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Synergistic effect of glutathione and IgG4 in immune evasion and the implication for cancer immunotherapy☆

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ABSTRACT

Background: We recently reported a novel IgG4-centered immune evasion mechanism in cancer, and this was achieved mostly through the Fc-Fc reaction of increased IgG4 to cancer-bound IgG in cancer microenvironment. The mechanism was suggested to be related to cancer hyperprogressive disease (HPD) which is a side-effect often associated to IgG4 subtype PD-1 antibody immunotherapy. HPD was reported to occur in cancers with certain mutated genes including *KRAS* and such mutations are often associated to glutathione (GSH) synthesis. Therefore, we hypothesize that IgG4 and GSH may play a synergistic role in local immunosuppression of cancer.
Methods: Quantitatively analyzed the distribution and abundance of GSH and IgG4 in human cancer samples with ELISA and immunohistochemistry. The interactions between GSH and IgG4 were examined with Electrophoresis and Western Blot. The synergistic effects of the two on classic immune responses were investigated in vitro. The combined effects were also tested in a lung cancer model and a skin graft model in mice.
Results: We detected significant increases of both GSH and IgG4 in the microenvironment of lung cancer, esophageal cancer, and colon cancer tissues. GSH disrupted the disulfide bond of IgG4 heavy chain and enhanced IgG4's ability of Fc-Fc reaction to immobilized IgG subtypes. Combined administration of IgG4 and GSH augmented the inhibitory effect of IgG4 on the classic ADCC, ADCP, and CDC reactions. Local administration of IgG4/GSH achieved the most obvious effect of accelerating cancer growth in the mouse lung cancer model. The same combination prolonged the survival of skin grafts between two different strains of mouse. In both models, immune cells and several cytokines were found to shift to the state of immune tolerance.
Conclusion: Combined application of GSH and IgG4 can promote tumor growth and protect skin graft. The mechanism may be achieved through the effect of the Fc-Fc reaction between IgG4 and other tissue-bound IgG subtypes resulting in local immunosuppression. This reaction was facilitated by increased GSH to dissociate the two heavy chains of IgG4 Fc fragment at its disulfide bonds. Our findings unveiled the interaction between the redox system and the immune systems in cancer microenvironment. It offers a sensible explanation for HPD and provides new possibilities for manipulating this mechanism for cancer immunotherapy.

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1. Introduction

Both the redox system and the immune systems are known to regulate cancer growth and affect cancer therapy. However, their relationship and interactions have not been well understood. IgG4 is the latest discovered immunoglobulin (Ig) subtype and constitutes the lowest concentration (3–5%) among the subtypes of IgG in the serum of healthy individuals [1]. It is the only antibody that can undergo Fab arm exchange (FAE) to form a new bispecific antibody with each half of the Fab recognizing one antigen [2,3]. This reaction between two IgG4 molecules occurs only in the liquid phase. In the solid phase, e.g., when one IgG is bound to tissue antigens and therefore is immobilized, the above FAE could not be completed due to space constraint [3] and result in IgG4 binding to tissue-bound IgG in a form of Fc-Fc reaction, i.e., IgG4 can bound to the Fc fragment of other immobilized IgG with its Fc fraction instead the antigen recognizing variable Fab fragment. This unique reaction blocks the ability of tissue-bound IgG to react to immune effector cells and complements. Therefore, IgG4 has been named "blocking antibody" [4]. It has been reported that IgG4 was significantly increased in cancer microenvironment and serum [5,6]. IgG4 could block the classic antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement dependent cytotoxicity (CDC) immune responses leading to immune evasion [7]. As cancer cells present a chronic antigen stimulant, they could induce increased IgG4 production by B lymphocytes and inhibit local immunity against cancer. The increase of IgG4 was a protective mechanism of the body to prevent chronic inflammation caused by prolonged antigen stimulation that may result in more damage to the body than the antigen itself [8].

Another feature of the cancer environment is the elevation of glutathione (GSH) levels. GSH, the most abundant thiol antioxidant in cells, is capable of preventing damage to important cellular components caused by reactive oxygen species [9]. Many chronic and age-related diseases are associated with decreased cellular GSH levels [9]. Elevated GSH levels have been observed in various cancer types and this helps to combat the constitutive oxidative stress in cancer cells and causes chemotherapy resistance [10]. Cancers with mutated genes including *KRAS, STK11, MDM2/MDM4* and *EGFR* tend to develop hyperprogressive disease (HPD) [11–13], whereas, these genes are known to be closely related to GSH synthesis and metabolism [11, 14–16]. HPD is a severe side-effect of cancer immunotherapy affecting about 15-20% of cancer patients receiving immunotherapy in which cancer cells have accelerated growth following therapy [17]. It has been speculated that HPD was caused by the binding of the Fc fragment of PD-1 antibody (an IgG4 protein itself) to the Fc receptor on macrophages [18]. Previously, we found that a S228P mutated IgG4 protein, a monoclonal antibody to PD-1 (Nivolumab), could facilitate Fc-Fc reaction like other IgG4 molecules and promote tumor growth in a colon cancer animal model by inhibiting local immune response [5].

The unique reactions of IgG4 to other IgG molecules including FAE and Fc-Fc reactions were enhanced by the presence of elevated GSH in vitro [3,19]. Therefore, a synergistic effect of GSH and IgG4 in inhibiting cancer immunity is likely and potentially important for cancer immunotherapy, but such a mechanism has not been investigated. We hypothesize that a simultaneous increase of GSH and IgG4 in cancer enhances the inhibitory effect of local immune reaction against cancer cells and leads to cancer growth. Because tumor and skin grafts share a similar microenvironment in immune response toward allogenic antigens, their fates are affected by local immunity. We recently reported an IgG4-driven mechanism of immune evasion unveiling a new and important aspect of cancer immunity [5]. In this study, we investigated the possible synergistic effect of GSH and IgG4 on immune evasion with a mouse lung cancer and a mouse skin graft model as well as with experiments in vitro. Interaction between redox and immune systems in inducing local immune inhibition was found.

2. Materials and methods

2.1. Key resources

Detailed information including antibodies, biological samples chemicals, assay kits, cell lines, and software is shown in Supplemental Table 1.

2.2. Patients and tumor tissues

Paraffin-embedded and frozen tumor tissues and matched normal tissues (n = 15/each type of cancer) from patients with esophageal, lung and colon cancer were retrospectively obtained from Shantou University Affiliated Tumor Hospital.

2.3. Technical protocols

On human and animal tissue samples, extracted proteins and molecules we performed protein affinity chromatography, non-reducing SDS–PAGE, Total Glutathione Assay, Western blot, ADCC, ADCP, CDC, cytokine assays, immunohistochemistry, multiple immunofluorescences, and ELISA. Detailed protocols are described in the supplements.

2.4. Mouse lung cancer model

All animals were obtained from Vital River Technical Co., LTD (Beijing, China) and maintained under specific pathogen-free (SPF) conditions. in the Experimental Animal Center of Shantou University Medical College. All animal experiments complied with the ARRIVE guidelines [20]. The sample size was guided by previous experience and preliminary data. Twenty-four (24) female C57BL/6 N mice aged between 6 and 8weeks and weight 20±2g were employed. Inoculated with Lewis Lung Cancer (LLC) cells subcutaneously at the right chest, 5×10^5 cells per mouse. One week after cell inoculation, the model was built and mice were divided randomly into four treatment groups (n = 6/group). Subcutaneously injected with corresponding protein or reagent in and around the tumor mass, including PBS, GSH, IgG4, and IgG4 + GSH. (2 mg/mL of protein, with or without 3 mM GSH in 100 μL PBS). All intervention reagents were pre-incubated in 37°C for 3 h before injection so that GSH could dissociate IgG4. The tumor size was measured three times per week with calipers, and tumor volume was calculated with the following formula: V (mm^3) = 1/2 ab^2 (a is the longest diameter of the tumor, and b is the length perpendicular to the longest diameter). The experiment was terminated on Day 24 after cancer cell inoculation.

2.5. Mouse skin graft model

Female C57BL/6 N and BALB/c mice aged 6∼8weeks and weight 20 ±2g were used. Back skin graft surgery was performed based on a previously reported protocol [21]. C57BL/6 N mice were used as skin donor and BALB/c mice as recipients. After transplantation, mice were divided randomly into 4 groups (n = 11/group). Each group was injected with a corresponding protein or reagent, including PBS, GSH, IgG4, and IgG4 + GSH (2 mg/mL of protein, with or without 3 mM GSH in 100 μL PBS). All intervention reagents were pre-incubated before injection in 37°C for 3 h so that GSH could dissociate IgG4. The grafts were observed daily and the experiments were terminated on Day 13.

2.6. Quantification and statistical analysis

Data were analyzed with GraphPad Prism. Statistical differences between groups were assessed with unpaired two-tailed Student's t-test or one-way analysis of variance (Tukey's multiple comparison test). Kaplan-Meier curves were produced from survival experiments and analyzed with log-rank (Mantel-Cox) test. Differences were considered statistically significant when $p<0.05$. Significance was indicated in the

figures as follows: * = p<0.05, ** = p<0.01 *** = p<0.001 and**** = p<0.0001.

3. Results

3.1. IgG4 and GSH were both significantly increased in cancer

The result showed that both IgG4-positive lymphocytes and GSH

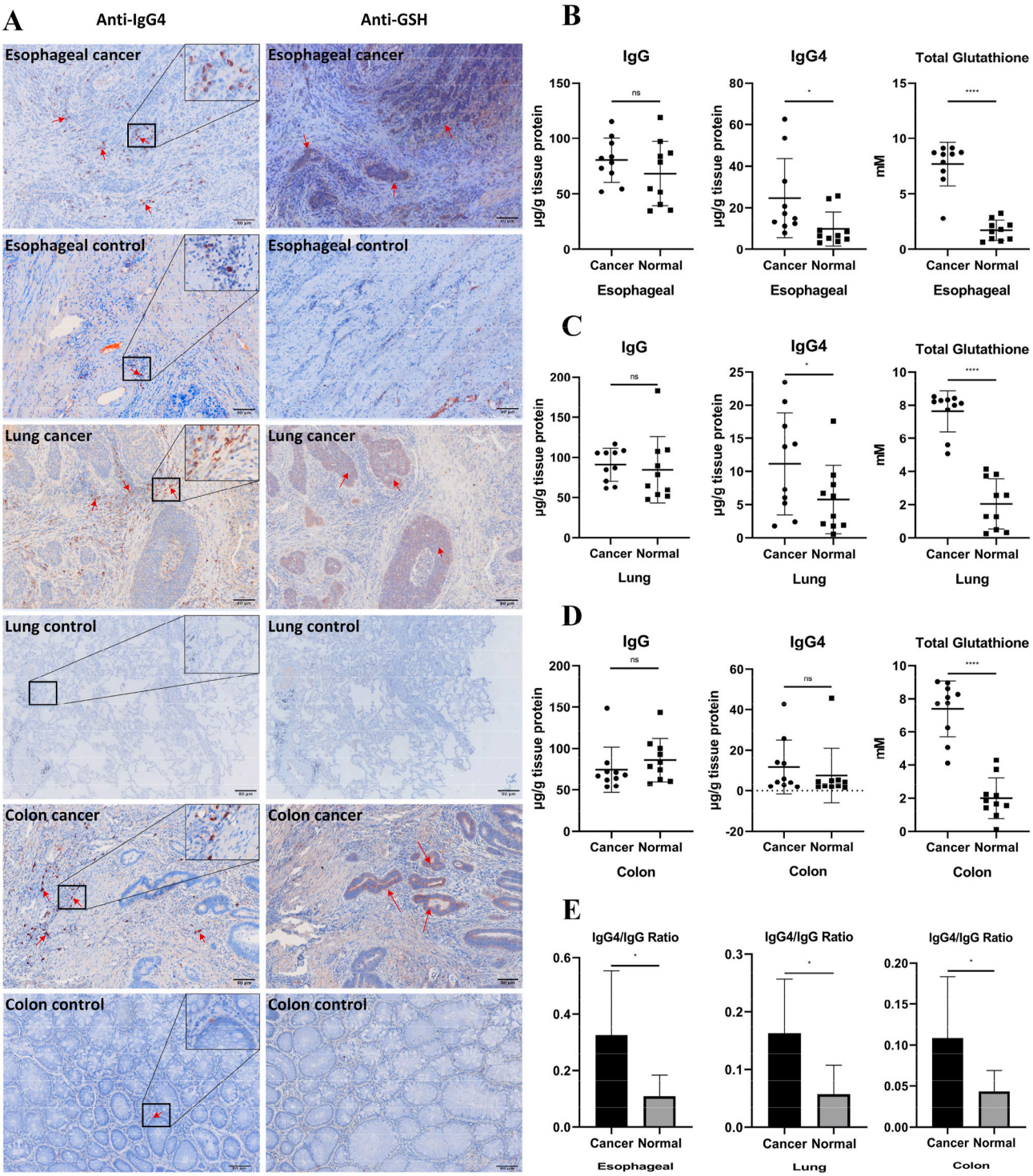

Fig. 1. IgG4 and GSH were significantly increased in tumor tissues of esophageal cancer, lung cancer, and colon cancer.
A. Immunohistochemical staining of IgG4 and GSH in esophageal cancer, lung cancer, colon cancer tissues and matched adjacent normal controls (n = 15/each cancer). A large number of IgG4-positive lymphocytes were found (red arrows) in cancer tissues compared to the adjacent normal controls (left column). GSH was also significantly increased in cancer tissues compared to control tissues (right column). (Magnification, 10 × . Scale bar: 80 μm). B-D. Determination of IgG, IgG4 and GSH content by ELISA (n = 10/each cancer) showed that IgG4 (except colon cancer) and GSH level were higher in cancer tissues than that in normal tissues, with no significant difference in IgG content. E. The IgG4/IgG ratio was significantly higher in cancer tissues compared to the matched normal tissues. * = p < 0.05, **** = p < 0.0001, by two-tailed Student's t-test, ns, not statistically significant.

were significantly increased in esophageal cancer, lung cancer and colon cancer tissues in comparison to the normal tissue controls adjacent to cancer mass (Fig. 1A). Quantitative image analysis indicated that the level of IgG4 in tumor tissues is correlated with the stages of esophageal cancer and lung cancer. The specificity of the immunostaining was verified with specific antigen pre-absorption controls. (Fig. S1). ELISA detection of tissue extract showed that the content of IgG4 (except no statistical difference in colon cancer), GSH and the ratio of IgG4/IgG in three cancer tissues were higher than those in normal tissues. No significant difference was observed in IgG content between cancer and normal tissues (Fig. 1B–E).

3.2. GSH denatured IgG4 and enhanced its Fc-Fc reaction to immobilized IgG in vitro

We added different concentrations of GSH into IgG1 or IgG4 and incubated them at 37°C for 6 h. The GSH was then inactivated with N-Ethylmaleimide (NEM). The samples were then run on an electrophoresis gel under non-reduced conditions. It was found that a 75 KD band appeared at the IgG4 but not the IgG1 channel and the thickness of the bands increased with the increase of GSH concentrations. This indicated that GSH could denature the molecule of IgG4 (MW 150 KD) into two half-molecules (MW 75 KD) in a dose-dependent manner, but IgG1 was not affected by GSH (Fig. 2A).

Previously we demonstrated that unspecific IgG4 could react to IgG that had been run on a nitrocellulose membrane (immobilized IgG) with

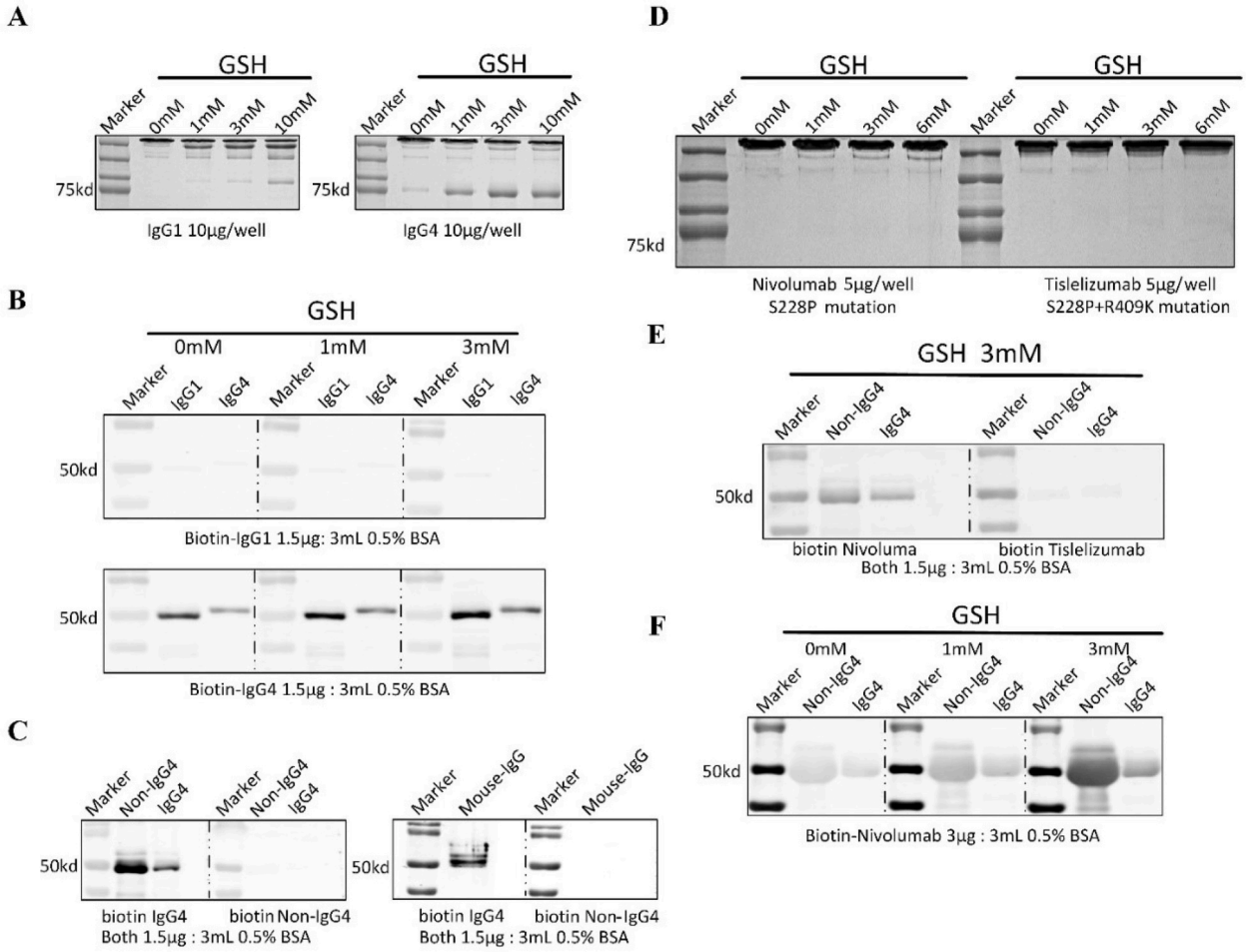

Fig. 2. GSH denatured IgG4 and enhanced its Fc-Fc reaction to immobilized IgG in vitro.
A. Commercial IgG4 (10ug/well) and IgG1(10ug/well) were incubated with different concentrations of GSH (0, 1, 3, 10 mM) at 37 °C for 2 h, and mixed equal volume of 2 × Tris-Glycine SDS sample buffer containing 20 mM N-ethylmaleimide, heated at 70°C for 10 min. The samples (20 μL each) were separated on 10% gels in non-reduced conditions. A molecular weight standard was also loaded and stained with Coomassie brilliant blue. The results showed that the half-molecular band of IgG4 (75kd) gradually increased with the increase of GSH concentrations, but no corresponding reaction was found for IgG1. B. The Fc-Fc reactions in biotin-IgG1/IgG and biotin-IgG4/IgG were detected. Commercial IgG1 and IgG4 were transferred onto nitrocellulose membranes of Western blot and incubated with biotin-IgG1 or biotin-IgG4 primary antibodies respectively, which contained different concentrations of GSH (0, 1, 3 mM). The result showed that the Fc-Fc reaction between biotin-IgG4 and IgG1/IgG4 was enhanced with the increase of GSH concentrations, and the reaction in biotin-IgG4/IgG1 was stronger than biotin-IgG4/IgG4, as shown on the bottom. But Fc-Fc reaction between biotin-IgG1 and IgG1/IgG4 was not detected, as shown on the top. C. Non-IgG4 is the component after IgG4 extraction from human IVIG that contained IgG1, 2, and 3. Human non-IgG4, IgG4, and mouse IgG were transferred onto nitrocellulose membranes of Western blot and incubated with biotin-IgG4 or biotin-non-IgG4 primary antibodies, respectively. It was found that biotin-IgG4 could react with immobilized non-IgG4, IgG4 (shown on the left), and mouse IgG (shown on the right), but biotin-non-IgG4 showed no such reaction D. Half-molecular band of IgG4 (75kd) was not detected in Nivolumab reaction with different concentrations of GSH (shown on the left), neither in Tislelizumab reaction (shown on the right). E. Non-IgG4 and IgG4 transferred onto nitrocellulose membranes were incubated with biotin-Nivolumab or biotin-Tislelizumab pre-treated with 3 mM GSH. Only the Fc-Fc reactions of biotin-Nivolumab with non-IgG4 and IgG4 were detected (shown on the left). F. The Fc-Fc reaction of biotin-Nivolumab with non-IgG4 and IgG4 under different concentrations of GSH was detected. The Fc-Fc reaction was gradually increased with the increase of GSH concentrations. (Nivolumab, S228P mutation IgG4 type PD-1. Tislelizumab, S228P + R409K double mutation IgG4 type PD-1).

its Fc fragment [5]. In this study, we tested the effect of GSH on this reaction. We labeled commercially purchased IgG1 and IgG4 with biotin and used them as the primary antibodies on a Western blot of which unlabeled IgG1 and IgG4 were run. We found that with gradually increased concentrations of GSH (0, 1, 3 mM), the reactions between IgG4 and immobilized IgG1 and IgG4 were also increased. The reaction between IgG4 and immobilized IgG1 was stronger than that between IgG4 and immobilized IgG4, indicating that GSH can indeed enhance the Fc-Fc reaction between IgG4 and immobilized IgG, particularly IgG1 (Fig. 2B). Our results further verified that biotin-IgG4 derived from intravenous immunoglobulin (IVIG) was also able to react with immobilized IgG4 and IgG1 (Fig. 2C).

In order to test the production of half molecules and the Fc-Fc reaction of PD-1 monoclonal antibodies, we tested two clinical PD-1 monoclonal antibodies Nivolumab (IgG4 with the S228P mutation) and Tislelizumab (IgG4 with the S228P + R409K mutations) respectively [22,23]. No half-molecule of IgG4 was produced by adding GSH. However, biotin-Nivolumab was able to bound to immobilized IgG with Fc-Fc reaction and the reaction was enhanced by GSH in a concentration-dependent manner. The Fc-Fc reaction was not found with S228P + R409K mutated Tislelizumab (Fig. 2D, E, F).

3.3. GSH enhanced the inhibition of IgG4 on ADCC, ADCP, and CDC

To verify the synergistic immunosuppressant effect of IgG4 and GSH on immune reactions against cancer cells, classic ADCC, ADCP and CDC experiments were performed. We found that the Fc-Fc reactions between biotin-IgG4 and cetuximab were enhanced by GSH (Fig. 3A). Cetuximab could effectively induce PBMC to kill cancer cells while IgG4, IgG1, or GSH alone had no such effect. The tests of Cetuximab + IgG1, Cetuximab + IgG4, Cetuximab + IgG1 + GSH, and Cetuximab + IgG4 + GSH revealed that IgG4 partially inhibited the cancer-killing effect of Cetuximab but IgG1 did not. We also compared Cetuximab + GSH, IgG1 + GSH and IgG4 + GSH, and confirmed that glutathione did not significantly weaken the ADCC and CDC effect mediated by cetuximab in the process (Fig. S2). The strongest inhibitory effect was achieved with the combination of IgG4 and GSH, which was stronger than that with IgG4 alone (Fig. 3B). In the ADCP experiment, we found that macrophages (labeled green) phagocytized tumor cells (labeled red) (Fig. 3C, yellow arrow indicated) with immunofluorescence staining. We also performed flow cytometry to detect macrophages' ability to phagocytize. The results indicated that IgG4 + GSH effectively inhibited cetuximab-mediated phagocytosis (Fig. 3D). We further studied the presence of IgG4-positive lymphocytes and complement components in esophageal cancers (n = 8), lung cancers (n = 6) and colon cancers (n = 6). A large number of IgG4-positive lymphocytes and C1q were seen around the tumor tissue while C1s and C4d were barely visible. Quantitative image analysis showed that there was a negative correlation between IgG4 and C1s. (Fig. S3). We used normal liver and tonsil as positive controls as most of the natural components of complement in normal body are synthesized in the liver, and a small part is produced by macrophages, while macrophages are the main source of complement in inflammatory areas.

3.4. IgG4 combined with GSH accelerated the growth of subcutaneously implanted lung cancer in mice

To explore the synergistic immunosuppressant effect of IgG4 and GSH in vivo, a mouse lung cancer model and a skin graft model were established. In the mouse lung cancer model, the IgG4 + GSH group showed the largest tumor mass in size and weight and also had the fastest growth rate, compared with those in PBS, GSH, and IgG4 groups (Fig. 4A, B, C). These results indicated that GSH enhanced the immune inhibitory effect of IgG4 in vivo.

We further examined the immune cells in the cancer tissue with immunohistochemistry with antibodies to CD3, CD4, CD8, F4-80, CD86, and CD163. Results showed that both infiltrating T lymphocytes (CD3 positive cells) and infiltrating macrophages (F4-80 positive cells) were significantly decreased in and around the tumor tissue in the groups of IgG4 + GSH, IgG4 alone, and GSH alone in comparison to the PBS control group. We found that the M1 macrophage subtype (CD86 positive cells) decreased and M2 macrophage subtypes (CD163 positive cells) increased in IgG4 alone, GSH alone, and IgG4 + GSH group, among which IgG4 + GSH group showed the most significant change. The M2/M1 ratio in the IgG4 + GSH group was almost 2 times of that in the IgG4 alone, 4 times of that in the GSH alone, and 8 times of that in the PBS groups. (Fig. 4D, E). The mouse serum was examined with the Luminex multifactor quantitative technique and was found that levels of IL-4 and EGF in the IgG4 + GSH group were significantly higher than that in the PBS control group.

3.5. IgG4 combined with GSH inhibited skin graft rejection and improved the survival of the graft in the mouse model

To investigate the synergistic effect of IgG4 and GSH in immune evasion, a mouse skin graft model was established. The mice were treated with PBS, IgG4, GSH, and IgG4 + GSH respectively after skin grafting. On day 7, blood vessels on the grafted skin tissue were examined. We found that the IgG4 + GSH group had the best blood supply as demonstrated by CD31 positive blood vessel endothelial cells in comparison to all the other groups (Fig. 5A, Supplemental Fig. S4). We performed multiple immunofluorescences staining to examine the infiltrating leukocytes in and around the skin grafts including subtypes of T lymphocytes (CD3, CD4, CD8) and macrophages (General marker/M2, F4/80, CD163). We found that the IgG4 + GSH group had a marked reduced in infiltrating T lymphocytes. CD8/CD3 ratio was also significantly decreased (Table S4). M2 macrophages were increased (Fig. 5B, C, D). On day13, significant grafted skin survival was observed in the IgG4 + GSH group, followed by GSH alone group, IgG4 alone group, and PBS group. On examination of gross appearance, the skin grafts of the IgG4 + GSH group had the best skin preservation with soft tissue texture and shinning hair without signs of infection or exudation (Fig. 5E). The quality of skin grafts was quantified by scoring which showed that the IgG4 + GSH group had the least necrosis and best graft survival among all the groups (Fig. 5F, G, H). The differences in the above parameters between IgG4 + GSH and other groups reached statistically significant levels.

On examination of cytokines in peripheral blood 14 days after skin transplant, proinflammatory cytokines IL-2 and IFN-γ were found significantly decreased in the IgG4 + GSH group in comparison to the PBS group while anti-inflammatory cytokines IL-10 and TGF-β were significantly increased. For groups treated with IgG4 alone or GSH alone, IL-2 was also decreased but IFN-γ, IL-10, and TGF-β showed no change compared to the PBS control group (Supplemental Fig. S5).

4. Discussion

In this study, we found that IgG4 and GSH were synchronously increased in the cancer microenvironment and exerted a powerful inhibitory effect on local immunity. We further found that inhibitory effect of IgG4 was facilitated by the increased presence of GSH. The mechanism is that GSH could denature IgG4 by dissociating its two heavy chains which would enhance the ability of IgG4 to react to immobilized anti-cancer IgG with Fc-Fc reaction and block the subsequent immune effector reactions. This was demonstrated in vitro and tested in vivo. In addition, we further proved that such an effect also took place in the immune reaction towards skin grafting thereby suggesting that this immune inhibitory mechanism may be universal. It appears that both detrimental effects, i.e., inhibiting local immune response against cancer and thereby promoting tumor growth, and beneficial effects, i.e., inhibiting local immune rejection against skin graft and thereby prolonging its survival, are mediated by the same

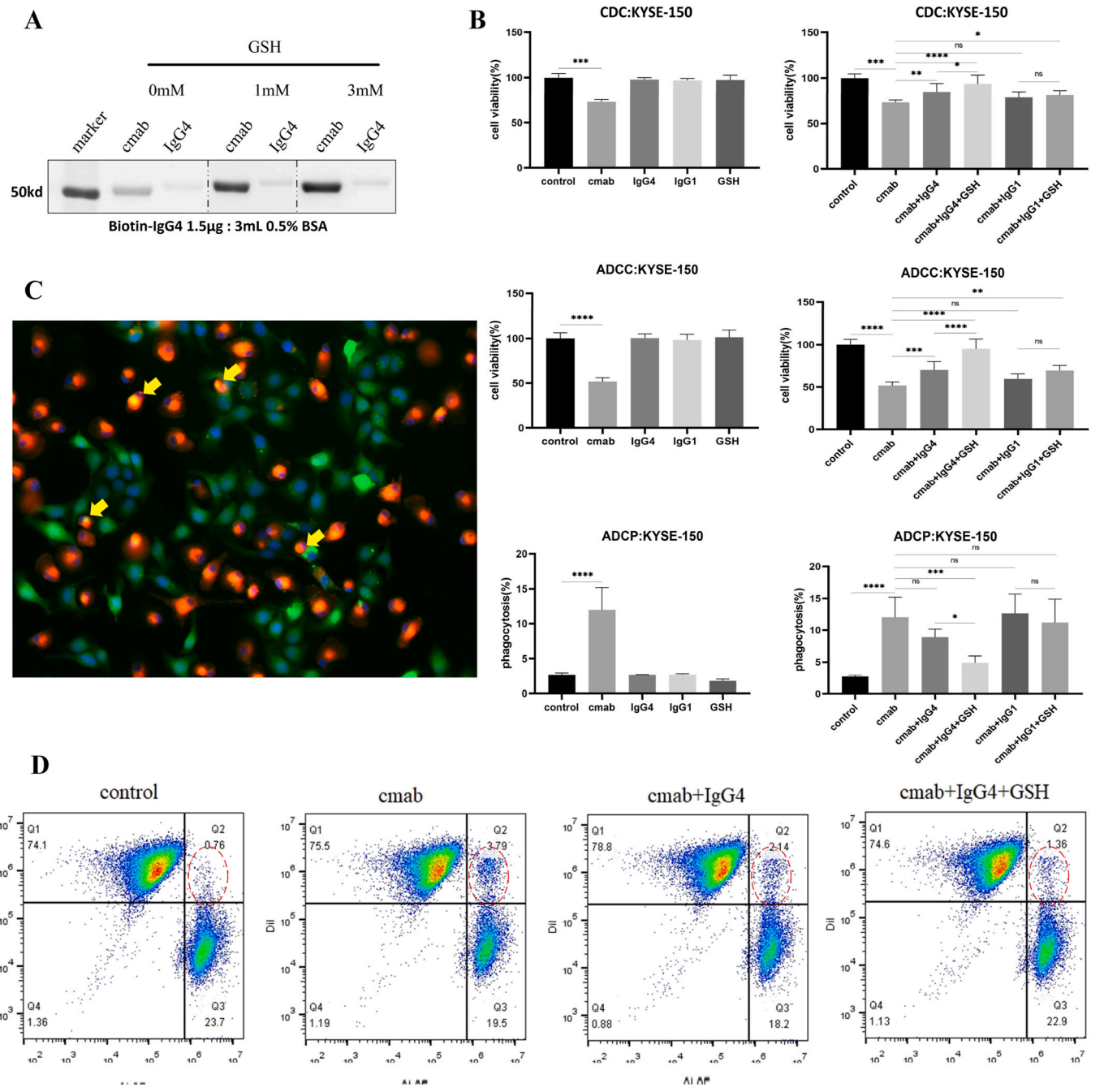

Fig. 3. Glutathione enhanced the inhibition of classical ADCC, ADCP, and CDC immune responses by IgG4.
A. Cetuximab (cmab, an anti-EGFR IgG1) and IgG4 were transferred onto nitrocellulose membranes of Western blot and incubated with biotin-IgG4 antibody pre-treated with different concentrations of GSH. The Fc-Fc reaction of cmab and biotin-IgG4 was detected and was significantly increased with the increase of GSH concentration. B. The effect of cmab with GSH and IgG4 in classical CDC, ADCC, and ADCP. In the CDC experiment, the results showed that the tumor cell activity was suppressed in cmab group (shown on the left side of the top row) but recovered in cmab + IgG4 and cmab + IgG4 + GSH (shown on the right side of the top row). Similar results were detected in ADCC (middle row). In the ADCP experiment (bottom row), the macrophage's phagocytosis was increased by cmab (shown on the left side of the bottom row), but the effect was weakened in cmab + IgG4 and cmab + IgG4 + GSH (showed on right side of bottom row). C. Macrophages phagocytized cancer cells. KYSE-150 esophageal cancer cells were labeled with CFSE (green), and macrophages derived from peripheral blood monocytes of healthy volunteers were labeled with DiI (red). Cancer cells were treated with Cetuximab and then incubated with macrophages. Yellow arrows indicate macrophages that devoured or were devouring tumor cells (nuclear were counter-stained with DAPI, blue). D. The phagocytosis of KYSE-150 esophageal cancer cells by macrophages was detected with FACS. The Q1 zone (DiI + CFSE-) represents macrophages that did not phagocytize tumor cells, the Q2 zone (DiI + CFSE+) represents macrophages of phagocytosis of tumor cells, and the Q3 zone (DiI- CFSE+) represents remaining tumor cells that have not been phagocytosed. The following formula was used to calculate the phagocytosis ratio: Phagocytosis% = [Q2/(Q2+Q3)] × 100%. CFSE, carboxyfluorescein diacetate succinimidyl ester; DiI, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate; DAPI, 4′,6-diamidino-2-phenylindole; cmab, cetuximab; **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, by one-way ANOVA, ns, not statistically significant.

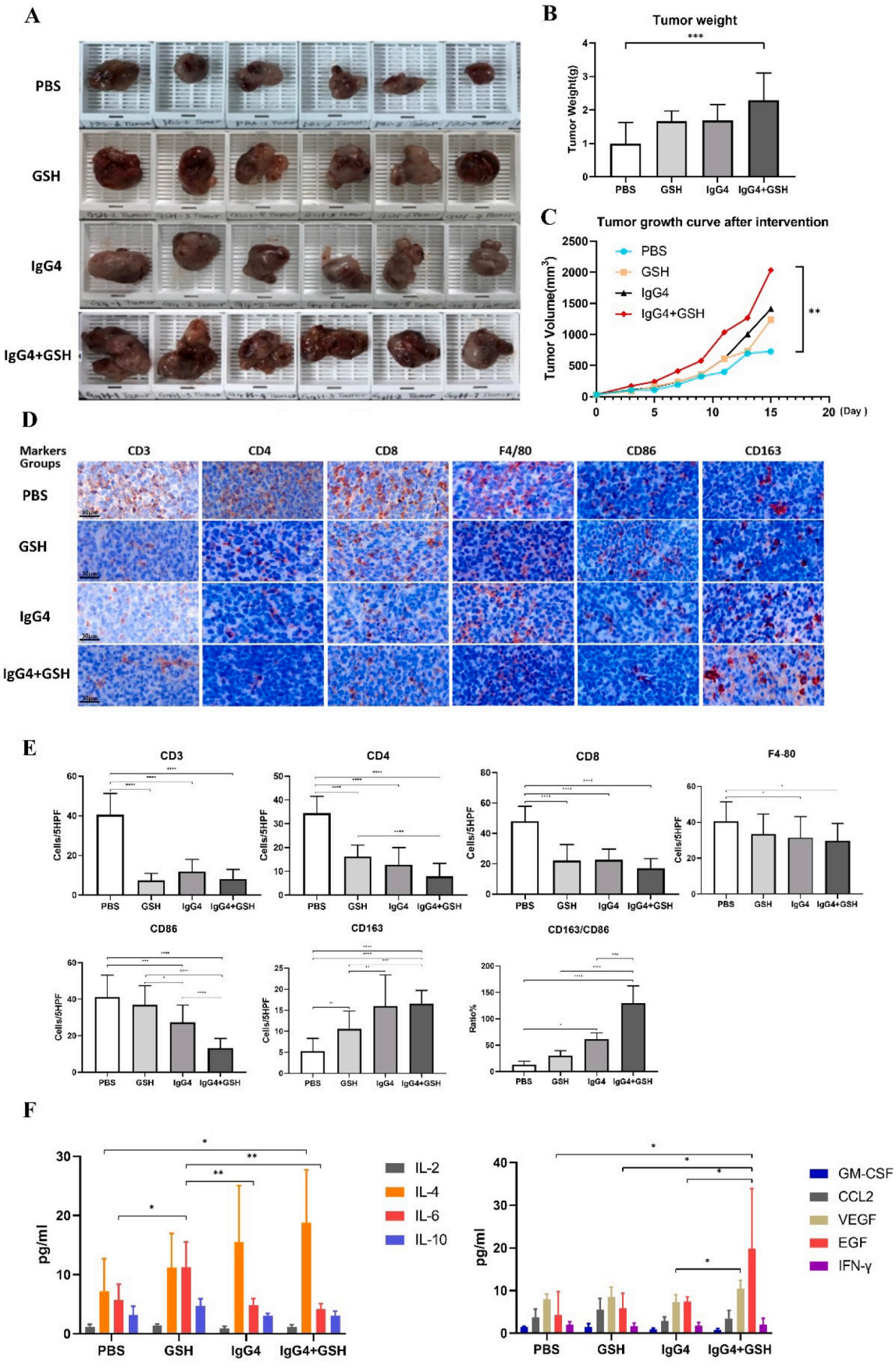

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Fig. 4. IgG4 combined with GSH accelerated growth of subcutaneously implanted lung cancer in mice.
A. Tumor size was measured on Day 24 in PBS, GSH, IgG4, and IgG4 + GSH groups. The tumor size of the IgG4 + GSH group was the largest, followed by GSH and IgG4 groups (n = 6/group). B. Tumor weight was measured and compared among PBS, GSH, IgG4, and IgG4 + GSH groups. The tumor weight of the GSH, IgG4, and IgG4 + GSH group was increased, compared to the PBS group. The tumor weight of the IgG4 + GSH group was nearly 3 times of that of the PBS group. C. Tumor growth curves after intervention were measured in vivo. It showed that the tumor growth rate was also significantly faster in the IgG4 + GSH group, almost 3 times faster, than that of the PBS group. D. Representative field of views of tumor-infiltrating T cells and macrophages in tissue immunohistochemical staining. $CD3^+$, $CD4^+$, $CD8^+$represent total T lymphocytes and its subtypes, and $F4/80^+$, $CD86^+$, $CD163^+$ represent total macrophages and M1/M2 subtypes. (Scale bar: 30 μm) E. The statistics of $CD3^+/CD4^+/CD8^+$ T cells indicated that IgG4 + GSH inhibited T lymphocyte infiltration in the tumor. The ratio of $CD163^+/CD86^+$ also suggested that IgG4 + GSH promoted the polarization of macrophages towards immunosuppressive M2 phenotype, i.e., $CD163^+$ phenotype. Average cell numbers of 5 high power field of views (HPF), cells/5HPF. F. The cytokines of mouse serum were examined with Luminex. The results showed that inhibitory inflammatory factor IL-4 (shown on the left) and the levels of EGF (shown on the right) were significantly increased in the IgG4 + GSH group in comparison to the control group. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ by one-way ANOVA; ns, not statistically significant.

mechanism. The original contribution of this study is that we found a potential synergistic effect of GSH and IgG4 in the cancer microenvironment that promotes cancer growth probably through accelerated denaturation of IgG4 and effective blockage of cancer-bound IgG. These observations connected two important mechanisms, i.e., the antioxidant system and the immune response into one and unveiled the interactions between the two. Based on our current and previous discoveries, the proposed mechanism of a synergistic effect of immune inhibition by IgG4 and GSH in cancer immune evasion is illustrated in Fig. 6.

It should be noted that since IgG4 or GSH alone could directly or indirectly induce tumor growth, the combined effect we observed could be an add-on effect rather than a synergistic effect. Indeed, we could not rule out the possibility of an add-on effect. The challenge is that interaction between GSH and IgG4 and the subsequent results in classic immune reactions could be demonstrated in vitro but difficult to observe directly in vivo. We tried to find a suitable animal model to demonstrate their direct interactions, but to our knowledge, there is no tool available to directly demonstrate the interactions of these two molecules and to examine ADCC, ADCP and CDC responses in vivo. However, as demonstrated in Fig. 2, once IgG4 and GSH were mixed before injection, the interaction between the two had occurred, which was also confirmed by other reports [19,22,23]. Full molecule of IgG4 had been dissociated and half molecules formed. The effect of IgG4 would have been weakened and the effect of Fc-Fc reaction and FAE of IgG4 facilitated by GSH would have been strengthened in the combined group of the tumor animal model. It was likely that such interaction between GSH and IgG4 would persist after injection of the mixture into the tumor tissue as we found that most IgG4 extracted from cancer tissue were not tumor specific [5] and therefore, the IgG4 we used is not tumor-specific and would not react with tumor antigens. The interaction between GSH and IgG4 should continue after injection as both would be available. As the immune inhibitory effect of each of the compounds alone was weak and simple add-on would not have produced the total effect we observed. Judging from the trend of the growth curve, the synergistic effect would be more obvious if the experiment was extended, but the tumor volumes of some animals had reached their limit, therefore we had to discontinue the experiment. The immune regulation mechanism of IgG4 and GSH in vivo is complex. Both GSH [10] and IgG4 [8] were reported to promote tumor growth and regulate immunity with different mechanisms. Glutathione may affect immunity by weakening antigen-antibody binding [24,25], inhibiting the complement system [24,26] and affecting the polarization of macrophages [27]. IgG4 also induces immune tolerance and promotes tumor growth through a variety of mechanisms such as inducing macrophage to polarize towards M2 subtype [6,28], competitive antigen binding [8,29] or Fc receptor binding [7,18] and preventing other IgG from activating complements [30]. In this study, we focused on demonstrating the role of IgG4 Fc-Fc reaction which could be facilitated by increased GSH concentration and has not been reported until now. We tried to minimize the effects of other possible mechanisms that might exert by these two molecules.

The significance of our findings is that both IgG4 and GSH are significantly increased in a variety of cancer types and therefore the mechanism that we discovered is likely to be operative in cancer immunity. As mentioned, chronic stimulation of antigen (including tumor antigens) or long-term inflammation would induce increased level of IgG4 [8,30,31], and 15%~20% of cancer cases have long-term infection, chronic inflammation, or autoimmune diseases before the affected organ develops cancer [32–34]. Patients with IgG4-related diseases have a higher cancer risk than general population [35], and the typical features of the disease are serum IgG4 elevation and higher IgG4/IgG ratio in the involved organs [36]. We observed increased number of IgG4 positive cells distributed in tumor stroma, and a higher proportion of IgG4/IgG in the tumor than that in normal tissue. Previously we measured the concentration of IgG4 in 86 esophageal patients and found that their serum IgG4 was significantly increased and negatively correlated to prognosis [5]. The result in this study also showed that the content of IgG4 in esophageal and lung cancer tissues is negatively correlated to cancer stage (Fig. S1). However, we were unable to measure GSH and IgG4 in peripheral blood as most of the patients enrolled in this retrospective study were already released from the hospital and no blood sample was available. IgG4 was found to negatively correlate with the number of cytotoxic T lymphocytes, and promote the transformation of macrophages to immunosuppressive M2 phenotype [6,37]. We observed that the infiltration of $CD8^+$ lymphocyte was decreased and macrophages polarized to M2 subtype in the intervention group containing IgG4 in the tumor and the graft skin tissues. It indicates that IgG4 could induce immune tolerance in the microenvironment of both tumor and skin graft. We noticed that the patterns of lymphocyte infiltration in the IgG4 and IgG4 + GSH groups were similar to one another, while the pattern in the GSH alone group was closer to that of the PBS group. This may suggest that when GSH was mixed with IgG4 it acted within the mechanisms of IgG4, i.e., Fc-Fc reaction, but GSH alone did not have this effect, further supporting interaction between GSH and IgG4 in vivo which is also consistent with previous reports [19,22,23]. Infection and inflammation can lead to adaptive upregulation of GSH [38,39]and the elevation of GSH in cancer is also closely related to prognosis [16,40]. Due to mitochondrial dysfunction, metabolic changes and frequent gene mutations, oxidative stress significantly increases in cancer cells, and maintains a complex redox balance with enhanced antioxidant pressure in which GSH plays an important role [10]. Blocking the synthesis of GSH can lead to oxidative stress damage and even the death of cancer cells [10,16,40]. In our study, we found that GSH increased in esophageal, lung, and colon cancer tissues similar to the increase of IgG4. In addition, immunostaining showed that GSH and IgG4 were distributed close to each other and even overlapping allowing interaction of the two. Therefore, chronic inflammation is not only an important inducer of tumor, but also a common inducing factor for the elevations of IgG4 and GSH, and facilitates the synergism of the two. The Fc-Fc reaction between IgG4 and anti-tumor IgG may hinder the formation of IgG polymers [3,41] as well as the activation of complements [30]. The results suggested that there was an interaction between tumor antioxidation mechanism and humoral immune evasion mechanism. Following the same logic, GSH would have dissociated IgG4 before injection into the tissue and GSH and IgG4 would be continuously available for interaction after injection, and therefore the blocking effect by a mixture of GSH and IgG4 on

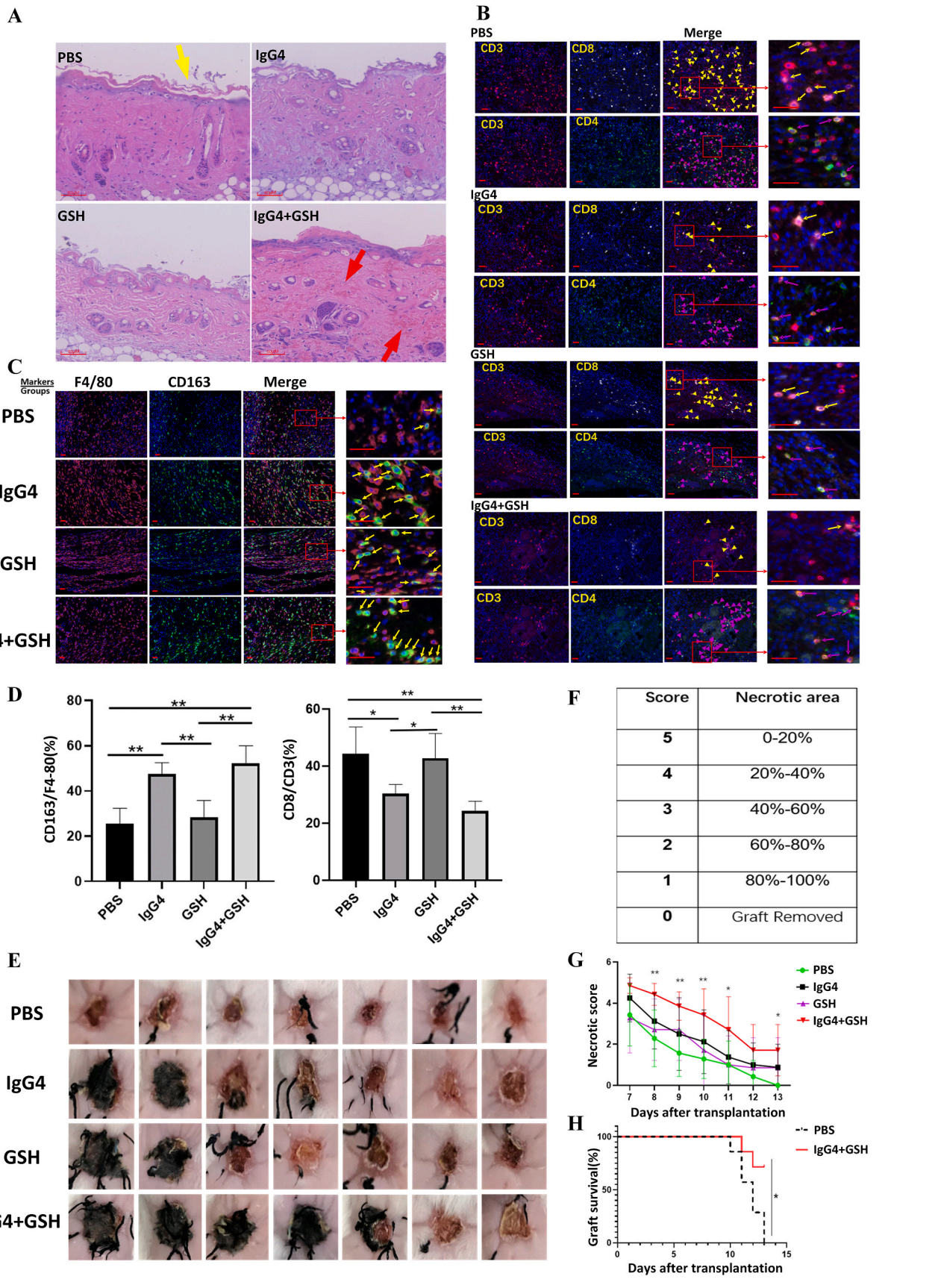

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Fig. 5. IgG4 combined with GSH inhibited skin graft rejection and improved survival of skin graft in a mouse model.
A. C57BL/6 mice were used as donors and BALB/c mice as recipients. Skin grafts were performed on the back of mice(n = 11/group). Four mice of each group were sacrificed on Day7 after skin transplantation, the grafted skin tissue was examined with HE staining (Scale bar: 60 μm). Extensive epidermal necrosis was seen in the PBS group (indicated with a yellow arrow). On the contrary, the squamous epidermis was intact and there were abundant blood vessels under the skin in the IgG4 + GSH group. (Indicated with red arrow). B, C. Representative fields of view of T cells and macrophages in skin graft with multiple immunofluorescence staining. T cells and its subtypes were represented with $CD3^+$ (red), $CD4^+$ (green), and $CD8^+$ (white). $F4/80^+$ (red) and $CD163^+$ (green) indicate macrophages, and nuclei were stained with DAPI (blue). Arrows indicate double-positive cells, i.e., $CD3^+$ $CD4^+$ subtype T cells, $CD3^+$ $CD8^+$ subtype T cells, and $F4/80^+$ $CD163^+$ subtype macrophage cell (M2 macrophage). D. The ratio of $CD8^+$ T cells and $F4/80^+$ $CD163^+$macrophages (M2) in each group (n = 4, average number/5 high power field) were statistically analyzed. The results showed that the ratio of $CD8^+$ T cells in the IgG4 + GSH group was significantly down-regulated, while the ratio of M2 macrophages was significantly up-regulated (Scale bar: 30 μm). E. The appearance of skin graft survivor on Day13 after surgery. It showed that almost no transplanted skin survived in the PBS group, partially survived in IgG4 and GSH groups, but mostly survived in the IgG4 + GSH group (n = 7/group). F. The skin necrosis ratio scale, and different necrotic areas corresponded to different scores. G. The grafted skin score was calculated from day7 to day13 in four groups based on F. It was found that the score was significantly higher in the IgG4 + GSH group, and no statistical difference among PBS, IgG4, and GSH groups. H. The survival curves of skin grafts in the PBS group and IgG4 + GSH group. *p < 0.05, **p < 0.01 by one-way ANOVA and Student's t-test.

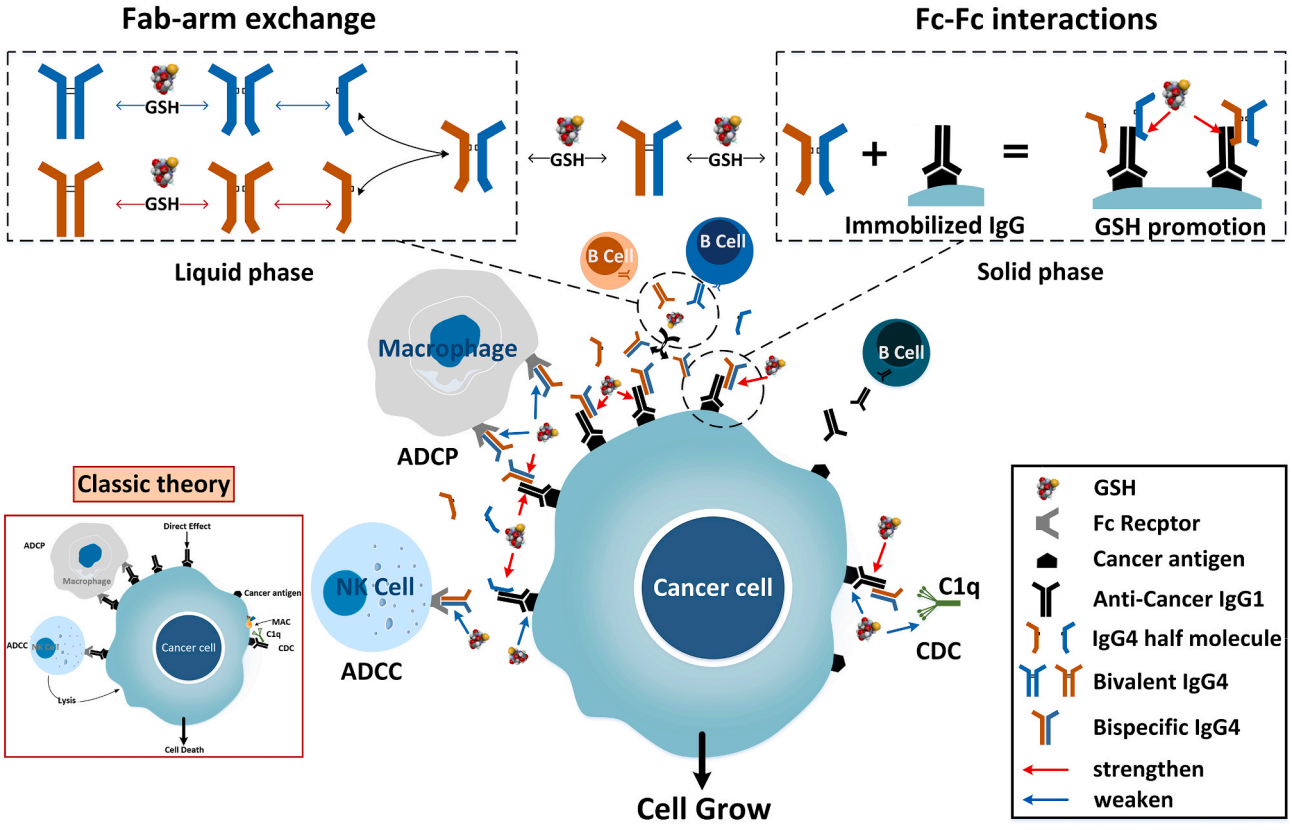

Fig. 6. The proposed mechanism of a synergistic effect of immune inhibition by IgG4 and GSH in cancer microenvironment.
In classic theory (shown on lower left), specific antibodies (mostly IgG1) against cancer cells induce ADCC, ADCP and CDC which leads to cancer cell death. In the present study, we found that increased GSH in the cancer microenvironment enhanced IgG4 Fab-arm exchange (occurs in liquid phase) and led to Fc-Fc reaction with tumor antigen-binding IgG (occurs in solid phase). This latter reaction would suppress IgG-mediated classic ADCC, ADCP and CDC immune reactions, and cause cancer cell to escape the immune attack. Therefore, the increased GSH and IgG4 in cancer microenvironment synergistically mediates an effective immune escape and promotes tumor growth. This mechanism was supported by our findings in vitro and in vivo of this study.

ADCC, ADCP and CDC demonstrated in vitro (Fig. 3) would also likely take place in vivo.

The implication of our observation to cancer immunotherapy is that the ability of Fc-Fc reaction retained by some PD-1 mAbs which are IgG4 in nature may play a harmful role in tumor patients who received the immunotherapy, especially those with elevated GSH level. The commonly used cancer immunotherapy drugs Pembrolizumab and Nivolumab are monoclonal antibodies modified from human IgG4 (both are S228P mutated) [42]. The common S228P mutation in the hinge region aims to stabilize the IgG4 molecular structure so that it could not cleave into half molecules and facilitate FAE [43]. However, this doesn't seem to stop the Fc-Fc reactions [22] and our results confirm this finding. We observed in this study that GSH could promote Fc-Fc reaction of S228P mutated Nivolumab but had no effect on S228P + R409K mutated Tislelizumab (Fig. 2). Accordingly, this effect may occur in some combined immunotherapy schemes that contain both IgG4 type PD-1 mAb and other IgG1 mAb such as Cetuximab and Trastuzumab. In a recent study, combined immunotherapy of Pembrolizumab (human IgG4) and Ipilimumab (IgG1 based CTLA-4 antibody) did not improve efficacy and was associated with greater toxicity and a higher death rate for metastatic Non–Small-Cell Lung Cancer with PD-L1 tumor proportion score ≥50% [44]. In an ongoing phase 2 clinical trial (NCT03082534), the therapeutic effect of Pembrolizumab combined with cetuximab on head and neck squamous cell carcinoma is under investigation, and up to 15% of patients developed serious treatment-related adverse events [45]. PD-1 mAb has greatly improved the efficiency of tumor treatment in the past decade [46]. However, the overall efficacy and long-term effectiveness are still undesirable. Even

among patients with positive PD-L1 expression, 55% of them did not benefit from PD-1 immunotherapy, indicating that there are still many unknowns [46,47]. HPD is the worst, high frequency (5~20%) adverse reaction of immunotherapy with rapid tumor progression and poor prognosis [48–51]. Several studies have found that patients with *KRAS, STK11/LKB1* [13], *MDM2/MDM4 and EGFR* [12] mutations were extremely prone to HPD after receiving PD-1 immunotherapy. These mutations frequently occur in lung cancer, of which *KRAS*-mutant is the most common oncogenic driver accounting for 15–30% [52]. Several clinical studies reported that *KRAS*-mutant with *STK11* (also called *LKB1*) co-mutations and concurrent mutations in *STK11/KEAP1* were associated with resistance to PD-1 blockade in lung cancer patients, and exhibited shorter progression-free and overall survival in comparison to mutant wild-type patients [53–55]. These mutations are all closely related to enhanced metabolism of GSH in tumors [11,14–16,56]. A recent study found that oxidized vitamin C selectively depletes GSH in *KRAS/BRAF* mutated colon cancer cells, resulting in tumor cell death due to oxidative stress injury, but this was not seen in wild-type tumor cells [57]. Based on what we found in this study, it appears that harboring certain gene mutations would increase local GSH in the mutant tumor. When these patients receive S228P mutated IgG4 antibodies such as Nivolumab or Pembrolizumab, GSH would promote the Fc-Fc reaction between these PD-1 mAbs and tumor-specific IgG. As a result, anti-tumor IgG-mediated immune responses would be blocked, and eventually lead to HPD. A supporting evidence for this mechanism was provided by Russo et al. who established a xenograft tumor model of human non-small cell lung cancer in immunodeficient mice and found that Nivolumab could promote the growth of LUAC with *EGFR L858R* mutation. They suggested that it was the Fc of Nivolumab rather than the Fab that played a key role in this effect [18].

Our results on the skin graft suggest that IgG4 might play a role in transplant immune tolerance similar to tumor immune evasion through Fc-Fc reaction, and GSH may enhance IgG4-induced transplant immune tolerance. B lymphocyte is known as the main initiators of rejection and mediate skin graft rejection by secreting antibodies or presenting antigens to activate T lymphocytes [58]. The antibody-mediated immune response serves as a key mechanism of graft injury [59]. Therefore, it is possible that IgG4 can block antibody-mediated immune rejection with Fc-Fc reaction. The results indicate the versatility of this mechanism. There were different degrees of rejection after skin transplantation in responses to different treatments, and IgG4 + GSH treatment produced the most significant protective effect. Immunostaining of grafted skin showed that the infiltration and differentiation of lymphocytes are similar to those of the lung cancer model with characteristics of immune suppression or tolerance in IgG4 + GSH group. Cytokine results showed that the levels of IL-10/TGF-β (anti-inflammatory factors) were elevated while IL-2/IFN-γ (pro-inflammatory factors) were decreased in plasma of mice treated with IgG4 + GSH compared to the control group. This may explain the M2 differentiation of macrophages and the less $CD8^{+}$T lymphocytes infiltration in the skin grafts of IgG4 + GSH group. Both cellular and humoral immune responses of graft injury were reduced by IgG4 + GSH. Therefore, local injection of IgG4 combined with GSH can significantly promote immune tolerance of transplanted skin grafts.

In summary, a synergistic effect of GSH and IgG4 in inducing immune inhibition was found in vitro and was likely in vivo. The possible mechanism of this effect was investigated with different animal models and we found that this could be a universal immunomodulatory mechanism. The implications of our findings are multi-fold. Theoretically, the results unveiled the interaction between two modulatory mechanisms in cancer. Clinically our research offers the possibility of manipulating the IgG4 structure of the immunotherapeutic drugs and GSH concentration in cancer that affects the IgG backbone of these drugs to avoid HPD side effects and to improve the treatment of cancer.

Ethics approval and consent to participate

The acquisition of clinical samples was approved by the Shantou University Research Ethics Committee (Reference Number: SUMC-2021-09) and fully informed the patients. All animal experiments were conducted according to the protocols approved by the Animal Care and Use Committee of Shantou University Medical College (Reference Number: SUMC2020-238) and complied with the ARRIVE guidelines [20].

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the authors on reasonable request.

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Authors' contributions

WFZ: designed and performed most of the experiments, analyzed the results, and wrote the manuscript; YQ: Performed the skin graft model related experiments, analyzed the skin graft model results, and wrote the manuscript; XNM: performed the experiments of ADCC, ADCP, CDC; LTZ: performed some of the animal model experiments and immunohistochemistry staining; JRL: performed tissue collection and processing; SQC: performed some of the animal model experiments and immunohistochemistry staining; MS: Manuscript improvement and analyzed the results; LLH: analyzed the results; PHL: performed tissue collection and processing; HW: result and literature analysis; QX: result and literature analysis; CYZ: performed some of the animal models and result analysis. XQZ: performed some cell experiments; YQG: result and literature analysis; XMY: performed some immunohistochemistry staining; ZF: image processing and analyzed the results; MWC: image processing and analyzed the results; DPT: collected and analyzed some pathologic tissue samples; MS: collected and analyzed some pathologic tissue samples; XLC: helped organizing and editing the manuscript; JG: designed and directed the experiment, analyzed the results and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.102608.

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