracranial LL/2 tumors on days 6 **(D)** and 14 **(E)**. Representative images (left) and summaries (right) are shown.

All *in vivo* experiments were performed at least twice and similar results were obtained. Two-way ANOVA with Bonferroni corrections was used in **(B)**, and one-way ANOVA with Bonferroni corrections were used in **(C)**, **(D)**, and **(E)**, respectively. Means and SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, NS, not significant.

Fig. 2. CTLA-4⁺ T cell subsets in tumor-infiltrating lymphocytes (TILs) from untreated intracranial and subcutaneous tumors

In vivo experiments were performed as described in Figure 1A using untreated control tumors. **(A)** CTLA-4 expression in each T cell subset of intracranial LL/2 TILs. Representative flow cytometric staining (left) and summary (right) are shown. Regulatory T cells (T_{reg} cells) and conventional CD4⁺ T cells (T_{conv} cells) were gated on Foxp3⁺CD4⁺ T cells and Foxp3⁻CD4⁺ T cells, respectively. **(B)** Frequency of T_{reg} cells in intracranial and subcutaneous LL/2 TILs. Representative flow cytometric staining (left) and summary (right) are shown. **(C)** CTLA-4, ICOS, and PD-1 expression in T_{reg} cells of intracranial and subcutaneous LL/2 TILs. Summary is shown. **(D)** PD-1 and ICOS expression in T_{conv} cells of intracranial LL/2 TILs according to CTLA-4 expression. Representative flow cytometric staining (left) and summary (right) are shown. **(E)** CTLA-4 expression in each T cell subset of intracranial LL/2 TILs. CD4⁺ T follicular helper (T_{FH}) cells were gated on PD-1⁺CXCR5⁺Foxp3⁻CD4⁺ T cells. Representative flow cytometric staining (left) and summary (right) are shown. **(F)** Frequency of T_{FH} cells in intracranial and subcutaneous LL/2 TILs. Representative flow cytometric staining (left) and summary (right) are shown. **(G)** CTLA-4 and ICOS expression in the T_{FH} cells of intracranial and subcutaneous LL/2 TILs. Summary is shown.

All *in vivo* experiments were performed at least twice and similar results were obtained. T-tests were used in **(B)**-(**G**), and one-way ANOVA with Bonferroni corrections were used in **(A)**, respectively. Means and SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, NS, not significant.

Fig. 3. CD8⁺ T cell and CD4⁺ T follicular helper (T_{FH}) cell activation in intracranial tumor-infiltrating lymphocytes (TILs) treated with PD-1 and/or CTLA-4 blockade *In vivo* experiments were performed as described in Figure 1A. **(A)-(C)** CD8⁺ T cell activation (A, PD-1⁺CD8⁺ T cell; B, IFN γ ⁺CD8⁺ T cell; C, Perforin⁺CD8⁺ T cell) in intracranial LL/2 TILs. Representative flow cytometric staining (left) and summary (right) are shown. **(D)** and **(E)** Frequency of T_{FH} cells in intracranial LL/2 TILs (D) and their IL-21 production (E). T_{FH} cells were gated on PD-1⁺CXCR5⁺Foxp3⁻CD4⁺ T cells. Representative flow cytometric staining (left) and summary (right) are shown.

All *in vivo* experiments were performed at least twice and similar results were obtained. One-way ANOVA with Bonferroni corrections were used in (A)-(E). Means and SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Fig. 4. Combination efficacy of PD-1 and CTLA-4 blockade and CD8⁺ T cell activation in the intracranial tumor model using CD4-cre/Bcl6^{fl/fl} mice *In vivo* experiments were performed as described in Figure 1A. **(A)** and **(B)** *In vivo* efficacy of PD-1 and/or CTLA-4 blockade against intracranial LL/2 tumors. Representative images (A) and summary (B) are shown. **(C)** and **(D)** CD8⁺ T cell activation (C, PD-1⁺CD8⁺ T cell; D, IFN γ ⁺CD8⁺ T cell) in intracranial LL/2 tumors. Representative flow cytometric staining (left) and summary (right) are shown.

All *in vivo* experiments were performed at least twice and similar results were obtained. One-way ANOVA with Bonferroni corrections were used in (B)-(D). Means and SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, NS, not significant.

Fig. 5. B cell interaction molecules with CD4⁺ T follicular helper (T_{FH}) cells in intracranial and subcutaneous tumor-infiltrating lymphocytes (TILs) **(A)** Frequency of B and plasma cells in intracranial and subcutaneous LL/2 TILs. *In vivo* experiments were performed as described in Figure 1A using untreated control tumors. B cells and plasma cells were gated on B220⁺CD3⁻CD45⁺ cells and CD138⁺CD3⁻CD45^{-/lo} cells, respectively. Summaries are shown. **(B)** Frequency of germinal center B cells (GC B cells) in intracranial and subcutaneous LL/2 TILs. *In vivo* experiments were performed as described in Figure 1A using untreated control tumors. GC B cells were gated on GL7⁺FAS⁺B220⁺CD3⁻CD45⁺ cells. Representative flow cytometric staining (left) and summary (right) are shown. **(C)** and **(D)** CD40, ICOSL, and MHC-II expression in B cells of intracranial and subcutaneous LL/2 TILs. *In vivo* experiments were performed as described in Figure 1A using untreated control tumors. Representative flow cytometric staining (C) and summary (D) are shown. **(E)** B and plasma cell infiltration in the intracranial LL/2 tumors treated with PLX3397. Tumor cells (2×10^5) were inoculated intracranially on day 0. PLX3397 was orally administered daily (day 0-13), and we analyzed TILs with CD79a immunohistochemistry on day 14. Summary is shown. **(F)** and **(G)** CD40, ICOSL,

and MHC-II expression in B cells from intracranial LL/2 TILs treated with PLX3397. *In vivo* experiments were performed as described in E and TILs were analyzed with flow cytometry. Representative flow cytometric staining (F) and summary (G) are shown.

All *in vivo* experiments were performed at least twice and similar results were obtained. T-tests were used in (A), (B), (D), (E) and (G). Means and SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

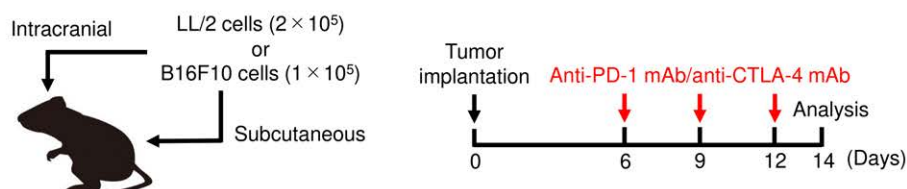
Fig. 6. Myeloid-B-CD4⁺ T follicular helper (T_{FH}) cell axis via B cell activating factor belonging to TNF family (BAFF)/a proliferation-inducing ligand (APRIL) in intracranial tumors.

(A) BAFF and APRIL expression in macrophages, dendritic cells, other CD45⁺ cells and CD45⁻ cells of intracranial LL/2 tumors. *In vivo* experiments were performed as described in Figure 1A using untreated control tumors. Macrophages and dendritic cells were gated on CD14⁺F4/80⁺CD11b⁺CD45⁺ and CD11c⁺CD45⁺ cells, respectively. Summaries of BAFF (left) and APRIL (right) expression are shown. **(B)** Frequency of B and plasma cells in intracranial LL/2 tumor-infiltrating lymphocytes (TILs) treated with TACI-Fc. Tumor cells (2×10^5) were inoculated intracranially on day 0. TACI-Fc was administered on days 0, 5, and 10, and we analyzed TILs with flow cytometry on day 14. B cells and plasma cells were gated on B220⁺CD3⁻CD45⁺ cells and CD138⁺CD3⁻CD45^{-/lo} cells, respectively. Summaries are shown. **(C)** and **(D)** CD40, ICOSL, and MHC-II expression in the B cells of intracranial LL/2 TILs treated with TACI-Fc. *In vivo* experiments were performed as described in (B). Representative flow cytometric staining (C) and summary (D) are shown. **(E)** Frequency of T_{FH} cells in intracranial LL/2 TILs treated with TACI-Fc. *In vivo* experiments were performed as described in (B) and T_{FH} cells were gated on PD-1⁺CXCR5⁺Foxp3⁻CD4⁺ T cells. Representative flow cytometric staining (left) and summary (right) are shown. **(F)** IL-21 production by T_{FH} cells in intracranial LL/2 TILs treated with TACI-Fc. *In vivo* experiments were performed as described in (B), and summary is shown. **(G)** **(H)** *In vivo* efficacy of PD-1 and/or CTLA-4 blockade combined with TACI-Fc against intracranial LL/2 tumors. *In vivo* experiments were performed as described in Figure 1A, and combined with TACI-Fc, as described in (B). Representative images (G) and summary (H) are shown.

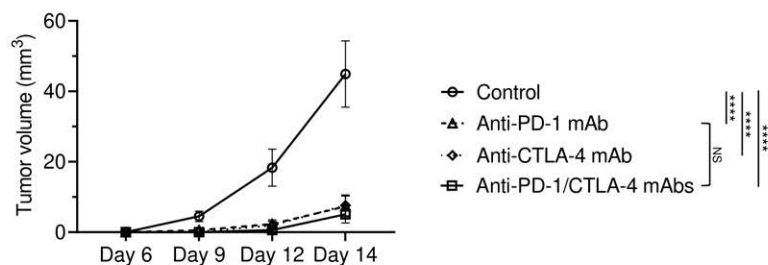
All *in vivo* experiments were performed at least twice and similar results were obtained. One-way ANOVA with Bonferroni corrections was used in (A) and (H), t-tests were used in (B), (D), (E), and (F), respectively. Means and SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, NS, not significant.

Figure 1

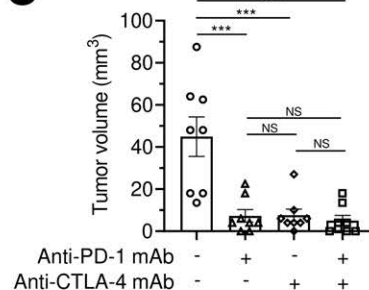
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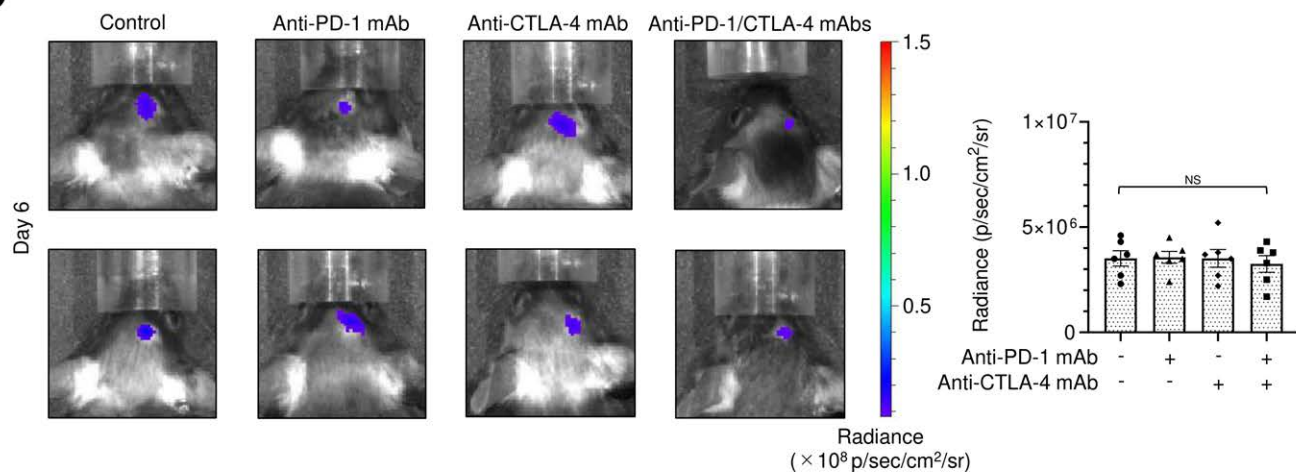
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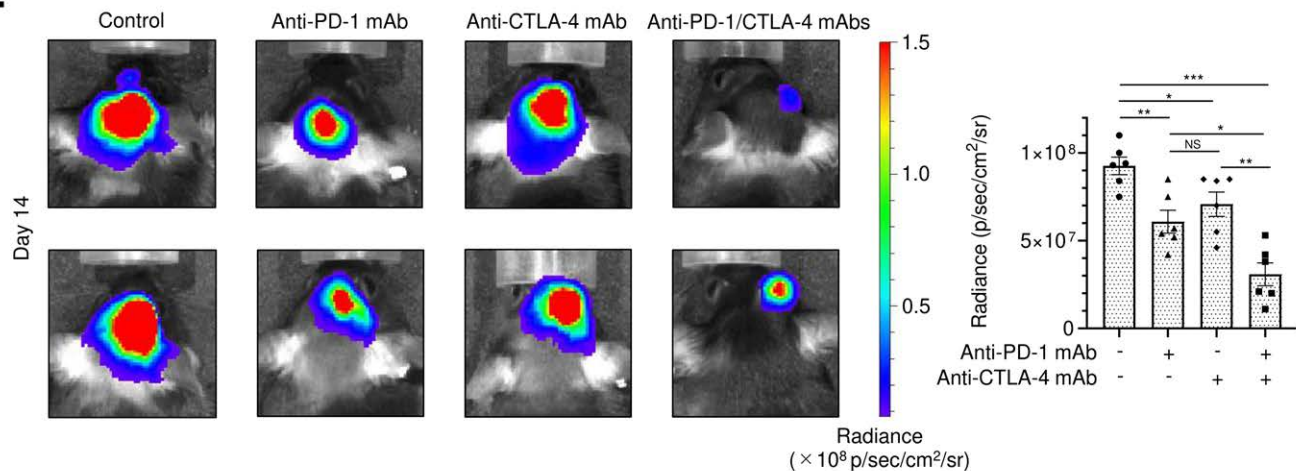
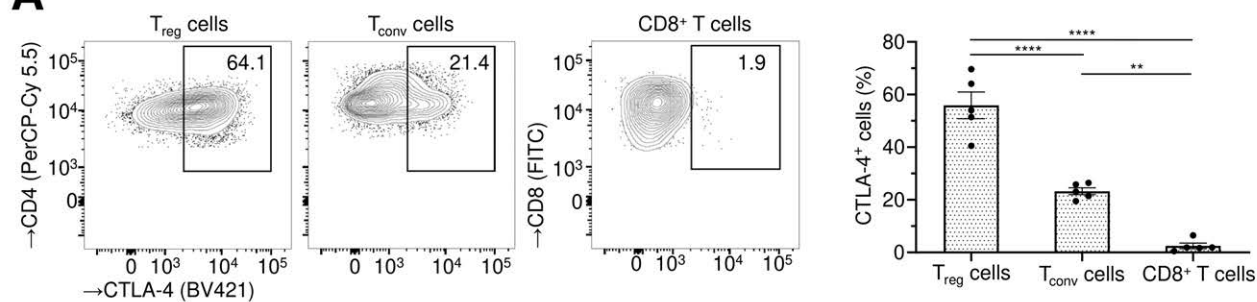
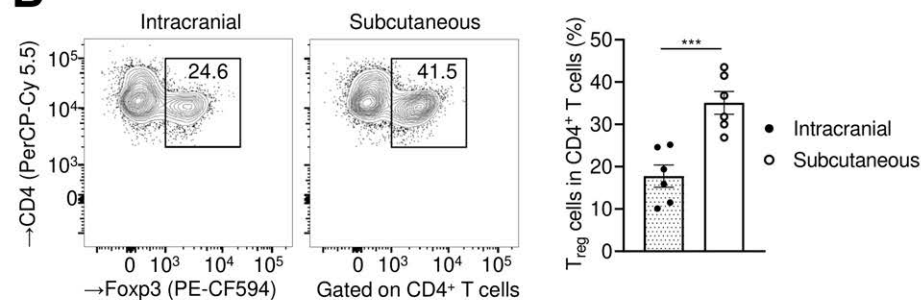


Figure 2

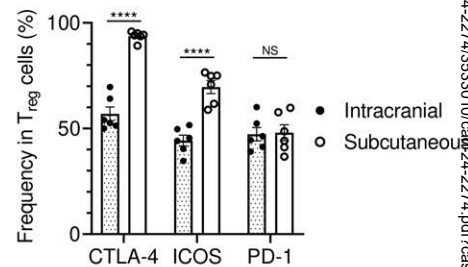
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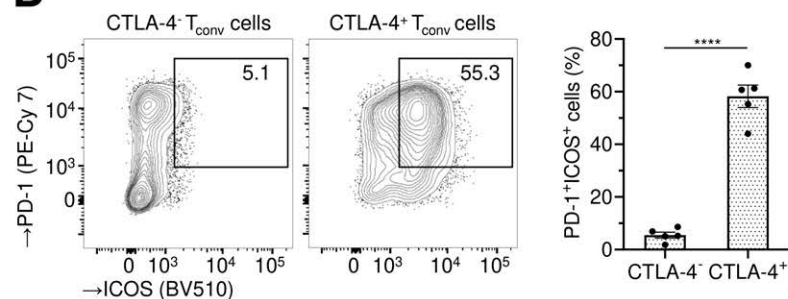
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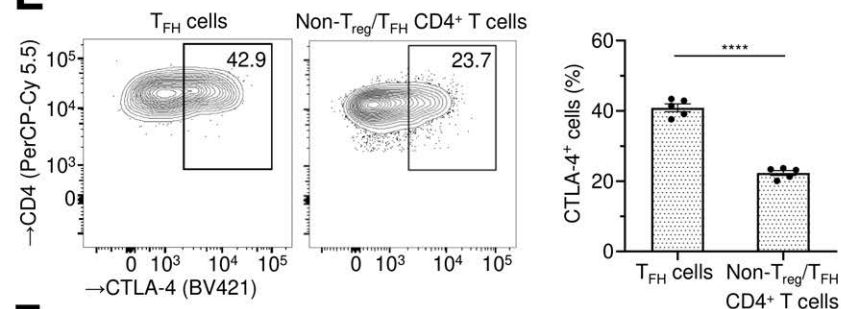
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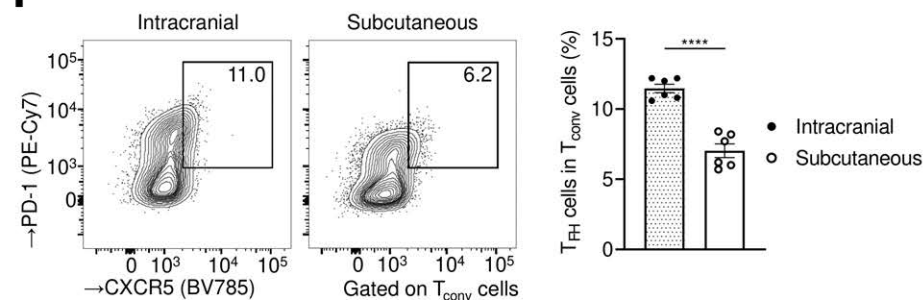
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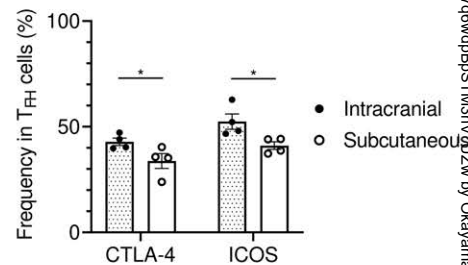
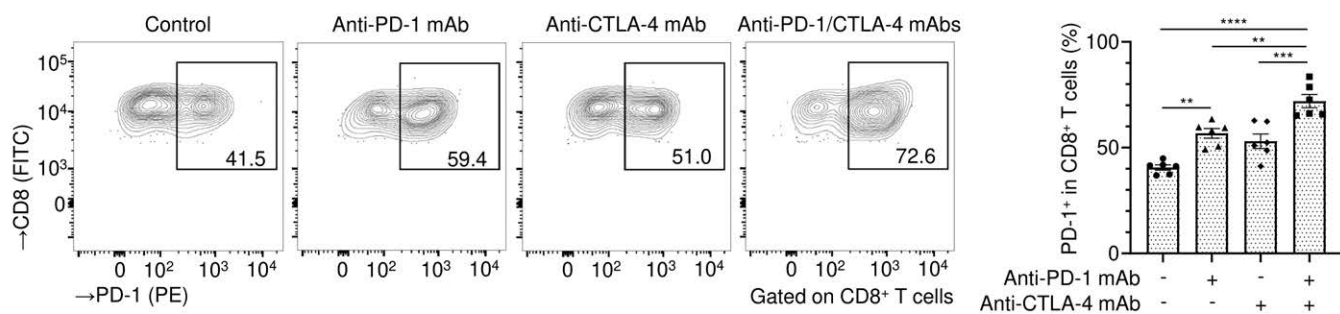
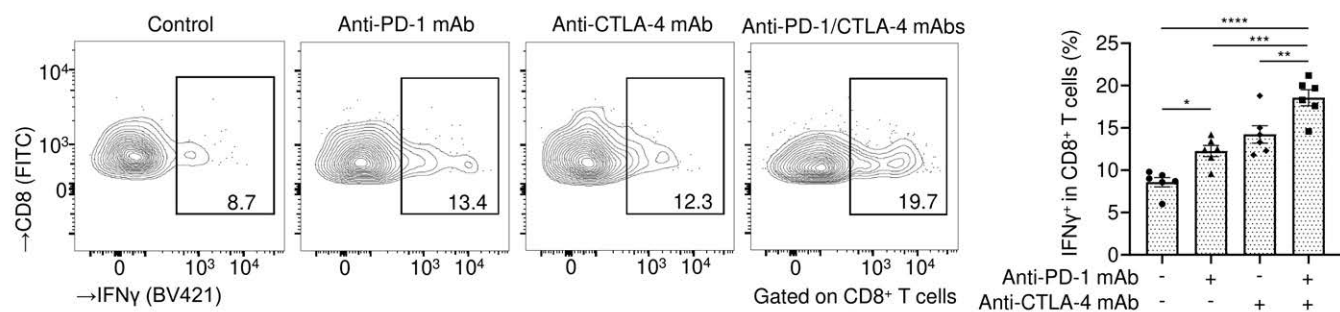


Figure 3

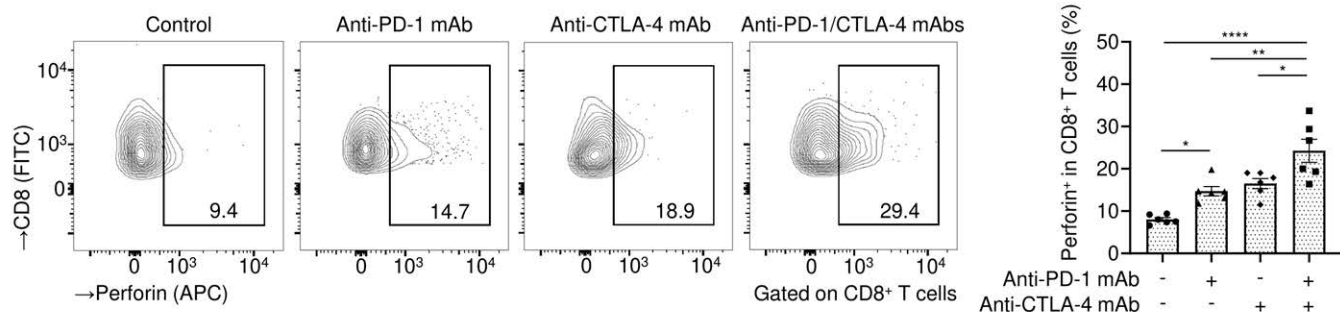
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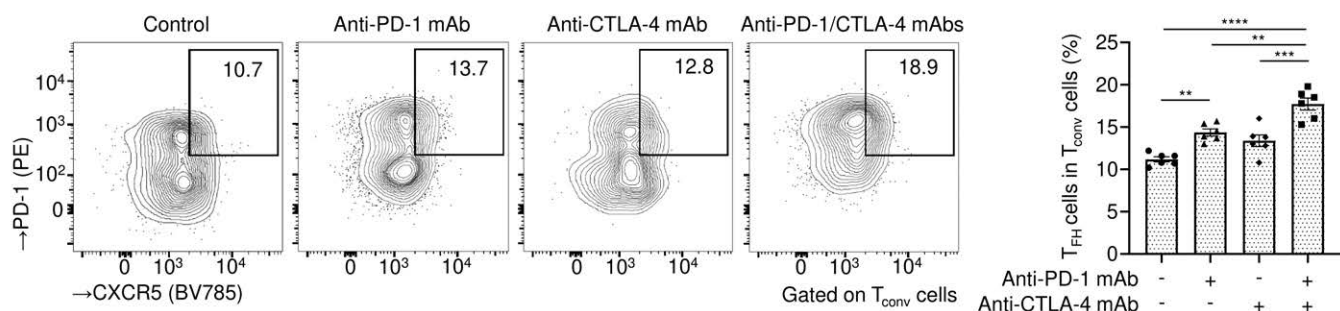
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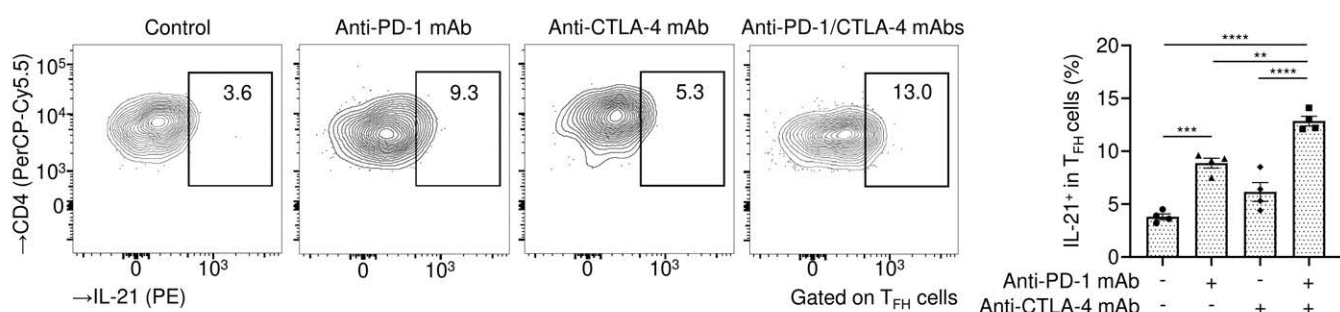
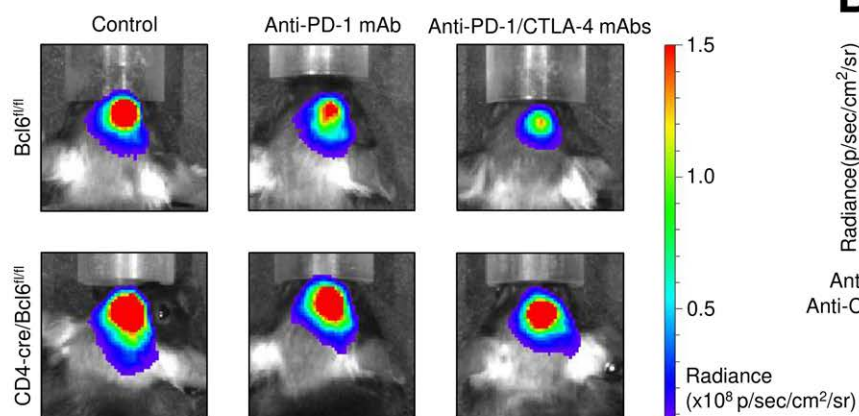
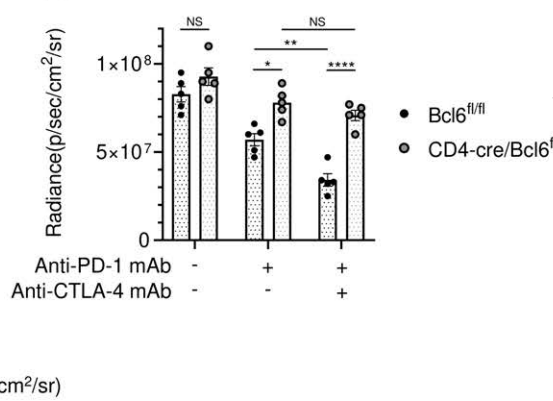


Figure 4

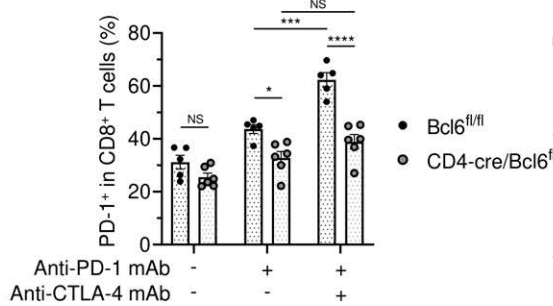
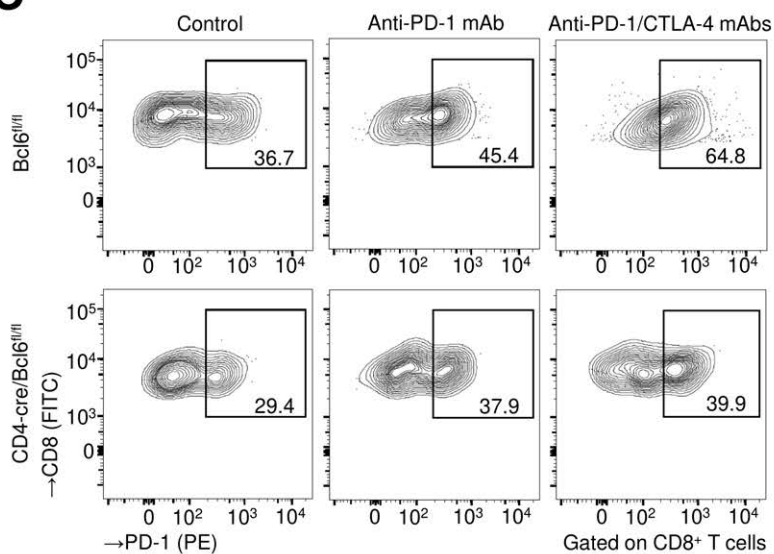
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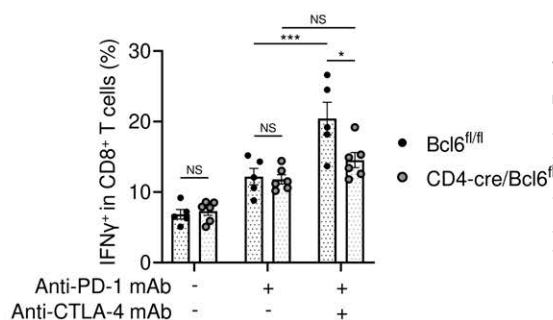
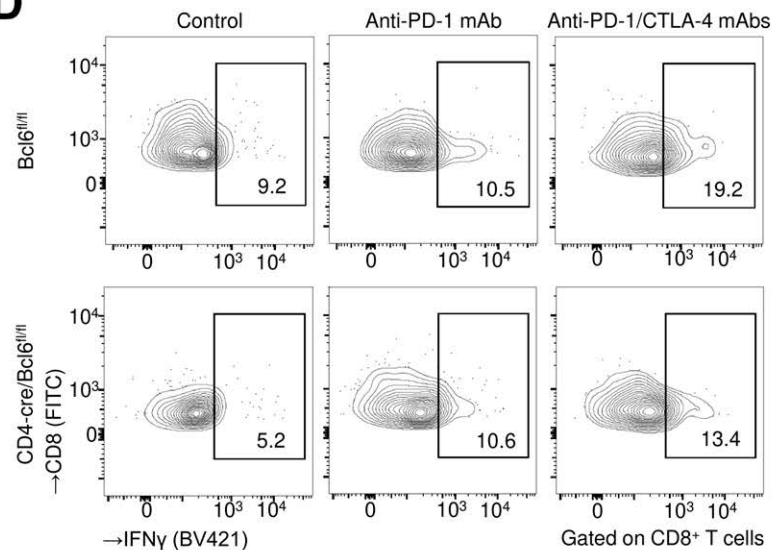


Figure 5

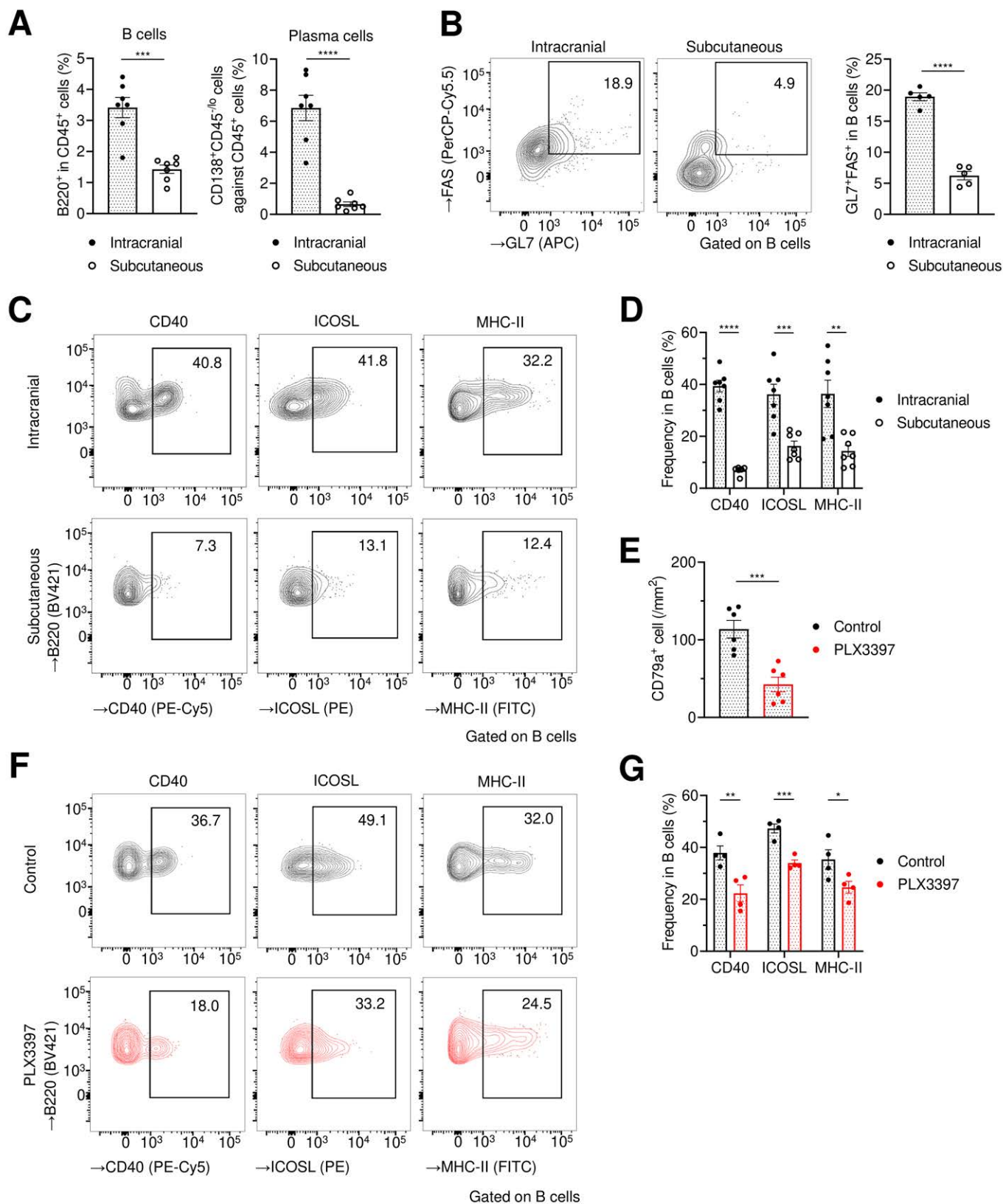


Figure 6

