

Fc–Fc interactions and immune inhibitory effects of IgG4: implications for anti-PD-1 immunotherapies

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ABSTRACT

Background The majority of anti-programmed cell-death 1 (PD-1) monoclonal antibodies (mAbs) use S₂₂₈P mutation IgG4 as the structural basis to avoid the activation of immune cells or complement. However, little attention has been paid to the Fc–Fc interactions between IgG4 and other IgG Fc fragments that could result in adverse effects. Fc-null IgG1 framework is a potential safer alternative to avoid the undesirable Fc–Fc interactions and Fc receptor binding derived effects observed with IgG4. This study provides a comprehensive evaluation of anti-PD-1 mAbs of these two frameworks.

Methods Trastuzumab and rituximab (both IgG1), wildtype IgG1 and IgG4 were immobilized on nitrocellulose membranes, coated to microplates and biosensor chips, and bound to tumor cells as targets for Fc–Fc interactions. Wildtype IgG1 and IgG4, anti-PD-1 mAb nivolumab (IgG4 S₂₂₈P), penpulimab (Fc-null IgG1), and tislelizumab (Fc-null IgG4 S₂₂₈P-R₄₀₉K) were assessed for their binding reactions to the immobilized IgG proteins and quantitative kinetic data were obtained. To evaluate the effects of the two anti-PD-1 mAbs on immune responses mediated by trastuzumab and rituximab in the context of combination therapy, we employed classic immune models for antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and complement dependent cytotoxicity. Tumor-bearing mouse models, both wildtype and humanized, were used for in vivo investigation. Furthermore, we also examined the effects of IgG1 and IgG4 on diverse immune cell populations.

Results Experiments demonstrated that wildtype IgG4 and nivolumab bound to immobilized IgG through Fc–Fc interactions, diminishing antibody-dependent cell-mediated cytotoxicity and phagocytosis reactions. Quantitative analysis of kinetic parameters suggests that nivolumab and wildtype IgG4 exhibit comparable binding affinities to immobilized IgG1 in both non-denatured and denatured states. IgG4 exerted inhibitory effects on various immune cell types. Wildtype IgG4 and nivolumab both promoted tumor growth in wildtype mouse models. Conversely, wildtype IgG1, penpulimab, and tislelizumab did not show similar adverse effects.

Conclusions Fc-null IgG1 represents a safer choice for anti-PD-1 immunotherapies by avoiding both the adverse Fc–Fc interactions and Fc-related immune inhibitory effects of IgG4. Fc-null IgG4 S₂₂₈P-R₄₀₉K and Fc-null IgG1

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The utilization of S₂₂₈P mutation IgG4 in anti-programmed cell-death 1 (PD-1) antibody is a common practice to mitigate the activation of immune cells or complement. However, the Fc–Fc interactions between IgG4 and other IgG Fc fragments can result in adverse effects such as tumor progression.

WHAT THIS STUDY ADDS

⇒ This study investigates the potential of employing an Fc-null IgG1 framework to prevent Fc–Fc interactions. Experiments on trastuzumab and rituximab (both IgG1-based) and various IgG types demonstrated that both S₂₂₈P mutation IgG4 anti-PD-1 monoclonal antibody (mAb) and wildtype IgG4 protein bound to other IgGs, diminishing antibody-dependent cell-mediated cytotoxicity and phagocytosis. Additionally, IgG4 exerted inhibitory effects on various immune cell types, and promoted tumor growth in mouse models. Conversely, wildtype IgG1 and Fc-null IgG1 anti-PD-1 mAb (penpulimab) did not show similar effects.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Fc-null IgG1 mAb framework represents a better option for anti-PD-1 drugs by avoiding the adverse Fc–Fc interactions and inhibitory effects of traditional IgG4 framework. This research contributes to the development of safer and more effective cancer immunotherapies.

displayed similar structural properties and benefits. This study contributes to the understanding of immunotherapy resistance and the advancement of safer immune therapies for cancer.

BACKGROUND

Programmed cell death protein 1 (PD-1) is a receptor predominantly expressed on activated CD8⁺ T cells that inhibits tumor-specific T-cell responses in cancer, and specific monoclonal antibodies (mAbs) blocking the

PD-1 receptor can relieve PD-1/programmed cell death protein ligand 1 (PD-L1) pathway mediated immune suppression.¹ Anti-PD-1 mAb immunotherapy has revolutionized cancer treatment by effectively treating a diverse range of cancer types.² Most clinically approved anti-PD-1 mAbs were engineered using the IgG4 framework^{3,4} as IgG4 does not facilitate classical antibody-dependent effector functions,^{5,6} which avoids autoimmune injury to the target immune cells. The natural IgG4 structure is instable and it can cleave into half molecules and rehybridize, known as the Fab-arm exchange (FAE). Therefore, IgG4 isotype with the S₂₂₈P mutation IgG4 isotype (IgG4 S₂₂₈P) is often used in anti-PD-1 mAbs to stabilize the molecule but still retaining high affinity to FcγRI and binding to FcγRIIb such as nivolumab and pembrolizumab.^{7,8} However, the improvement is insufficient as IgG4 induces immune inhibition through various mechanisms, particularly with its Fc. The known mechanisms including but not limited to the obstruction of antibody-mediated effector functions through competitive binding antigens or Fc receptors (FcRs),^{5,6,9} and inducing immunosuppressive M2 macrophage phenotypes through FcR interactions.^{10,11} The S₂₂₈P mutated anti-PD-1 mAbs were also found to retained similar characteristics of IgG4. Previous studies found that binding interactions between IgG4 Fc and FcγR could lead to nivolumab (an IgG4 S₂₂₈P anti-PD-1 mAb) transfer from CD8+ T cells to macrophage,¹² induce M2-like macrophage phenotype and tumor progression.¹³ Fc:FcγR interactions activated by IgG4 anti-PD-1 mAbs also led to depletion of activated CD8+ T cells and abrogate their therapeutic activity while its engineered Fc-null counterpart enhanced endogenous CD8+ T cell expansion.^{14,15} These mechanisms are speculated to be associated with therapeutic resistance and the occurrence of hyperprogressive disease (HPD) in anti-PD-1 mAb cancer immunotherapy, with an incidence ranging from 5.9% to 43.1%.^{4,16}

The Fc–Fc interactions are another property unique to IgG4, where the Fc fragment of IgG4 binds to the Fc fragment of other immobilized IgGs and reacts more strongly with IgG1.¹⁷ Our recent investigations found that non-specific IgG4 can inhibit antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) mediated by IgG1 antitumor antibodies through Fc–Fc interactions. This interaction can be augmented by elevated levels of glutathione (GSH) in the tumor microenvironment.^{18,19} As IgG²⁰ and GSH²¹ were found abundantly distributed in various tumors, the Fc–Fc interactions between IgG4 and immobilized IgG may be further facilitated in the cancer microenvironment. Notably, the S₂₂₈P mutation has minimal impact on the Fc–Fc interactions of IgG4, but the R₄₀₉K mutation at the Fc terminal is crucial for such reaction.¹⁷ We found that the nivolumab still retained Fc–Fc interactions¹⁹ like wildtype IgG4 which could lead to antibody blocking and off-target effects, possibly causing resistance to anti-PD-1 mAb therapy. In clinical practice, there is ongoing

research on combining IgG4 S₂₂₈P anti-PD-1 mAb with other IgG1 antibodies, such as nivolumab with trastuzumab,²² pembrolizumab with rituximab,²³ nivolumab with cetuximab,²⁴ and nivolumab with ipilimumab.²⁵ While some studies showed promising efficacy, it is important to further evaluate the risks of dual antibody interaction between IgG4 and IgG1 due to their strong tendency for Fc–Fc reaction. To mitigate these risks, newer versions of PD-1 antibodies like penpulimab²⁶ and tislelizumab²⁷ have incorporated optimization strategies such as framework replacements with Fc-null IgG1 and IgG4 S₂₂₈P+R₄₀₉K isotype. By implementing Fc-null modifications, interactions between Fc of anti-PD-1 mAb and FcR of immune cells can be eliminated, resulting in the absence of ADCC, ADCP, and antibody-dependent cytokine release effects.²⁶ The Fc-null IgG1 backbone offers a more structurally stable framework and solution stability²⁸ without the Fc–Fc interactions or FAE. Therefore, Fc-null IgG1 anti-PD-1 mAb has the potential to improve the efficacy and safety of PD-1 blockade combination therapies. In this study, we analyzed the structure and function of IgG4 S₂₂₈P and Fc-null IgG1 anti-PD-1 mAb and evaluated how their frameworks could affect the safety and effectiveness of dual-antibody combination therapies through Fc–Fc interactions. The study also examined how IgG4 and IgG1 affect the growth and suppression of immune cells commonly found in cancer environments.

METHODS

Key resources

Online supplemental table S1 provides a comprehensive list of the essential resources used in this study, including antibodies, chemicals, detection kits, cell lines, and software.

Healthy volunteers and tissue samples

Healthy volunteers provided blood samples, which were used to isolate and differentiate various immune cell types. Cancer tissues from the lung, esophagus, and colon (six samples each) were collected from the Shantou University Affiliated Tumor Hospital. The levels of IgG, IgG1, and GSH were assessed.

Immunohistochemistry and antigen preabsorption tests

Cancer tissues from the esophagus, lungs, and colon were fixed in formalin, embedded in paraffin, and cut into 4 μm thick sections. The sections were then dried at 65°C for 3 hours. Deparaffinization was done by incubating the sections in xylene three times for 10 min each, followed by rehydration using graded alcohol solutions. Immunohistochemistry (IHC) was performed using anti-IgG, IgG1, and GSH antibodies (Abcam) to study the expression and location of IgG, IgG1, and GSH in the tumor microenvironment. Confirm the specificity of the staining, we performed antigen preabsorption tests using antibodies that were preincubated with specific antigens. Specifically, 100 μL of antibody solutions containing a gradient

concentration of IgG (Solarbio), IgG1 (Athens Research), and GSH (Sigma-Aldrich) antigens were preincubated at 4°C for 3 hours. These preincubated solutions were then used as primary antibodies for the staining process.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining

To test antibody tolerance to GSH and detect IgG half-molecule, both reduced and non-reduced sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining were used. The samples included wildtype IgG1 (IgG1wt) and IgG4 (IgG4wt), various versions of IgG1 and IgG4 anti-PD-1 mAbs including nivolumab (IgG4 S₂₂₈P), pembrolizumab (IgG4 S₂₂₈P), and penpulimab (AK 105, Fc-null IgG1), tislelizumab (IgG4 S₂₂₈P+R₄₀₉K), as well as antitumor IgG1 antibodies trastuzumab, rituximab, and cetuximab. Detailed protocols and specific experimental conditions are shown in online supplemental information.

Papain digestion

The Fc fragments of IgG4wt, nivolumab, and penpulimab were obtained using the Pierce Fab Preparation Kit (Thermo Scientific) and labeled with biotin for Fc–Fc binding tests. Additional information can be found in online supplemental information.

Protein labeling

Biotin labeling was performed following the instructions provided by Biotin Protein Labeling Kit (Ana spec) and described in online supplemental methods. IgG1wt, IgG4wt, penpulimab, nivolumab, tislelizumab, rituximab, trastuzumab, inetetamab, bevacizumab, pembrolizumab, sintilimab, and Fc fragments of IgG4wt, penpulimab, and nivolumab were all biotin-labeled as primary antibodies for Western blot, ELISA tests, or immunocytochemistry.

Western blot

Various types of proteins including subtypes of standard IgG proteins, mAbs, and standard IgGs from human, mouse, rabbit, and goat were immobilized on nitrocellulose membranes using electrophoretic transfer as target proteins. Biotin-labeled IgG4wt and IgG1wt, along with IgG4 and IgG1 backbone mAbs, were used as primary antibodies to assess their binding interactions with the proteins on the membrane. To confirm the Fc–Fc interactions, IgG Fc fragments were obtained by digesting IgGs with papain. The Fc fragments were then labeled with biotin and employed as primary antibodies to validate the presence of Fc–Fc interactions. Detailed methods can be found in online supplemental information.

ELISA and surface plasmon resonance assays

To further analyze the characteristics of Fc–Fc interactions and determine the kinetics, ELISA and biacore analysis (surface plasmon resonance, SPR) measurements were conducted. Briefly, IgG1wt was immobilized on high-binding microplates or biacore sensor chips as the target. Serial dilutions of IgG1wt, IgG4wt, penpulimab,

nivolumab, or tislelizumab were added to the ELISA wells or flowed through the sensor chip to assess binding. Quantitative data was collected using a microplate reader (BioTek, EPOCH 2) or a Biacore T200 SPR system. Detailed methods can be found in online supplemental information.

Cell lines

THP-1, Raji, and BT-474 cell lines were obtained from Procell Life Science & Technology Co., Ltd. The THP-1 cells were cultivated in complete medium (Procell Life Science) containing RPMI 1640 media supplemented with 10% fetal bovine serum, 0.05 mM β-mercaptoethanol, and 100 U/mL penicillin-streptomycin. Raji cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin (all Gibco). BT-474 cells were cultured in complete medium (Procell Life Science) consisting of RPMI 1640 media supplemented with 10 μg/mL insulin, 2 mM L-glutamine, 20% fetal bovine serum, and 100 U/mL penicillin-streptomycin. All cells were cultured under standard conditions of 37°C and 5% CO₂. The cell lines were cultured specifically for ADCC, ADCP, and CDC cell model tests.

Immunocytochemistry and immunofluorescence

BT-474 and Raji cells were attached to glass slides, and trastuzumab and rituximab were bound to these cells through specific interactions with their corresponding cell antigens. Biotin-labeled IgG1wt and IgG4wt, along with IgG1 and IgG4 backbone anti-PD-1 mAbs, were employed to assess their binding interactions with the cells or the cell-bound trastuzumab or rituximab. More details can be found in online supplemental information.

Immune cells isolation and preparation

Immune cells (CD8+ T cells, natural killer (NK) cells, and monocytes) were isolated from healthy donors' peripheral blood using immune cell separation kits. THP-1 cells were stimulated and differentiated to create M1 macrophages. These immune cells and M1 macrophages were then cultured and prepared for in vitro cellular tests. Detailed procedures can be found in online supplemental information.

ADCC, ADCP, and CDC assays

Two cellular models (Raji and BT-474) were used to study the effects of Fc-null IgG1 and IgG4 S₂₂₈P anti-PD-1 antibodies on immune responses mediated by trastuzumab and rituximab. Tumor cells were treated with antibodies, washed to remove excess antibodies, and then tested for killing ability with immune cells or serum. IgG1wt and IgG4wt were used as isotype controls. Time-lapse fluorescence microphotography was conducted for multiple wells in ADCP experiments lasting 12 hours. Further details can be found in online supplemental information.

Analysis of cytokines

The supernatant samples obtained from BT-474 ADCC cell experiments were divided into aliquots and immediately frozen at -80°C following centrifugation. The analysis of cytokines was conducted using a multiplex kit (Merck Millipore, HSTCMAG-28SK) and the Luminex MAGPIX System (Luminex Corporation). The cohort samples were assessed for the presence of various cytokines, chemokines, and growth factors, including interleukin (IL)- 1β , IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-21, IL-23, tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony-stimulating factor, interferon- γ (IFN- γ), macrophage inflammatory protein-1 beta, and macrophage inflammatory protein-3 alpha.

Tests of IgG subtypes on immune cells

We examined how different IgG subtypes affect cytokine secretion and protein expression in CD8+ T cells, monocytes, and NK cells. This was done by culturing immune cells with different subtypes of IgG and using techniques including ELISA, flow cytometry, and RT-PCR to assess cytokine secretion, protein expression, and mRNA profiles. An FcR blockade test was also conducted to confirm the binding of IgG4 to FcR and its interaction with CD8+ T cells and NK cells. More information can be found in online supplemental methods.

Animal models

4T1 Breast cancer and CT26 colon cancer tumor bearing models were established in wildtype BALB/c mice ($n=45$, female) or humanized PD-1 BALB/c-hPD1 mice ($n=20$, female). Experimental mice were obtained from Vital River Technology (Beijing, China) and GemPharmatech (Nanjing, China). Mice aged between 8 and 10 weeks, weighing $20\pm 2\text{g}$, were selected for all experimental procedures and maintained in specific pathogen-free conditions. The mice were assigned to separate treatment groups by random-numbers table, where they received either IgG1wt, IgG4wt, penpulimab, or nivolumab, with the phosphate-buffered saline (PBS) group designated as the control. Proteins were dissolved in PBS at 1 mg/mL and administered peritumorally at a dose of $100\text{ }\mu\text{g}$ every 5 days for four injections. Cancer models were induced by injecting 4T1 ($n=5/\text{group}$, 1×10^5 cells for wild-type and 5×10^4 cells for BALB/c-hPD1 model per mouse) or CT26 ($n=4/\text{group}$, 5×10^4 cells per mouse) cells subcutaneously under the left forearm, with tumors becoming measurable within 5–7 days after the initial protein injection. The longest diameter (a) and the shortest diameter (b) of the tumor were measured every 3 days using a caliper, and the volume (V) was then calculated using the formula: $V=1/2\times a\times b^2$. If the tumor volume exceeds 2000 mm^3 or the longest diameter exceeds 2 cm, mice would be humanely euthanized. After 3 weeks, the mice were anesthetized and euthanized by intraperitoneal overdose of 0.1% sodium pentobarbital. The tumor tissues were weighed and fixed in 10% formalin solution for 24 hours, followed by dehydration and embedding in paraffin. The

tissue blocks were cut into $4\text{ }\mu\text{m}$ sections and stained with antibodies for CD3, CD4, CD8, CD31, F4/80, and CD163. T cell and macrophage infiltration, as well as tumor angiogenesis, were quantitatively analyzed by capturing five high-power views of the slides and processing the images with ImageJ software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software V.9 (GraphPad Software, La Jolla, CA, USA). Results are shown as mean $\pm$ SD unless stated otherwise. Statistical significance is shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and ns=not statistically significant. Statistical significance was determined using a one-way analysis of variance with Bonferroni's correction for multiple comparisons.

RESULTS

Tumor microenvironment creates a conducive environment for the Fc–Fc interactions of IgG4

IHC analysis of esophageal, lung, and colon cancer tissues ($n=6$ per type) showed significant presence and distribution of IgG, IgG1, and GSH within tumor tissues (figure 1A). Stronger staining in cancer nests compared with stroma suggests these antibodies may be tumor-specific and able to bind to tumor cells. Antigen preabsorption experiments confirmed the specificity of immunohistochemical staining (figure 1B). These findings showed that the tumor microenvironment is conducive for the Fc–Fc interactions of IgG4 or IgG4-type mAbs. This highlights the potential impact of IgG4-mediated immunosuppressive effects in cancer immunotherapy as the abundance of IgG, IgG1, and GSH in the tumor microenvironment creates a favorable setting for the Fc–Fc interactions involves IgG4 or IgG4 $S_{228}\text{P}$ anti-PD-1 mAbs.

IgG4 and clinical therapeutic IgG4 $S_{228}\text{P}$ anti-PD-1 mAb bind to immobilized IgGs through Fc–Fc interactions

Figure 2 shows that both wildtype IgG4 and $S_{228}\text{P}$ mutated IgG4 anti-PD-1 mAb can bind to immobilized IgGs through Fc–Fc interactions. The Western blot analysis in figure 2A demonstrates that biotin-labeled IgG4wt, IgG4 $S_{228}\text{P}$ variant penpulimab, and nivolumab bind to immobilized IgGs, including IgG1-type antibodies like trastuzumab and rituximab. The strength of Fc–Fc interactions between IgG4 and IgG1 is stronger than between IgG4 and IgG4. Figure 2B provides additional evidence supporting the ability of IgG4wt and nivolumab to interact with four different IgG subtypes and IgG derived from various species (such as human, mouse, rabbit, and goat). This finding suggests that the Fc–Fc interactions of IgG4 demonstrates a trait that is evolutionarily conserved.

Figure 2C illustrates the papain digestion assays and Western blot results that validate the mechanism by which IgG4 binds to immobilized IgG. Both IgG4wt and nivolumab demonstrate the ability to bind to the

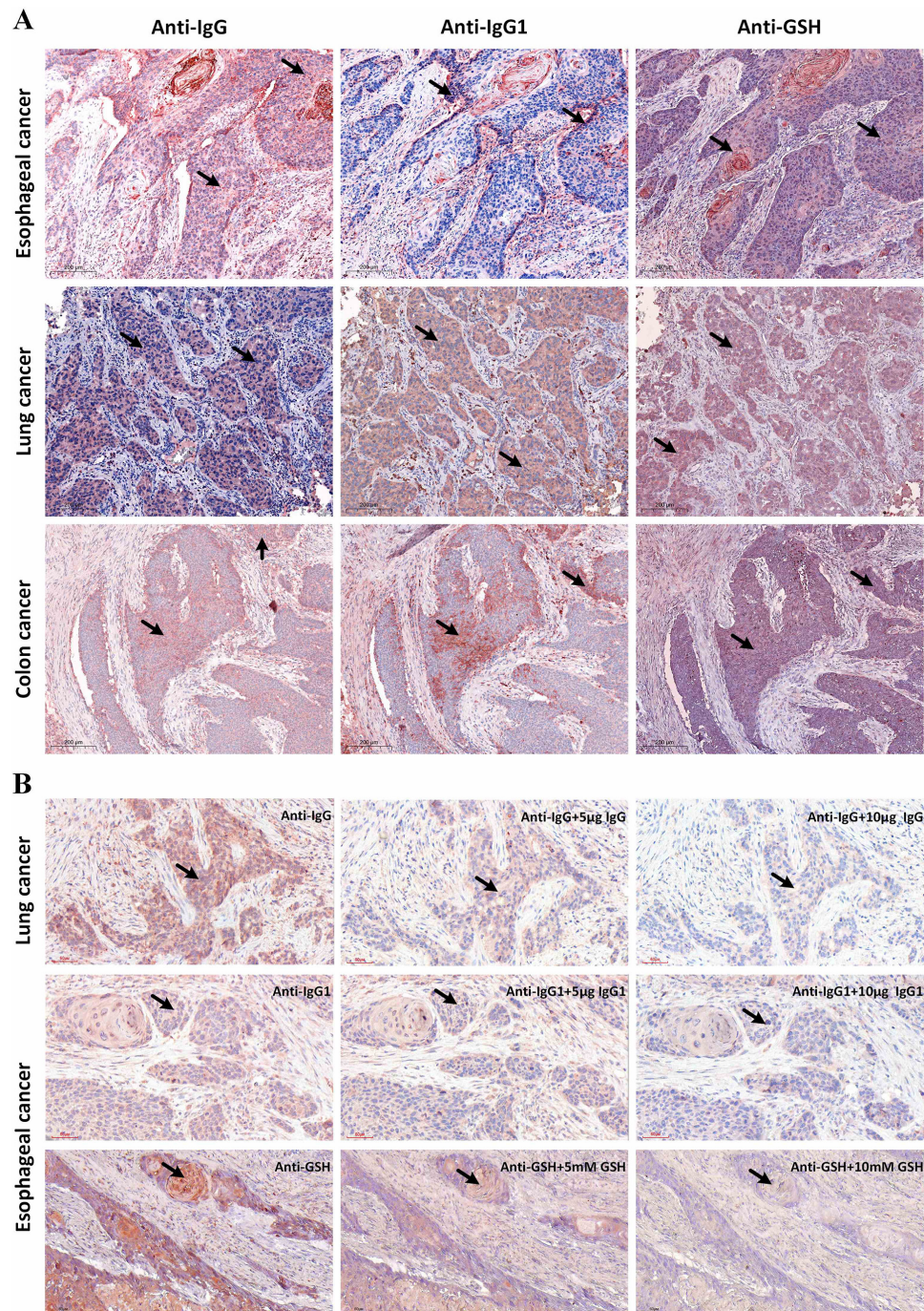


Figure 1 Immunohistochemistry staining results of IgG, IgG1, and glutathione (GSH) in various cancer tissues. (A) Immunohistochemistry staining showed significant presence of IgG, IgG1, and GSH in esophageal, lung, and colon cancer tissues (n=6/type). Scale bar=200 μm. (B) The specificity of IgG, IgG1, and GSH immunohistochemical staining in tumor tissues was confirmed by antigen preabsorption tests. Higher concentrations of specific antigens led to less staining on the tumor tissue. Scale bar=60 μm.

immobilized IgG Fc fragment through Fc–Fc interactions. [Figure 2D](#) shows the Fc–Fc interactions under varying concentrations of GSH to simulate elevated GSH levels within the tumor microenvironment. The findings indicate a direct relationship between the strength of Fc–Fc interactions involving IgG4wt, nivolumab, and immobilized IgG on the membrane, and the rising levels of GSH. Furthermore, half molecules were observed in IgG4wt and IgG4 S₂₂₈P PD-1 mAbs in the presence of gradient

concentration of GSH, whereas IgG1wt and IgG1 type antibodies were more stable under the same conditions (online supplemental figure S1). Experiments were also conducted on the other IgG1 (inertamab, bevacizumab) or IgG4 S₂₂₈P (sintilimab, pembrolizumab) mAbs. Results indicated that the IgG4 antibody displayed similarities to IgG4wt and demonstrated the capacity to produce Fc–Fc interactions, while the IgG1 antibodies did not exhibit these characteristics, as illustrated in [figure 2E](#).

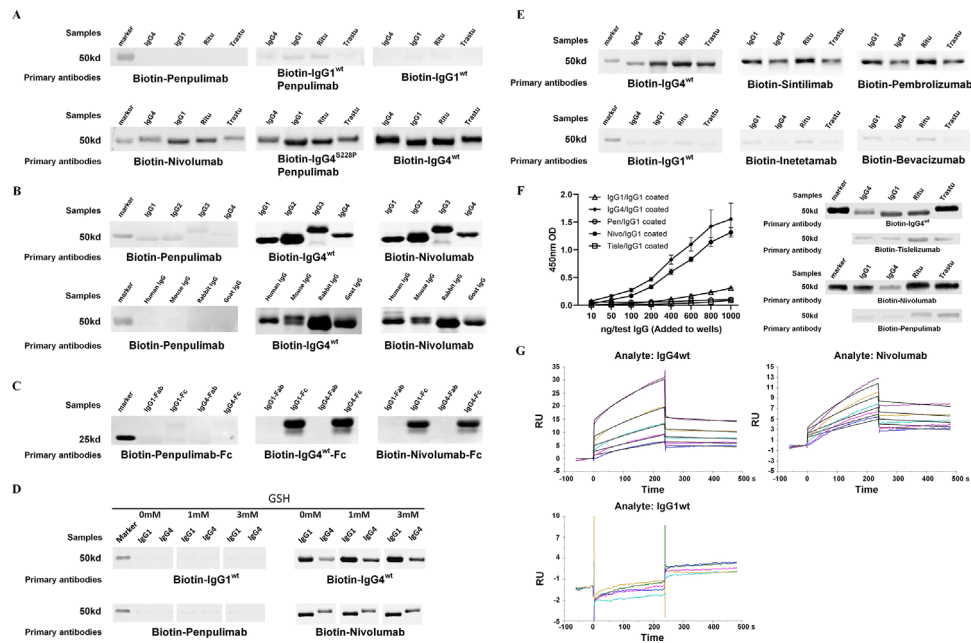


Figure 2 IgG4 and IgG4 S₂₂₈P anti-programmed cell-death 1 monoclonal antibody (mAb) bind to immobilized IgG through Fc–Fc interactions. (A) The Western blots demonstrate that biotin-labeled IgG4wt, nivolumab and IgG4 S₂₂₈P variant penpulimab demonstrate binding capability to immobilized IgG on the membranes (1 µg/well). Biotin-labeled IgG1wt, penpulimab (Fc-null IgG1), and Fc functional IgG1 variant penpulimab (similar to IgG1wt) do not exhibit a similar binding reaction. (B) IgG4wt and nivolumab bound to all IgG subtypes and cross-species IgGs (sample loading 2 µg/well), with stronger binding to IgG1 than IgG4. Penpulimab does not show the same binding pattern. (C) The binding mode involves Fc–Fc interactions between IgG4wt, nivolumab, and immobilized IgG on the membrane. (D) Increasing GSH concentration from 0 to 3 mM enhanced Fc–Fc interactions between IgG4wt/nivolumab and immobilized IgG1 or IgG4 on the membrane, with stronger reactions observed with IgG1. (E) Sintilimab and pembrolizumab, both IgG4 S₂₂₈P mutated variants, showed similar Fc–Fc interactions to IgG4wt, while IgG1wt, inetetamab and bevacizumab (both IgG1 mAb) did not bind. (F) Left, IgG1wt was immobilized on ELISA plate wells at a concentration of 10 µg/mL in 100 µL PBS. Various concentrations of biotin-labeled IgG1wt, IgG4wt, penpulimab, nivolumab, and tislelizumab were introduced to assess their binding affinity to the immobilized IgG1wt. Right, biotin-labeled penpulimab and tislelizumab exhibit a lack of Fc–Fc interactions like IgG4wt and nivolumab (sample loading 2 µg/well). (G) IgG1wt was immobilized on CM5 biocore sensor chips at a coupling level of 8000RU. IgG4wt and nivolumab at concentrations ranging from 312 to 5000 nM at a flow rate of 2 µL/min on the chips. IgG1wt was set as the control. T25°C, association time 240 s and dissociation time 240 s. GSH, glutathione; Nivo, nivolumab; Pen, penpulimab; Ritu, rituximab; Tisle, tislelizumab; Trastu, trastuzumab.

The Fc–Fc interaction pattern was also examined under non-denatured conditions using ELISA experiments. The results in [figure 2F](#) show that IgG4wt and nivolumab have comparable reaction capabilities, with reactions increasing in intensity as the dosage increases. Notably, both penpulimab and tislelizumab exhibit a lack of Fc–Fc interactions as seen for IgG4wt indicating that these two antibodies might be similar in eliminating the Fc–Fc interactions. Subsequent analysis of the kinetic data of Fc–Fc interactions between IgG4wt and nivolumab to immobilized IgG1wt was conducted through SPR

testing ([figure 2G](#)). The estimated apparent dissociation constants were 0.63 µM for IgG4 and 0.81 µM for nivolumab ([table 1](#)).

IgG4 and IgG4 S₂₂₈P anti-PD-1 mAb can bind to cancer cells through the Fc–Fc interactions with tumor-targeting antibody

Based on previous findings, we conducted experiments using immunocytochemistry and immunofluorescence to study the potential binding of IgG4 to IgG1 antibodies on tumor cells through Fc–Fc interactions ([figure 3A](#)). The expression of HER-2 antigens in breast cancer cells (BT-474) or CD20 antigens in lymphoma cells (Raji) were

Table 1 Apparent dissociation constants of IgG4wt and nivolumab to immobilized IgG1wt, measured with surface plasmon resonance

Solid phase(RU)	Analyte	K _D (µM)	k _a (s ⁻¹ M ⁻¹)	k _d (s ⁻¹)
IgG1wt (8000)	IgG4wt	0.63	692.1	4.3×10 ⁻⁴
	Nivolumab	0.81	672.3	5.4×10 ⁻⁴

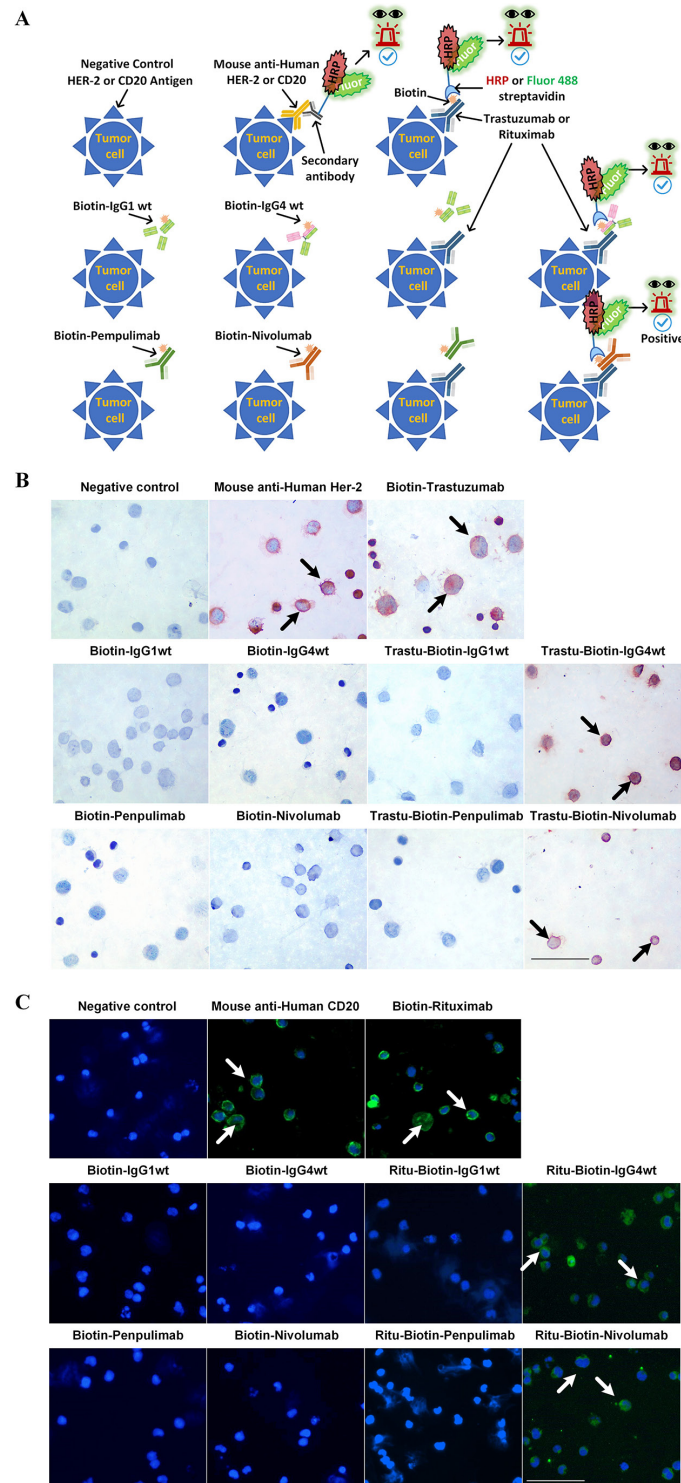


Figure 3 The binding of IgG4wt and IgG4 S₂₂₈P anti-programmed cell-death 1 monoclonal antibody to IgG1 antibodies immobilized on cancer cells. (A) Schematic representation of the immunocytochemistry and immunofluorescence experiments. The presence of HER-2 or CD20 antigens on cancer cell lines (BT-474/Raji) was confirmed using mouse anti-human antibodies. Cancer cells were fixed on slides and incubated with trastuzumab or rituximab to immobilize the antibodies. Biotin-labeled IgG4wt, IgG1wt, pemulimab, and nivolumab were tested for binding to the immobilized IgG1 on the cells. (B) Immunocytochemistry results show Fc-Fc binding reaction between biotin-labeled IgG4wt/nivolumab and trastuzumab immobilized on BT-474 cells. (C) Similar immunofluorescence results seen with rituximab on Raji cells. Positive binding indicated by brown staining or green fluorescence. Scale bar=60µm. Ritu, rituximab; Trastu, trastuzumab.

confirmed with mouse anti-human HER-2 or CD20 antibodies. Biotin-labeled trastuzumab (anti-HER-2 IgG1) and rituximab (anti-CD20 IgG1) showed specific binding to tumor antigens while biotin-labeled IgG1wt, IgG4wt, penpulimab, and nivolumab did not bind. Immunocytochemistry and immunofluorescence confirmed binding of IgG4wt and nivolumab to tumor cells (BT-474 and Raji) after these cell pre-incubation with trastuzumab or rituximab. These results indicate that IgG4 antibodies interact with IgG1 antibodies on tumor cells through Fc–Fc interactions, blocking antitumor antibodies. Conversely, biotin-labeled IgG1wt and penpulimab do not exhibit comparable binding to the tumor cells (figure 3B,C).

IgG4 S₂₂₈P anti-PD-1 mAb attenuates the tumor killing effects of tumor-targeting antibodies in vitro

In cell experiments, the effects of IgG1 and IgG4 subtype anti-PD-1 mAbs on trastuzumab and rituximab-mediated ADCC, ADCP, and CDC immune responses were investigated using BT-474 and Raji cell models. Results showed that IgG4wt and nivolumab inhibited the ADCC and ADCP effects mediated by trastuzumab and rituximab, whereas no attenuation was observed in penpulimab groups (figure 4A,B). The ADCC tests were extended to include various other IgG4 and IgG1-type antibodies and we found that all IgG4 S₂₂₈P anti-PD-1 mAb, except tislelizumab (IgG4 S₂₂₈P+R₄₀₉K), inhibited the ADCC effect of trastuzumab, while IgG1-type antibodies like penpulimab, bevacizumab did not have the same effect (online supplemental figure S2). The CDC tests showed that trastuzumab alone did not elicit complement activation, except when combined with pertuzumab, which is consistent with previous reports.²⁹ In a separate experiment, we observed the inhibitory effects of IgG4wt on the trastuzumab plus pertuzumab-mediated CDC effect and IgG1wt not (online supplemental figure S2). In rituximab-mediated CDC tests, only the IgG4wt group showed reduced activity, while the other groups did not. In ADCP experiments, a time-lapse imaging technique using multiple wells and fluorescence microscopy was employed to visually capture the dynamic process of cellular phagocytosis. The recordings documented the phagocytic activity of BT-474 cell models over a 12-hour period for each subgroup. Analysis of the results revealed that in the IgG4wt and nivolumab groups, macrophages had reduced ability to engulf tumor cells, allowing tumor cells to grow and adhere well (online supplemental video 1). Phagocytosis was measured by quantifying overlapping 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil)/+carboxyfluorescein diacetate succinimidyl ester (CFSE)+ cells using fluorescence microscopy and flow cytometry. Dil labeled macrophages and CFSE labeled tumor cells were observed as red and green fluorescent cells under fluorescence microscopy. (online supplemental figure S2).

Analysis of cytokines in cell culture supernatants of ADCC in the BT-474 model showed that both IgG4wt and nivolumab inhibited cytokine release in ADCC mediated

by trastuzumab. Penpulimab treated groups had similar cytokine release patterns to trastuzumab treated groups (figure 4C), suggesting that IgG4 S₂₂₈P anti-PD-1 mAb may interfere with immune responses mediated by IgG1 antibodies through Fc–Fc interactions, diminishing their effectiveness in combine immunotherapy.

IgG4 but not IgG1, inhibited the viability and cell function of immune cells in vitro

Numerous antibodies targeting multiple antigens are generated using IgG1 or IgG4 frameworks. The impact of varying concentrations of these IgG subtypes on immune cells was investigated in this study, revealing divergent effects when cocultured with immune cells. Figure 5A shows the effects of four IgG subtypes on activated CD8+ T cells. IgG4 decreased cell viability in a dose-dependent manner after 2 days, while the other subtypes did not. Coculturing with 0.5 mg/mL IgG4 increased CTLA4 and PD-1 mRNA expression in CD8+ T cells. Higher concentrations of IgG1, IgG3, and IgG4 hindered Granzyme B secretion, while IgG4, IgG1, and IVIG inhibited IFN- γ secretion compared with control group. TNF α secretion was significantly inhibited by higher concentrations of IgG4. IgG4 has a stronger inhibitory effect on cytokine secretion by activated CD8+ T lymphocytes compared with other IgG subtypes. IgG4 exerts such immunosuppressive effects by binding to FcRs, and the binding reaction can be inhibited by FcR blocker (figure 5B). Flow cytometry confirmed the purity of CD8+ T cells, and activated cells treated with antibodies showed irregular shapes and clustered together (online supplemental figure S3A,B). Similar tests on NK cells showed that IgG4 at 0.1 mg/mL also significantly inhibited NK cell activity, secretion of Granzyme B and IFN- γ , and downregulated the expression of CD69, CD314, and CD226 on NK cells (online supplemental figure S3C,D).

The results of monocytes tests are depicted in figure 5C,D. The presence of IgG1 induced the differentiation of monocytes into macrophages exhibiting an M1 phenotype, while the presence of IgG4 led to monocytes showing a propensity for differentiation into macrophages with an M2 phenotype. The morphological features of the differentiated cells are illustrated in figure 5D. Flow cytometry analysis confirmed that IgG4 treatment decreased M1 marker expression (IL-1 β , IL-12, TNF α) and increased M2 marker expression (CD163, IL-10, CD206, ARG1), suggesting that IgG4 promotes M2 macrophage polarization in human monocytes, while IgG1 promotes M1 macrophage polarization. These findings suggest that IgG4 inhibits the growth and immune function of CD8+ T cells and monocytes in vitro by suppressing cytokine secretion and inducing inhibitory phenotype, potentially affecting immune responses.

IgG4 S₂₂₈P anti-PD-1 mAb but not Fc-null IgG1 antibody promotes tumor growth in vivo

In tumor-bearing mouse models experiments, the tests were conducted in accordance with the methodology

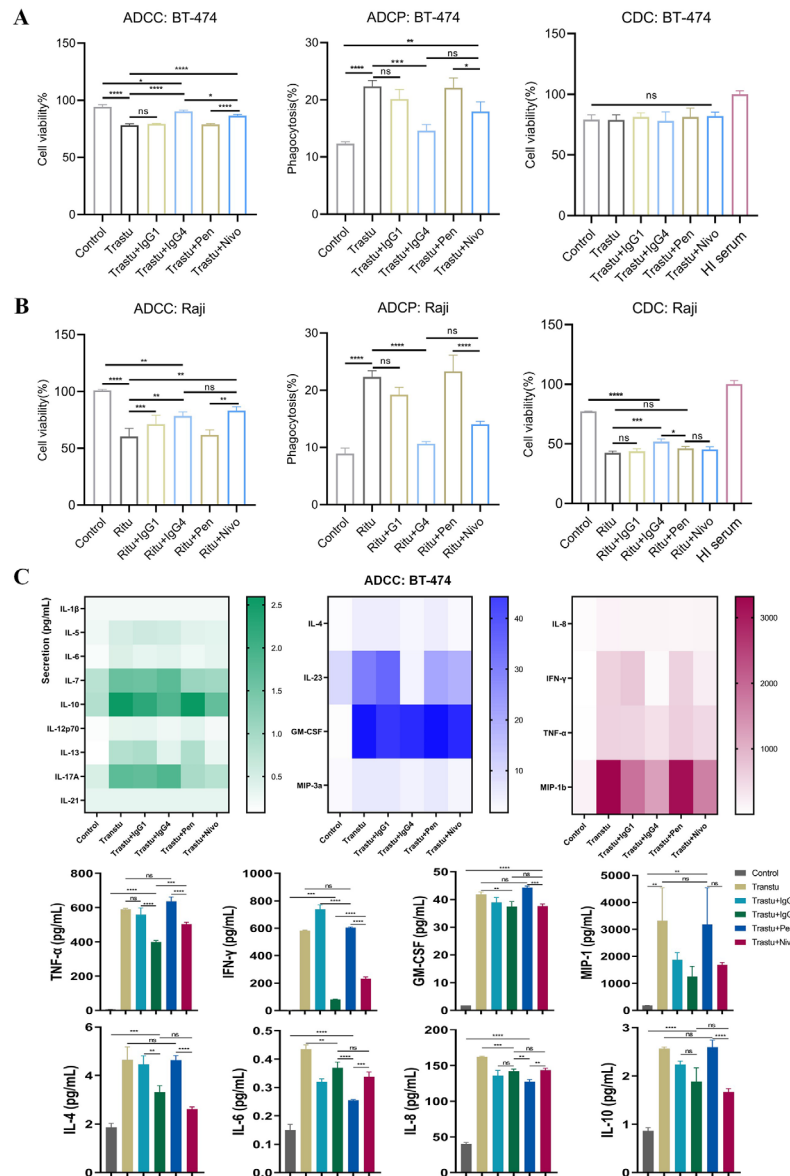


Figure 4 IgG4 S₂₂₈P anti-programmed cell-death 1 monoclonal antibody impedes classical immune effect mediated by antitumor antibodies and hinders cytokine secretion in vitro. (A–B) Left, BT474 or Raji cells were seeded at a density of 1×10^4 cells/well and pretreated with various antibodies. ADCC effect was induced using trastuzumab (2 μ g/mL) or rituximab (1 μ g/mL). Peripheral blood mononuclear cell-derived natural killer cells (2×10^4 cells/well) were added to the well and cocultured with tumor cells for 2 hours at 37°C. IgG4wt and nivolumab were found to reduce the ADCC effect mediated by the IgG1 antibodies; middle, M1 macrophages were labeled with Dil, while BT474 breast cancer cells or Raji lymphoma cells were labeled with CFSE. Phagocytosis was mediated with trastuzumab or rituximab at a 1:1 effector-target ratio. The ADCP effect was weakened by IgG4wt and nivolumab; right, Trastuzumab did not activate complement or cause CDC on BT-474 cells. Rituximab activated complement and damaged Raji cells. IgG4wt reduced CDC effect of rituximab, while IgG1wt, penpulimab, and nivolumab did not. (C) The Luminex test results showed that IgG4wt and nivolumab inhibited cytokine release in the ADCC experiment with the BT-474 model. Trastuzumab and trastuzumab plus penpulimab had similar effects on cytokine release. Nivo, nivolumab; Pen, penpulimab; Ritu, rituximab; Trastu, trastuzumab; HI serum, heat-inactivated serum; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; CFSE, carboxyfluorescein diacetate succinimidyl ester; Dil, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF α , tumor necrosis factor α ; IFN- γ , interferon γ ; IL, interleukin; MIP, macrophage inflammatory protein.

depicted in figure 6A. We observed that both nivolumab and IgG4wt displayed comparable effects in promoting tumor growth in 4T1 breast cancer and CT26 colon cancer wildtype mouse models (figure 6B,C). Tumor weight and volume in both groups were similar and significantly

better than the control groups at harvest (figure 6D–G). In the humanized PD-1 4T1 mouse model, both penpulimab and nivolumab exhibited obvious efficacy. Although penpulimab displayed a tendency towards superior efficacy compared with nivolumab, no statistically significant

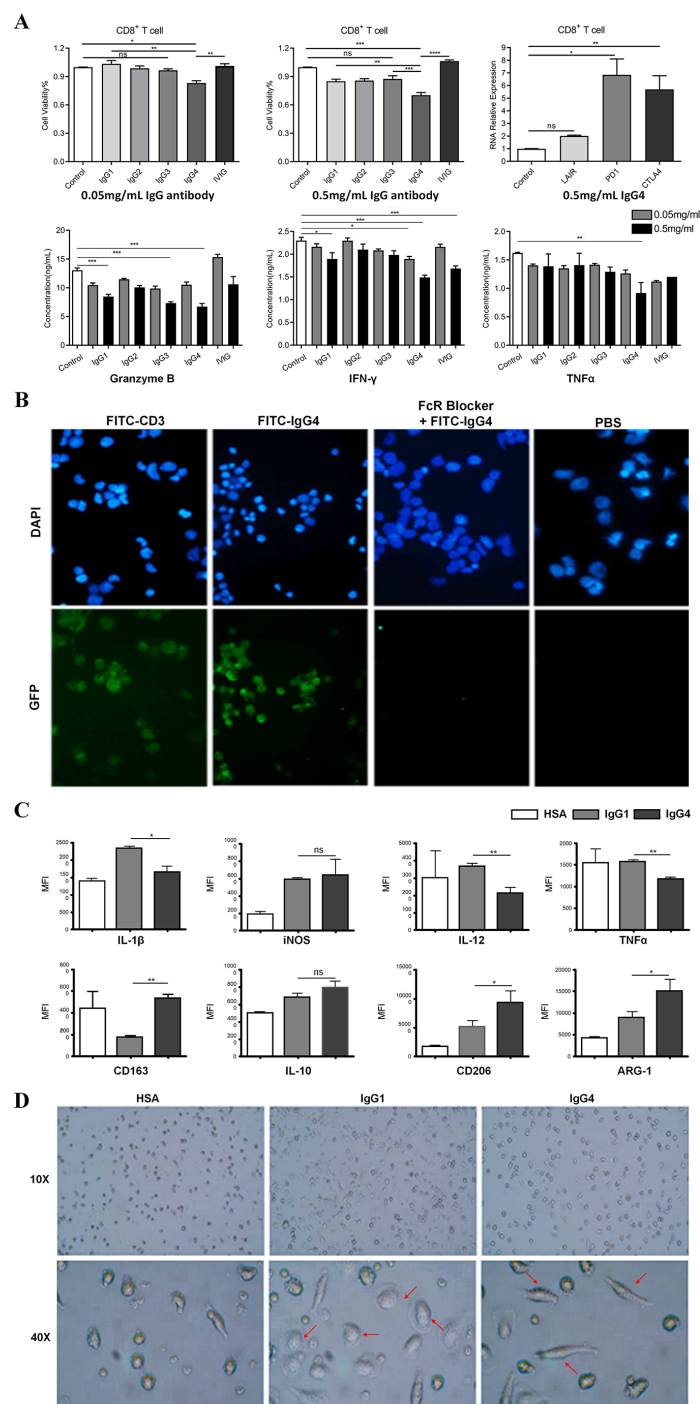


Figure 5 Comparison of the direct effects of different IgG subtype proteins on CD8⁺ T cells and monocytes. (A) The upper panel, the cell viability of activated CD8⁺ T cells treated with lower (0.05 mg/mL) and higher concentrations (0.5 mg/mL) of IgG subtypes or IVIG was assessed using CCK-8 kits. PD-1 and CTLA4 mRNA expression in CD8⁺ T cells treated with IgG4 was measured with real-time qPCR. The lower panel, the secretion of TNF- α , IFN- γ , and Granzyme B in CD8⁺ T cells treated with IgG subtypes and IVIG was measured with ELISA kits; (B) FITC-labeled IgG4 can bind to activated CD8⁺ T cells or NK cells, and FcR blockers can block the binding reaction. The PBS group was set as a negative control; (C) flow cytometry was used to assess the expression of CD163, CD206, ARG-1, IL-10, IL-1 β , IL-12, and TNF- α in human monocytes following treatment with IgG1 or IgG4; (D) monocytes were treated with IgG1 or IgG4 (250 μ g/mL) for a week. IgG1 led to M1-like macrophages, while IgG4 led to M2-like macrophages. HSA was used as the control. DAPI, 4',6-diamidino-2-phenylindole; FcR, Fc receptor; HSA, human serum albumin; CTLA-4, Cytotoxic T lymphocyte antigen-4; IFN- γ , interferon γ ; IL, interleukin; PBS, phosphate-buffered saline; TNF α , tumor necrosis factor α .

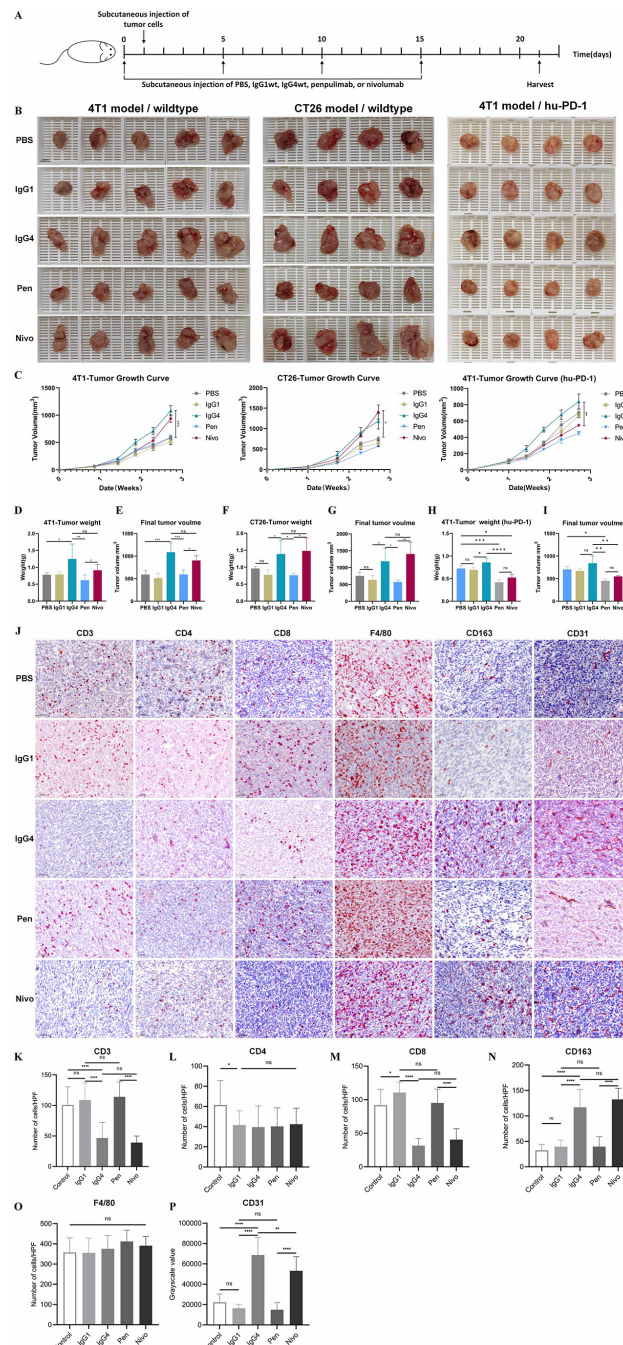


Figure 6 IgG4 proteins promote tumor growth on mouse tumor-bearing models. (A) BALB/c mice with subcutaneous tumors were randomly assigned to groups and given different treatments, including IgG1wt, IgG4wt, penpulimab, or nivolumab. The proteins were dissolved in PBS at 2 mg/mL and given at a dose of 200 μ g peritumorally every 5 days for four injections. The control group received PBS. Cancer models were created by injecting 4T1 cells or CT26 cells under the left forearm after the initial protein injection. (B–C) Tumor tissues were analyzed in groups of 4T1 breast cancer (n=5/group for wild-type, n=4/group for humanized PD-1 model) and CT26 colon cancer (n=4/group) mouse models. Tumor growth curves were plotted for each treatment group in both models. (D–I) Tumor weight results and final tumor volume in each treatment group of the three models at harvest. (J) Tumor tissues from the 4T1 model in each treatment group were analyzed using immunohistochemical staining with antibodies against CD3, CD4, CD8, CD163, F4/80, and CD31. Scale bar=50 μ m. (K–O) The quantitative analysis of T cell subtype (CD3, CD4, CD8) and M2 phenotype macrophage (CD163, F4/80) infiltration was performed by randomly selecting five high power visual fields of staining tissues. (P) Tumor angiogenesis was assessed by measuring the average gray value of anti-CD31 staining. PBS, phosphate-buffered saline; PD-1, programmed cell-death 1.

difference was observed between the two within the observation period (figure 6B,C and H,I). Immunohistochemical staining was performed on tumor tissues from

the wild-type 4T1 model using antibodies against CD3, CD4, CD8, CD163, F4/80, and CD31 (see figure 6J). The analysis of immunohistochemical staining revealed that

the presence of IgG4wt and nivolumab impeded the infiltration of T cells, specifically CD8⁺ T cells, into the tumor, promoted the polarization of macrophages towards the M2 phenotype, and increased the level of vascularization within the tumor. In contrast, no notable differences were observed among the IgG1wt, penpulimab treated groups, and the control group (figure 6K–P). These results suggest that the S₂₂₈P mutated IgG4-type PD-1 mAb maintains the immunosuppressive properties of IgG4wt.

DISCUSSION

This study compared strategies for developing cancer immunotherapy drugs using IgG4 S₂₂₈P and Fc-null IgG1 frameworks. The findings show how these strategies can affect cancer treatment, including efficacy, safety, and potential risks of combination therapy with anti-PD-1 mAb-based dual antibodies. Choosing the right IgG subtype is important for the stability of anti-PD-1 mAb and for reducing Fc–Fc interactions related off-target effects in tumor microenvironment. The complex redox environment in cancer often results in elevated levels of GSH,²¹ which is known to promote FAE⁷ and enhance the Fc–Fc interactions¹⁷ of IgG4. The S₂₂₈P mutation in the core hinge maintains the structure of IgG4 anti-PD-1 mAbs and prevents FAE.^{30–31} Analysis showed no half molecule in S₂₂₈P mutated IgG4 and IgG1 mAbs under non-reduced condition. However, exposure to 6 mM GSH resulted in the persistence of half molecules in the IgG4 S₂₂₈P anti-PD-1 mAbs, as opposed to IgG1 mAbs, indicating a potential variance in structural stability among them (online supplemental figure S1). Moreover, the Fc–Fc interactions only required partial dissociation of IgG4 Fc terminus on which S₂₂₈P mutation has little effect.¹⁷ We observed that IgG4 S₂₂₈P mAbs including nivolumab, pembrolizumab, and sintilimab were all able to bind to immobilized IgG via the Fc–Fc interactions and showed inhibitory effects like IgG4wt (figure 2A,E, online supplemental figure S2). Such interactions showed consistency in both denatured (Western blot) and non-denatured conditions (ELISA) as demonstrated in figure 2F. The IgG4 S₂₂₈P mutant (nivolumab) and IgG4wt showed comparable binding affinity (figure 2G). Notably, the IgG4 S₂₂₈P-R₄₀₉K mutant anti-PD-1 is similar to IgG1 particularly in their ability for Fc–Fc interactions and FAE.^{17–28–32–33} Our tests confirmed that both penpulimab (Fc-null IgG1) and tislelizumab (Fc-null IgG4 S₂₂₈P-R₄₀₉K mutant) are lack of Fc–Fc interactions (figure 2F) and do not impede the ADCC effect facilitated by IgG1 antibody in cellular tests (online supplemental figure S2D). They also demonstrate comparable structural stability when exposed to GSH (online supplemental figure S1A). Further analysis may be needed to verified whether the two structures are completely equivalent in their actions. The synchronous abundant distribution of GSH and IgG in various cancer tissues (figure 1) suggests that IgG4 is prone to Fc–Fc interactions in the tumor microenvironment as illustrated in figure 2D. Previously we demonstrated

that IgG1 derived from tumor patients specifically binds to their own tumor tissues thereby becomes the immobilized target for IgG4 to bind to in an Fc–Fc manner.¹⁸ The presence of GSH and immobilized IgG could also promote the Fc–Fc interactions of IgG4 S₂₂₈P anti-PD-1 mAbs to immobilized IgG. This interaction may hinder antitumor immune responses and promote tumor growth as we observed in the animal models (figure 6), while potentially causing off-target effects, resistance to immunotherapy, and immune evasion for IgG4-type anti-PD-1 mAbs in clinical therapy. This could lead to side effects such as HPD. HPD manifests as accelerated tumor growth with poor prognosis seen in patients after immune checkpoint inhibitor therapy at an incidence ranging from 6% to 43%.¹⁶ Researches have linked HPD to dysregulated tumor signaling pathways^{34–35} and mutations in genes like *KRAS*, *STK11/LKB1*,^{36–37} *MDM2/MDM4*, and *EGFR* in patients on PD-1 blockade.³⁵ These mutations were also found affecting synthesis and metabolism of GSH in tumor microenvironment, leading to increased GSH levels.^{37–40} Tumors that possess these mutations may exhibit resistance to IgG4 S₂₂₈P anti-PD-1 mAb immunotherapy by promoting the dissociation of IgG4 and Fc–Fc interactions through increased levels of GSH.

Contrary to the prevalent occurrence of HPD, the efficacy of anti-PD-1 mAb immunotherapy in diverse cancers is moderate. Only about 30% of patients exhibited favorable responses to these treatments, and even fewer experienced sustained benefits.⁴¹ Most patients ultimately face disease progression, suggesting the emergence of potential secondary resistance mechanisms.^{41–42} Various approaches are currently being tested to overcome these challenges, with one extensively researched strategy involving enhancing efficacy through the combination of anti-PD-1 mAb with other IgG1 mAbs. However, previous studies had found a higher prevalence of HPD in patients undergoing combination immunotherapy as opposed to monotherapy (15.4% vs 4.4%),⁴³ with the proportion up to 30%.⁴⁴ While the underlying mechanisms remain unclear, the Fc–Fc interactions could be a potential contributing factor. Our cellular tests showed that choosing the IgG framework is crucial for the effectiveness and safety of combination therapies involving “anti-PD-1 mAb plus antitumor IgG1 mAb.” Administrating IgG4 S₂₂₈P anti-PD-1 mAb with antitumor IgG1 mAb may lead to Fc–Fc interactions, particularly in the presence of increased GSH levels in the tumor microenvironment. This can reduce the antitumor effectiveness of the IgG1 antibody and increase off-target effects of the anti-PD-1 mAb. Fc-null IgG1 anti-PD-1 mAb could be a safer option for these combination treatments, as it avoids the detrimental Fc–Fc interaction. Nevertheless, IgG4-type anti-PD-1 mAb can still be effective in cancer treatment. In the humanized PD-1 mouse model (figure 6), nivolumab demonstrated favorable efficacy and was not significantly inferior to penpulimab. The mice were in good condition with robust immune reserves, akin to patients with early stage cancer. Under such condition, the inhibitory effect

of Fc–Fc interactions are likely to be overshadowed by the immune-promoting effect brought by PD-1 blockade, thereby slightly weakens its efficacy. Previous studies^{45 46} indicated that patients with advanced tumor stage, poor physical condition, and old age (>65 years) were prone to HPD after treatment with anti-PD-1 mAbs (typically nivolumab or pembrolizumab). For advanced cancer patients with lower immune capacity, the beneficial effect of PD-1 blockade is limited while the immunosuppression of IgG4 anti-PD-1 mAbs Fc–Fc interactions still exists, and even is enhanced by elevated GSH.^{19 47} The Fc–Fc interactions may affect its efficacy and potentially lead to tumor progression in such conditions. However, due to animal welfare issues caused by excessive tumor burden, we were unable to use this model to simulate advanced tumors patients with poor physical condition.

IgG4 also exerts inhibitory effects through interactions between its Fc region and FcRs on various human immune cells and showed a dose-dependent trend, as demonstrated in our observations. Although the tests are similar to, but not exactly the same as, the in vivo environment, where other IgG subtypes may compete with endogenous IgG4 for binding to FcRs, potentially reducing its immunosuppressive effectiveness. However, studies^{10 11 18 19 48 49} have shown that IgG4 levels within the tumor microenvironment are typically higher than normal (approximately 0.5 mg/mL) and contribute to an immunosuppressive environment. Injectable PD-1 mAbs are typically administered at doses of 200–480 mg every 2–3 weeks with terminal half-life of about 27 days.⁵⁰ The cumulative presence of IgG4 mAbs from repeated injections could elevate the total IgG4 levels in the patient's system and lead to a concentration-dependent inhibitory effects in vivo. These effects were evident in the Fc-functional IgG4 S₂₂₈P anti-PD-1 mAb, leading to the inhibition of its functional activity. IgG4 has a high affinity to FcγRIIB, and a recent study found that FcγRIIB positive CD8⁺ T cells exhibited reduced responsiveness to Fc-functional anti-PD-1 mAb. This reduced responsiveness can be reversed by devoicing the Fc of anti-PD-1 mAb or blocking the FcγRIIB.¹⁵ Our tests showed that IgG4wt administration can increase PD-1 and CTLA4 in CD8⁺T cells and using an FcR blocker can prevent IgG4 from binding to immune cells. This indicates that IgG4 may affect immune suppressive markers and cause CD8⁺ T cell dysfunction through Fc:FcR interactions. The amplification of PD-1⁺/CTLA4⁺ T cell clusters promoted by nivolumab was proposed to be a mechanism to explain HPD in PD-1/PD-L1 blockage therapy.⁵¹ Several studies have revealed that the simultaneous inhibition of PD-1 and CTLA4 can effectively reverse the dysfunction of CD8⁺ tumor-infiltrating T cells, characterized by their impaired capacity to proliferate and secrete effector cytokines, ultimately resulting in tumor rejection,⁵² and promising outcomes have been reported in preliminary trials.^{53 54} Additionally, IgG4 prompts the polarization of macrophages into immunosuppressive M2 phenotypes, leading to the release of inhibitory cytokines (figure 5C,D, figure 6J), which is consistent with previous

studies^{10 11 55} and is applicable to IgG4 S₂₂₈P PD-1 mAbs. Previous studies have shown that nivolumab's Fc binding to FcRs on tumor-associated macrophages (TAM) leads to autoimmune phagocytic effects¹³ and off-target effects,¹² contributing to HPD development. HPD patients and xenograft mouse models treated with nivolumab¹³ have shown an abundance of TAMs with an immunosuppressive M2 phenotype. In contrast, the Fc-null IgG1 framework has proven to be an effective strategy to avoid FcR binding for anti-PD-1 mAb,²⁶ and Fc-null anti-PD-1 mAb deliver optimal clinical benefit by enhancing endogenous CD8⁺ T-cell expansion.¹⁴ Therefore, current anti-PD-1 mAbs with IgG4 S₂₂₈P framework may have an immunoregulatory effect in the tumor microenvironment, potentially leading to resistance to PD-1 blockade. This was further evidenced by findings of the in vivo experiments. We observed that both IgG4wt and nivolumab promoted tumor growth, suppressed CD8⁺ T cell infiltration, and induced M2-like phenotype macrophage (figure 6).

There are also limitations in our study. First, the Fc–Fc response in vivo cannot be intuitively observed and quantified, and it is still unclear what proportion of IgG4 is involved in these Fc–Fc interactions in vivo. This could be unveiled with an in vivo fluorescence imaging platform in combination immunotherapy animal models. Furthermore, due to the limitations of animal models, we were unable to compare the effects of IgG1 and IgG4 PD-1 mAbs in advanced tumor animal model, and the effect of Fc–Fc response on the efficacy of PD-1 mAbs is likely to be very important for the treatment of advanced tumor patients. In addition, the Fc–Fc interactions tested in vitro cannot completely simulate the in vivo environment. Fc–Fc interactions are affected by many factors, including but not limited to GSH concentration, pH, etc. Further tests are necessary to provide more definite answers.

Overall, this study provides a comprehensive comparison of PD-1 mAbs in the IgG4 S₂₂₈P and Fc-null IgG1 as well as Fc-null IgG4 S₂₂₈P-R₄₀₉K frameworks, with a specific emphasis on the differing Fc–Fc interactions capabilities and their potential implications for immunotherapeutic applications based on the findings of previous reports.^{18 19} Additionally, we also studied the direct impacts of IgG1 and IgG4 on various immune cells, given that the two antibodies under investigation were designed based on their frameworks and with their inherent properties. These findings reveal the mechanisms by which IgG4 inhibits classical immune responses, promotes an immunosuppressive microenvironment, and enables tumor immune evasion. This study proposes a novel mechanism of HPD in the context of the persistent IgG4 Fc–Fc interactions observed in the traditional IgG4 S₂₂₈P anti-PD-1 mAb as depicted in figure 7. This additional mechanism helps to understand how immunotherapy resistance and HPD are influenced by the Fc fragment of anti-PD-1 mAb, providing insights into possible alternative mechanisms. The Fc-null IgG1 framework appears to present several advantages in comparison to the traditional IgG4 framework for developing anti-PD-1 mAb. These advantages

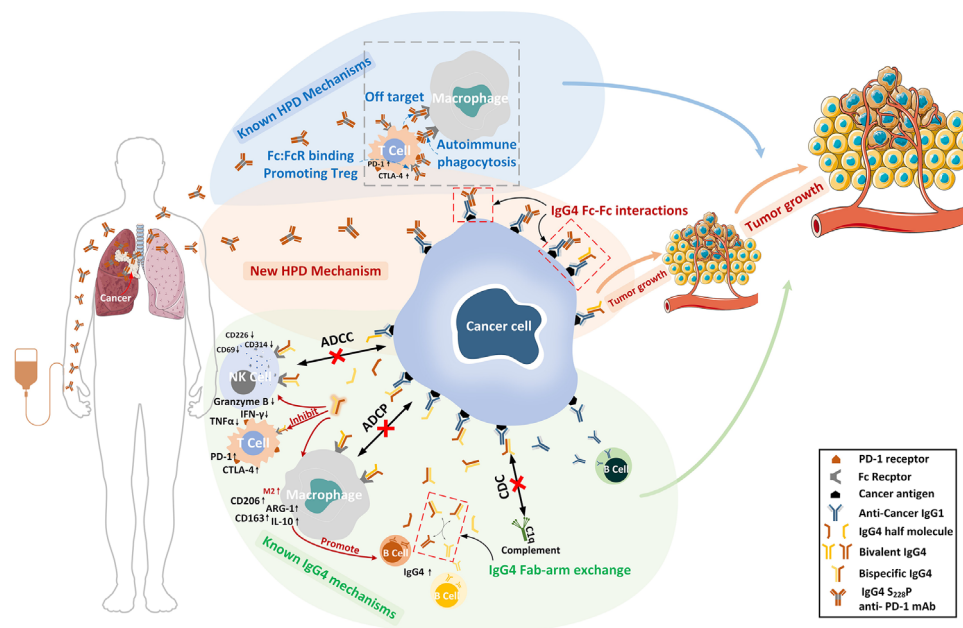


Figure 7 The proposed mechanisms of IgG4 and IgG4-type anti-PD-1 mAb suppress the immune reactions and promote tumor immune escape. Initially, B cells produce bivalent IgG4 antibodies that can be exchanged to form bispecific IgG4. In the tumor microenvironment, IgG4 can react with anticancer IgG antibodies through Fc–Fc interactions and lead to the blocking of ADCC, ADCP, and CDC effects, promoting tumor growth.^{18,19} Additionally, IgG4 inhibits CD8+ T cells and natural killer cells, impeding their ability to target and eliminate tumor cells. It also promotes M2 macrophage polarization, creating an immunosuppressive tumor environment. The Fc-functional IgG4 S₂₂₈P anti-PD-1 mAb retains Fc–Fc interactions and Fc:FcR interaction like wildtype IgG4. In vivo, this mutated mAb may block anticancer IgG-mediated immune response through Fc–Fc interactions, promoting tumor growth. We proposed this Fc–Fc interactions of IgG4 S₂₂₈P anti-PD-1 mAb as a new and additional mechanism potentially causing hyper-progressive disease. This severe side effect has been reported in previous studies to be triggered by the Fc:FcR interaction among IgG4-type anti-PD-1 mAb and the Fc receptor of macrophages and T cells.^{12–15,51} ADCC, antibody-dependent cellular cytotoxicity; ADCP; antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; FcR, Fc receptor; HPD, hyperprogressive disease; IFN- γ , interferon γ ; IL, interleukin; mAb, monoclonal antibody; PD-1, programmed cell-death 1; CTLA-4, Cytotoxic T lymphocyte antigen-4; TNF α , tumor necrosis factor α .

include the absence of Fc–Fc interactions preventing the antibody from binding to other immobilized IgG, thereby avoiding off-target effects and attenuating the immune response of antitumor antibodies. Furthermore, the elimination of Fc:FcR interactions would prevent off-target effects and autoimmune phagocytosis injury, as well as inhibitory effects on immune cells mediated by Fc:FcR binding. Furthermore, the Fc-null IgG1 framework exhibits a more stable structural form with improved solution stability²⁶ and the ability to withstand higher redox stress in the tumor microenvironment. Moreover, Fc-null IgG1 and Fc-null IgG4 S₂₂₈P-R₄₀₉K showed almost equivalent structural properties during testing. Using Fc-null IgG frameworks without Fc–Fc interactions (ie, either adopting IgG1 backbone or IgG4 S₂₂₈P-R₄₀₉K mutant) could be safer and more effective choices for developing anti-PD-1 immunotherapy mAbs, reducing side effects associated with traditional IgG4 framework with greater potential therapeutic benefits. This study highlights the importance of taking Fc–Fc interactions into consideration in designing cancer immunotherapy strategies.

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Contributors WZ: conceptualization, writing original draft, visualization, in vitro and in vivo experiments, methodology and data analysis; XuellingC: protein electrophoresis, Western blot, papain digestion, T cell tests, flow cytometry, and RT-PCR experiments, methodology and data analysis; XingxingC: cellular and mouse model experiments and data analysis; JL: immunohistochemistry, tissue sample preprocessing, antibody preabsorption tests; HW: monocytes experiments, flow cytometry; XY: data curation; HZ: investigation, review and editing; XM: NK and macrophage experiments; CZ: papain digestion; MS, LH, PL, YL, WZ: data curation, writing–review and editing; YX, BL, TZ: production of multiple versions of penpulimab and IgG proteins, manuscript review and editing; JG: conceptualization, funding acquisition, supervision, project administration, writing review and editing. All authors contributed to the final manuscript and approved the submission with the intent to publish. JG: guarantor.

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Competing interests TZ is an employee of Chia Tai Tianqing Pharmaceutical Group and YX and BL are employees of Akeso Biopharma.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Ethics Committee of Shantou University Medical College (Reference Number: SUMC-2021-09). Participants gave informed consent to participate in the study before taking part. The animal study was reviewed and approved by the Animal Care and Use Committee of the Shantou University Medical College (Reference Number: SUMC2023-151) and complied with the ARRIVE guidelines.⁵⁶

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Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

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REFERENCES

- Constantinidou A, Alifiridis C, Trafalis DT. Targeting programmed cell death -1 (PD-1) and ligand (PD-L1): a new era in cancer active immunotherapy. *Pharmacol Ther* 2019;194:84–106.
- Sznol M, Chen L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. *Clin Cancer Res* 2013;19:1021–34.
- Dumet C, Pottier J, Gouilleux-Gruart V, et al. Insights into the IgG heavy chain engineering patent landscape as applied to IgG4 antibody development. *MAbs* 2019;11:1341–50.
- Chen X, Song X, Li K, et al. Fcγ-macrophage binding is an important functional attribute for immune checkpoint antibodies in cancer immunotherapy. *Front Immunol* 2019;10:292.
- Aalberse RC, Dieges PH, Knul-Bretlova V, et al. IgG4 as a blocking antibody. *Clin Rev Allergy* 1983;1:289–302.
- Bianchini R, Karagiannis SN, Jordakieva G, et al. The role of IgG4 in the fine tuning of tolerance in IGE-mediated allergy and cancer. *Int J Mol Sci* 2020;21:5017.
- Rispens T, Ooijsaar-de Heer P, Bende O, et al. Mechanism of immunoglobulin G4 Fab-arm exchange. *J Am Chem Soc* 2011;133:10302–11.
- Silva J-P, Vetterlein O, Jose J, et al. The S228P Mutation prevents in vivo and in vitro IgG4 Fab-arm exchange as demonstrated using a combination of novel quantitative immunoassays and physiological matrix preparation. *J Biol Chem* 2015;290:5462–9.
- Karagiannis P, Gilbert AE, Josephs DH, et al. IgG4 Subclass antibodies impair antitumor immunity in melanoma. *J Clin Invest* 2013;123:1457–74.
- Bianchini R, Roth-Walter F, Ohradanova-Repic A, et al. IgG4 drives M2A macrophages to a regulatory M2B-like phenotype: potential implication in immune tolerance. *Allergy* 2019;74:483–94.
- Jordakieva G, Bianchini R, Reichhold D, et al. IgG4 induces tolerogenic M2-like macrophages and correlates with disease progression in colon cancer. *Oncoimmunology* 2021;10:1880687.
- Arlaukas SP, Garris CS, Kohler RH, et al. In vivo imaging reveals a tumor-associated macrophage-mediated resistance pathway in anti-PD-1 therapy. *Sci Transl Med* 2017;9:eaa13604.
- Lo Russo G, Moro M, Sommariva M, et al. Antibody-FC/FCR interaction on Macrophages as a mechanism for Hyperprogressive disease in non-small cell lung cancer subsequent to PD-1/PD-L1 blockade. *Clin Cancer Res* 2019;25:989–99.
- Moreno-Vicente J, Willoughby JE, Taylor MC, et al. Fc-null anti-PD-1 Monoclonal antibodies deliver optimal checkpoint blockade in diverse immune environments. *J Immunother Cancer* 2022;10:e003735.
- Bennion KB, Tariq M, Wyatt MM, et al. Fcγ-macrophage binding expressed on Cd8(+) T cells limits responsiveness to PD-1 checkpoint inhibition in cancer. *Sci Transl Med* 2023;15:eadd1868.
- Park HJ, Kim KW, Won SE, et al. Definition, incidence, and challenges for assessment of hyperprogressive disease during cancer treatment with immune Checkpoint inhibitors: a systematic review and meta-analysis. *JAMA Netw Open* 2021;4:e211136.
- Rispens T, Meesters J, den Bleker TH, et al. Fc-FC interactions of human IgG4 require dissociation of heavy chains and are formed predominantly by the intra-chain hinge isomer. *Mol Immunol* 2013;53:35–42.
- Wang H, Xu Q, Zhao C, et al. An immune evasion mechanism with IgG4 playing an essential role in cancer and implication for immunotherapy. *J Immunother Cancer* 2020;8:e000661.
- Zhang W, Quan Y, Ma X, et al. Synergistic effect of glutathione and IgG4 in immune evasion and the implication for cancer immunotherapy. *Redox Biology* 2023;60:102608.
- Zhao J, Peng H, Gao J, et al. Current insights into the expression and functions of tumor-derived immunoglobulins. *Cell Death Discov* 2021;7:148.
- Gamsik MP, Kasibhatla MS, Teeter SD, et al. Glutathione levels in human tumors. *Biomarkers* 2012;17:671–91.
- Janjigian YY, Kawazoe A, Yañez P, et al. The KEYNOTE-811 trial of dual PD-1 and Her2 blockade in Her2-positive gastric cancer. *Nature* 2021;600:727–30.
- Nastoupil LJ, Chin CK, Westin JR, et al. Safety and activity of Pembrolizumab in combination with Rituximab in relapsed or refractory follicular lymphoma. *Blood Adv* 2022;6:1143–51.
- Chung CH, Li J, Steuer CE, et al. Phase II multi-institutional clinical trial result of concurrent cetuximab and Nivolumab in recurrent and/or metastatic head and neck squamous cell carcinoma. *Clin Cancer Res* 2022;28:2329–38.
- Baas P, Scherpereel A, Nowak AK, et al. First-line Nivolumab plus Ipilimumab in unresectable malignant pleural mesothelioma (Checkmate 743): a Multicentre, randomised, open-label, phase 3 trial. *Lancet* 2021;397:375–86.
- Huang Z, Pang X, Zhong T, et al. Penpulimab, an FC-engineered IgG1 anti-PD-1 antibody, with improved efficacy and low incidence of immune-related adverse events. *Front Immunol* 2022;13:924542.
- Song Y, Gao Q, Zhang H, et al. Treatment of relapsed or refractory classical Hodgkin lymphoma with the anti-PD-1, tislelizumab: results of a phase 2, single-arm, multicenter study. *Leukemia* 2020;34:533–42.
- Labrijn AF, Rispens T, Meesters J, et al. Species-specific determinants in the IgG Ch3 domain enable Fab-arm exchange by affecting the noncovalent Ch3-Ch3 interaction strength. *J Immunol* 2011;187:3238–46.
- Tsao L-C, Crosby EJ, Trotter TN, et al. Trastuzumab/Pertuzumab combination therapy stimulates antitumor responses through complement-dependent cytotoxicity and phagocytosis. *JCI Insight* 2022;7:e155636.
- Labrijn AF, Buijsse AO, van den Bremer ETJ, et al. Therapeutic IgG4 antibodies engage in Fab-arm exchange with endogenous human IgG4 in vivo. *Nat Biotechnol* 2009;27:767–71.
- Yang X, Wang F, Zhang Y, et al. Comprehensive analysis of the therapeutic IgG4 antibody pembrolizumab: hinge modification blocks half molecule exchange in vitro and in vivo. *J Pharm Sci* 2015;104:4002–14.
- Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 2014;5:520.
- van der Neut Kolfschoten M, Schuurman J, Losen M, et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science* 2007;317:1554–7.
- Wang X, Wang F, Zhong M, et al. The biomarkers of hyperprogressive disease in PD-1/PD-L1 blockade therapy. *Mol Cancer* 2020;19:81.
- Kato S, Goodman A, Walavalkar V, et al. Hyperprogressors after immunotherapy: analysis of genomic alterations associated with accelerated growth rate. *Clin Cancer Res* 2017;23:4242–50.
- Kim Y, Kim CH, Lee HY, et al. Comprehensive clinical and genetic characterization of hyperprogression based on volumetry in advanced non-small cell lung cancer treated with immune checkpoint inhibitor. *J Thorac Oncol* 2019;14:1608–18.
- Galan-Cobo A, Sitthideatphai boon P, Qu X, et al. LKB1 and KEAP1/NRF2 pathways cooperatively promote metabolic Reprogramming

- with enhanced glutamine dependence in KRAS-mutant lung adenocarcinoma. *Cancer Res* 2019;79:3251–67.
- 38 Zulato E, Ciccarese F, Agnusdei V, *et al.* Lkb1 loss is associated with glutathione deficiency under oxidative stress and sensitivity of cancer cells to cytotoxic drugs and gamma-irradiation. *Biochem Pharmacol* 2018;156:479–90.
 - 39 Riscal R, Schrepfer E, Arena G, *et al.* Chromatin-bound MDM2 regulates Serine metabolism and redox homeostasis independently of P53. *Mol Cell* 2016;62:890–902.
 - 40 Hu K, Li K, Lv J, *et al.* Suppression of the SLC7A11/glutathione axis causes synthetic lethality in KRAS-mutant lung adenocarcinoma. *J Clin Invest* 2020;130:1752–66.
 - 41 Pérez-Ruiz E, Melero I, Kopecka J, *et al.* Cancer Immunotherapy resistance based on immune checkpoints inhibitors: targets, biomarkers, and remedies. *Drug Resist Updat* 2020;53:100718.
 - 42 Kim TK, Vandsemb EN, Herbst RS, *et al.* Adaptive immune resistance at the tumour site: mechanisms and therapeutic opportunities. *Nat Rev Drug Discov* 2022;21:529–40.
 - 43 Matos I, Martin-Liberal J, García-Ruiz A, *et al.* Capturing hyperprogressive disease with immune-checkpoint inhibitors using RECIST 1.1 criteria. *Clin Cancer Res* 2020;26:1846–55.
 - 44 Kanjanapan Y, Day D, Wang L, *et al.* Hyperprogressive disease in early-phase immunotherapy trials: clinical predictors and association with immune-related toxicities. *Cancer* 2019;125:1341–9.
 - 45 Arasanz H, Zuazo M, Bocanegra A, *et al.* Hyperprogressive disease: main features and key controversies. *Int J Mol Sci* 2021;22:3736.
 - 46 Shen P, Han L, Ba X, *et al.* Hyperprogressive disease in cancers treated with immune checkpoint inhibitors. *Front Pharmacol* 2021;12:678409.
 - 47 Kennedy L, Sandhu JK, Harper M-E, *et al.* Role of glutathione in cancer: from mechanisms to therapies. *Biomolecules* 2020;10:1429.
 - 48 Rispens T, Huijbers MG. The unique properties of IgG4 and its roles in health and disease. *Nat Rev Immunol* 2023;23:763–78.
 - 49 Crescioli S, Correa I, Karagiannis P, *et al.* IgG4 characteristics and functions in cancer immunity. *Curr Allergy Asthma Rep* 2016;16:7.
 - 50 Yan T, Yu L, Shangguan D, *et al.* Advances in pharmacokinetics and pharmacodynamics of PD-1/PD-L1 inhibitors. *Int Immunopharmacol* 2023;115:109638.
 - 51 Kamada T, Togashi Y, Tay C, *et al.* PD-1(+) regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. *Proc Natl Acad Sci U S A* 2019;116:9999–10008.
 - 52 Duraiswamy J, Kaluza KM, Freeman GJ, *et al.* Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. *Cancer Res* 2013;73:3591–603.
 - 53 Mai H, Lin S, Chen D, *et al.* 436 A phase II study of Ak104, a Bispecific antibody targeting PD-1 and CTLA-4, in patients with metastatic Nasopharyngeal carcinoma (NPC) who had progressed after two or more lines of chemotherapy. *J Immunother Cancer* 2021;9:A466.
 - 54 Ji J, Shen L, Li Z, *et al.* Ak104 (PD-1/CTLA-4 Bispecific) combined with chemotherapy as first-line therapy for advanced gastric (G) or gastroesophageal junction (GEJ) cancer: updated results from a phase IB study. *JCO* 2021;39:232.
 - 55 Swisher JFA, Haddad DA, McGrath AG, *et al.* IgG4 can induce an M2-like phenotype in human monocyte-derived Macrophages through Fcγγmari. *MAbs* 2014;6:1377–84.
 - 56 Percie du Sert N, Ahluwalia A, Alam S, *et al.* Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biol* 2020;18:e3000411.