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CD103$^+$ Cell Growth Factor Flt3L Enhances the Efficacy of Immune Checkpoint Blockades in Murine Glioblastoma Model

Xiaolin Miao,*[1] Yiqi Chen,*[1] Ke Hao,†[1] Meiqin Zheng,‡ Bingyu Chen,† Kaiqiang Li,† Ying Wang,† Wei Zhang,§ Yu Zhang,§ Xiaozhou Mou,§ Shanshan Jiang,¶ and Zhen Wang‡§

*Eye Hospital of Wenzhou Medical University, Zhejiang, P.R. China
†Research Center of Blood Transfusion Medicine, Education Ministry Key Laboratory of Laboratory Medicine, Zhejiang Provincial People's Hospital, Zhejiang, P.R. China
‡School of Laboratory Medicine and Life Science, Wenzhou Medical University, Zhejiang, P.R. China
§Clinical Research Institute, Zhejiang Provincial People's Hospital, Zhejiang, P.R. China
¶Department of Gynecology, Zhejiang Provincial People's Hospital, Zhejiang, P.R. China

Glioblastoma is a lethal disease featuring a high proliferation of tumor cells, excessive angiogenesis, and heavy drug resistance. The overall survival of glioblastoma patients has been dismal, even with an intensive standard of care. Recent advances in immune checkpoint blockades are changing the treatment of cancers. However, the efficacy of immune checkpoint blockades in glioblastoma is still unclear. Here we investigated the roles of CD103$^+$ cells in regulating the effect of immune checkpoint blockades in glioblastoma mouse models. Our findings indicated that the murine glioblastoma model was not sensitive to immune checkpoint blockades. Flt3L, a growth factor for CD103$^+$ cells, could significantly increase the number of CD103$^+$ dendritic cells in the murine glioblastoma model and, thus, sensitize murine glioblastoma to immune checkpoint blockades. Downstream analysis indicated that the Flt3L and immune checkpoint blockade combination increased the number of tumor-infiltrating CD8$^+$ cells, decreased immune checkpoint expression, and therefore enhanced the antitumor immune response in the murine glioblastoma model. These findings suggested that Flt3L could enhance the efficacy of immune checkpoint blockades in glioblastoma via expanding CD103$^+$ dendritic cells and downstream antitumor immune response.

Key words: Glioblastoma; Immune checkpoints; Flt3L; CD103; Mouse model

INTRODUCTION

Glioblastoma, a subtype of glioma, is the most common primary brain tumor in adults, representing about 15% of all primary brain tumors[1–3]. The prognosis for glioblastoma is poor[1]. The median overall survival is around 15 months for patients receiving the standard first-line treatment, including maximal safe resection and a combination of radiotherapy with temozolomide chemotherapy[1,4]. The primary reasons leading to this dismal prognosis include the aggressive biological nature of glioblastoma and its adaptive drug resistance to chemotherapy[5,6]. Recent attempts with targeted agents have also failed to improve patient outcomes in glioblastoma[7].

The interaction of tumor cells with immune cells is a major determinant of cancer progression[8,9]. Immune cells, dependent on their functions, are either tumor suppressive or promoting. Studies showed that neutralizing inflammation induced by myeloid-derived immune-suppressive cells could inhibit tumor development[10,11]. On the other hand, recent studies also indicated that the boosting antitumor immune response would be a cure for certain cancer patients[12]. The antitumor immune response is controlled by several factors, including the immunogenicity feature of tumors, antigen-presenting intensity, immune cell infiltration, and immune cell activation and cytotoxicity[13,14]. Immune checkpoints, such as CD28, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and programmed cell death protein 1 (PD-1), are molecules that are expressed on immune cells to either enhance or inhibit immune responses[15,16]. The immune

[1]These authors provided equal contribution to this work.
Address correspondence to Shanshan Jiang, Department of Gynecology, Zhejiang Provincial People's Hospital, 5th Floor, Tower III, 3 East Qingchuan Road, Hangzhou Medical University, Zhejiang, P.R. China. E-mail: shashaj5488@163.com *or* Zhen Wang, School of Laboratory Medicine and Life Science, Wenzhou Medical University, University Town, Chashan, Wenzhou, Zhejiang, P.R. China and Clinical Research Institute, Zhejiang Provincial People's Hospital, 158 Shangtang Road, Hangzhou, Zhejiang, P.R. China. E-mail: zhenww157@126.com

checkpoint signaling pathways, together with the primary signaling pathway (antigen presented through the MHC to the T-cell receptor), determine the overall activation and cytotoxicity of immune cells[16,17].

Recent advances in antitumor immunity regulation by immune checkpoint mechanisms resulted in exciting novel immunotherapies: immune checkpoint blockade-based immunotherapies[15,16]. Immune checkpoint blockades have been approved by the FDA for certain melanoma, lung cancer, and renal cancer patients[16,18]. However, the overall response rate of immune checkpoint blockades in solid tumors is low due to the abnormal immune microenvironment of tumors[16,18]. The efficacy of immune checkpoint blockades on glioblastoma is still unclear. $CD103^+$ cells are identified as either dendritic cells (DCs) or $CD8^+$ T cells, both having critical roles in antitumor immunity[19,20]. In the current study, we aimed to investigate the efficacy of immune checkpoint blockades in glioblastoma preclinical models and the roles of $CD103^+$ cells in regulating immune checkpoint blockade's efficacy.

MATERIALS AND METHODS

Cell Culture

The glioma 261 (GL261) cell line (ATCC, Manassas, VA, USA) was cultured in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were passed for subculture when they reached 90%–100% confluence.

Animal Model

Mouse GL261 is frequently used to study glioblastoma therapy[21]. We used the GL261 cell line (Cell Bank of Chinese Academy of Sciences, Shanghai, P.R. China) and C57BL/6 mice (female; 6 weeks old; 19–21 g; SLAC Laboratory, Shanghai, P.R. China) to establish the orthotopic murine glioblastoma model. We followed the method developed by Jouanneau et al.[22]. Briefly, the mice were anesthetized by intraperitoneal injection with the mixture of 6.5% ketamine and 2.2% xylazine (0.02 ml/g). The GL261 cell suspension (10^5 cells in 10 μl) was then injected into the frontal lobe very slowly. The mice were raised in a clean environment with free access to standard food and water. The treatments were started 1 week after inoculation with GL261 cells. The mice were randomly divided into three groups to accept different treatments (12 mice per group): IgG (control group); three doses per week immune checkpoint blockades [anti-PD-1 antibody (10 mg/kg) plus anti-CTLA-4 antibody (5 mg/kg)]; and six doses per week immune checkpoint blockades. We also explored the effect of anti-CD103 treatment in combination with immune checkpoint blockades: IgG (control group), anti-CD103 antibody plus three doses per week immune checkpoint blockades; and 0.1% Flt3L (30 μg/dose; PeproTech Inc. Rocky Hill, NJ, USA) plus three doses per week immune checkpoint blockades. All the antibodies were purchased from Bio X Cell (West Lebanon, NH, USA). The survival status of these mice was checked every day. The mice were euthanized when adverse symptoms occurred such as rapid body weight loss, hunched posture, and apparent reduced mobility. Tumor volume ($\text{length} \times \text{width}^2\pi/2$) was measured at the end point.

Immunostaining

In order to detect the number of $CD8^+$ or $CD103^+$ cells in the mouse model tumor, we performed immunofluorescence (IF) staining. Common standard procedures were followed. Briefly, the formalin-fixed paraffin-embedded (FFPE) sections were deparaffinized by xylene and rehydrated with ethanol. The antigen was retrieved by heating the citric acid buffer (pH 6.0) and submerging the slides in a water bath for 15 min. Incubation with 5% BSA–PBS for 30 min at room temperature was performed for blocking nonspecific antibodies. Primary antibodies (diluted 1:100) were added to the slides to incubate overnight at 4°C. Fluorescent secondary antibodies (for IF; R&D Systems) were then added to incubate for 1 h at room temperature. The slides were then washed with PBS and stained with DAPI for 1 min at room temperature. Pictures were taken for further evaluation. The average positive number of five random fields under 400× magnification was calculated as the final score for each case.

Enzyme-Linked Immunosorbent Assay

Expression of multiple proteins of tumor tissues from the mouse model was evaluated by enzyme-linked immunosorbent assay (ELISA), including TNF-α, IFN-γ, granzyme B, and IL-2. All ELISA kits were purchased from Sigma-Aldrich.

Flow Cytometry Analysis

Flow cytometry analysis was performed to evaluate the key immune cells in the tumor tissue of the glioblastoma mouse model, including the $CD11c^+MHCII^+CD103^+$ cells, $CD3^+CD4^+CD103^+$ cells, and $CD3^+CD8^+CD103^+$ cells. Expression of immune checkpoints, including PD-1, CTLA-4, PD-L1, and PD-L2, was also measured by flow cytometry. Primary antibodies were purchased from BioLegend (San Diego, CA, USA). For staining, the fresh tumor tissues were minced into small fragments and dissociated into single-cell suspension by collagenase type I. The cells were then incubated with the primary antibodies for 20 min at 4°C and washed with PBS twice. Finally, the cells were analyzed on a FACSCanto II machine (Becton Dickinson, San Jose, CA, USA). FlowJo software was used to quantify the data.

Statistical Analysis

Statistical analysis and data visualization were performed using GraphPad Prism 7.0 (La Jolla, CA, USA). Statistical difference between multiple groups was analyzed by one-way ANOVA. Tukey's multiple-comparison test was performed for comparison of two groups after one-way ANOVA. The Kaplan–Meier method was used for survival analysis, and the log-rank test was used to compare survival curves. A value of $p<0.05$ (two-tailed) was considered to be statistically significant. Data are shown as mean and standard deviations in the figures and text.

RESULTS

Murine Glioblastoma Model Was Resistant to Immune Checkpoint Blockades

To examine the effects of immune checkpoint therapy on the glioblastoma mouse model, we treated the mice with anti-CTLA-4 and anti-PD-1 Abs and measured their

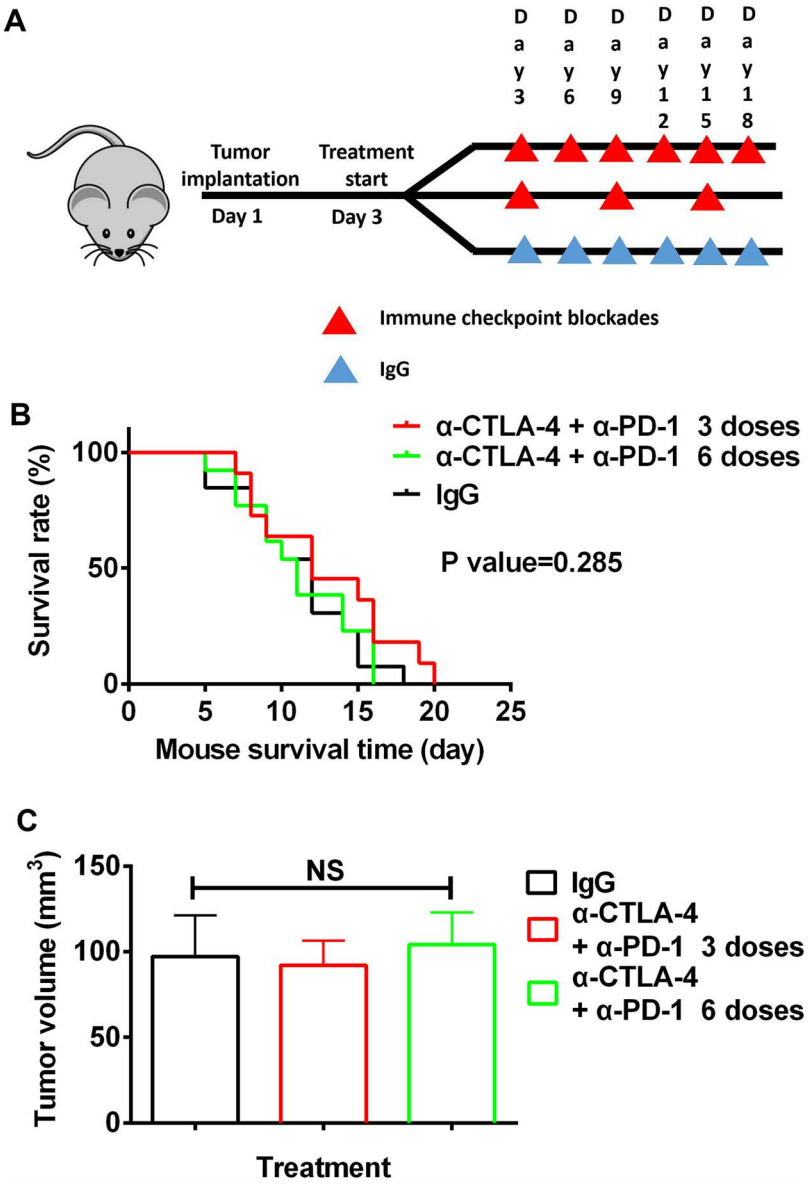

Figure 1. Immune checkpoint blockades on the glioblastoma mouse model. The orthotopic murine glioblastoma model was established using the glioma 261 (GL261) cell line. (A) Treatment strategies of the glioblastoma mouse model. The triangles indicate treatment time points. (B) Survival curves of the mice accepting different treatments: anti-CTLA-4 Abs + anti-PD-1 Abs treatment (three doses/week), anti-CTLA-4 Abs + anti-PD-1 Abs treatment (six doses/week), and IgG (as control). These three groups did not show apparent differences in overall survival time. The value of $p=0.285$ was the log-rank test p value of the survival curves. (C) Observation end point tumor volume of the mice from different treatment groups. No significant difference was observed. α-CTLA-4, anti-CTLA-4 Abs; α-PD-1, anti-PD-1 Abs.

tumor growth and survival time. Two treatment schedules were performed: six doses per week and three doses per week (Fig. 1A). Unfortunately, there was no big difference in survival time observed among the experimental and control groups (Fig. 1B). The end point tumor volume of these groups was also similar within all groups (Fig. 1C). This evidence suggested that this glioblastoma mouse model was not sensitive to the immune checkpoint blockades.

Expanding CD103$^+$ DCs by Flt3L Synergized With Immune Checkpoint Blockades in the Murine Glioblastoma Model

To sensitize the murine glioblastoma model to immune checkpoint blockades, we combined the CD103$^+$ cells regulating treatment with immune checkpoint blockades. Survival time of the glioblastoma mouse model was recorded, and their tumor tissue was collected to measure the CD103$^+$ cell numbers (Fig. 2A–C). The survival analysis showed that the mice accepting the CD103$^+$ cell growth factor (Flt3L)+anti-CTLA-4 Abs+anti-PD-1 Abs treatment had the highest survival rate compared with other groups (Fig. 2D). Importantly, the number of tumor-infiltrating CD103$^+$ cells was consistent with the trend in survival time: the mice who had a longer survival time had a higher number of CD103$^+$ cells in tumor tissues (Fig. 2E).

We further analyzed the subtypes of CD103$^+$ cells that were expanded by Flt3L. We measured the numbers of CD11c$^+$/CD11b$^+$MHCII$^+$CD103$^+$ DCs, CD3$^+$CD4$^+$CD103$^+$ T cells, and CD3$^+$CD8$^+$CD103$^+$ T cells within the glioblastoma tissue from the mice by flow cytometry. We found that the numbers of DCs were expanded significantly by Flt3L (Fig. 3B). However, the CD3$^+$CD4$^+$CD103$^+$ T cells and CD3$^+$CD8$^+$CD103$^+$ T cells did not change much with this treatment (Fig. 3C and D). These data highlighted that CD103$^+$ DCs could be most efficiently expanded by Flt3L, therefore leading to a strong antitumor immune response.

Response of Tumor-Infiltrating CD8$^+$ Cells and Immune Checkpoints to Flt3L and Immune Checkpoint Blockade Combination

To understand the mechanisms by which the Flt3L and immune checkpoint blockade combination renders the

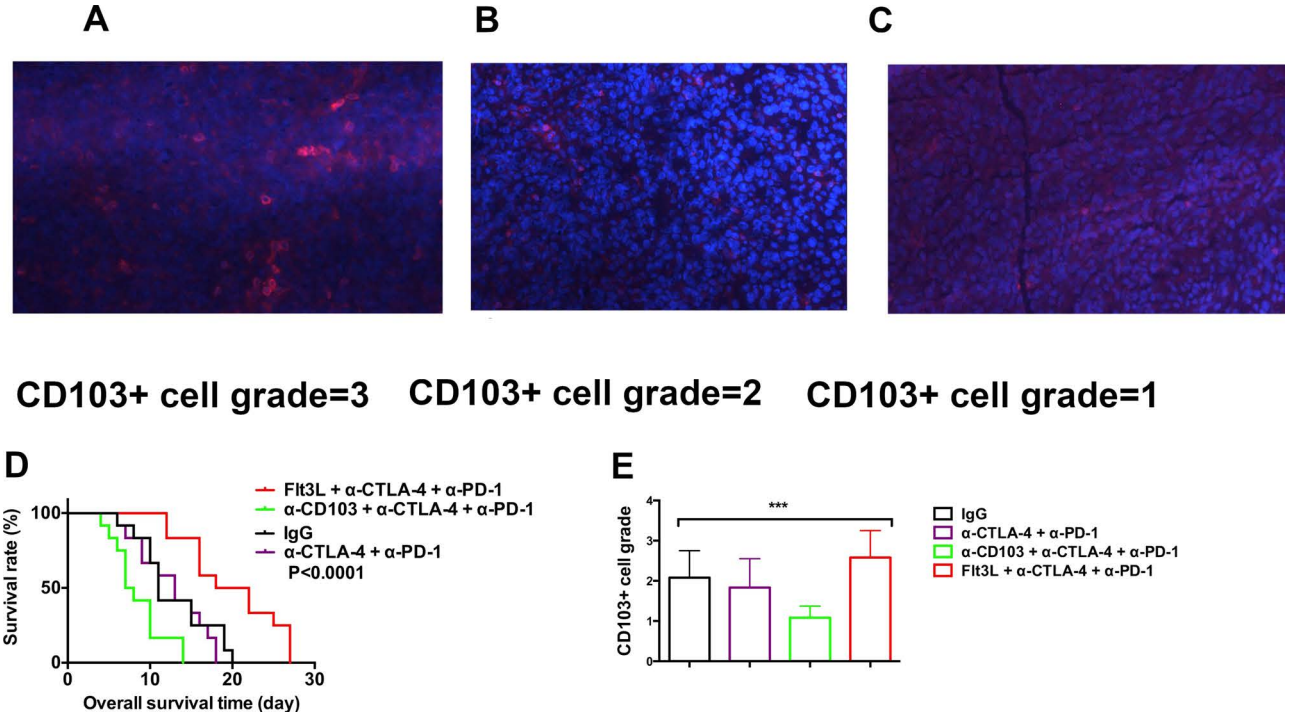

Figure 2. The effects of combining CD103 growth factor Flt3L with immune checkpoint blockades in the glioblastoma mouse model. (A–C) Representative pictures showing high (grade 3), median (grade 2), and low (grade 1) CD103$^+$ cell numbers in the glioblastoma tumor tissues from the mouse model. (D) Survival curves of the glioblastoma mouse models accepting different treatments ($n=12$ per group, three doses per week): IgG, anti-CTLA-4 Abs+anti+PD-1 Abs, anti-CD103 Abs+anti-CTLA-4 Abs+anti+PD-1 Abs, and Flt3L+anti-CD103 Abs+anti-CTLA-4 Abs+anti+PD-1 Abs. Mice treated with Flt3L+anti-CTLA-4 Abs+anti+PD-1 Abs had the longest survival time, whereas mice treated with anti-CD103 Abs+anti-CTLA-4 Abs+anti+PD-1 Abs had the shortest overall survival time. (E) Mean CD103$^+$ cell grade in the tumor tissue from the glioblastoma mouse models. Mice treated with Flt3L+anti-CTLA-4 Abs+anti+PD-1 Abs had the highest mean grade of CD103$^+$ cells, whereas mice treated with anti-CD103 Abs+anti-CTLA-4 Abs+anti+PD-1 Abs had the lowest mean grade. ***$p<0.001$.

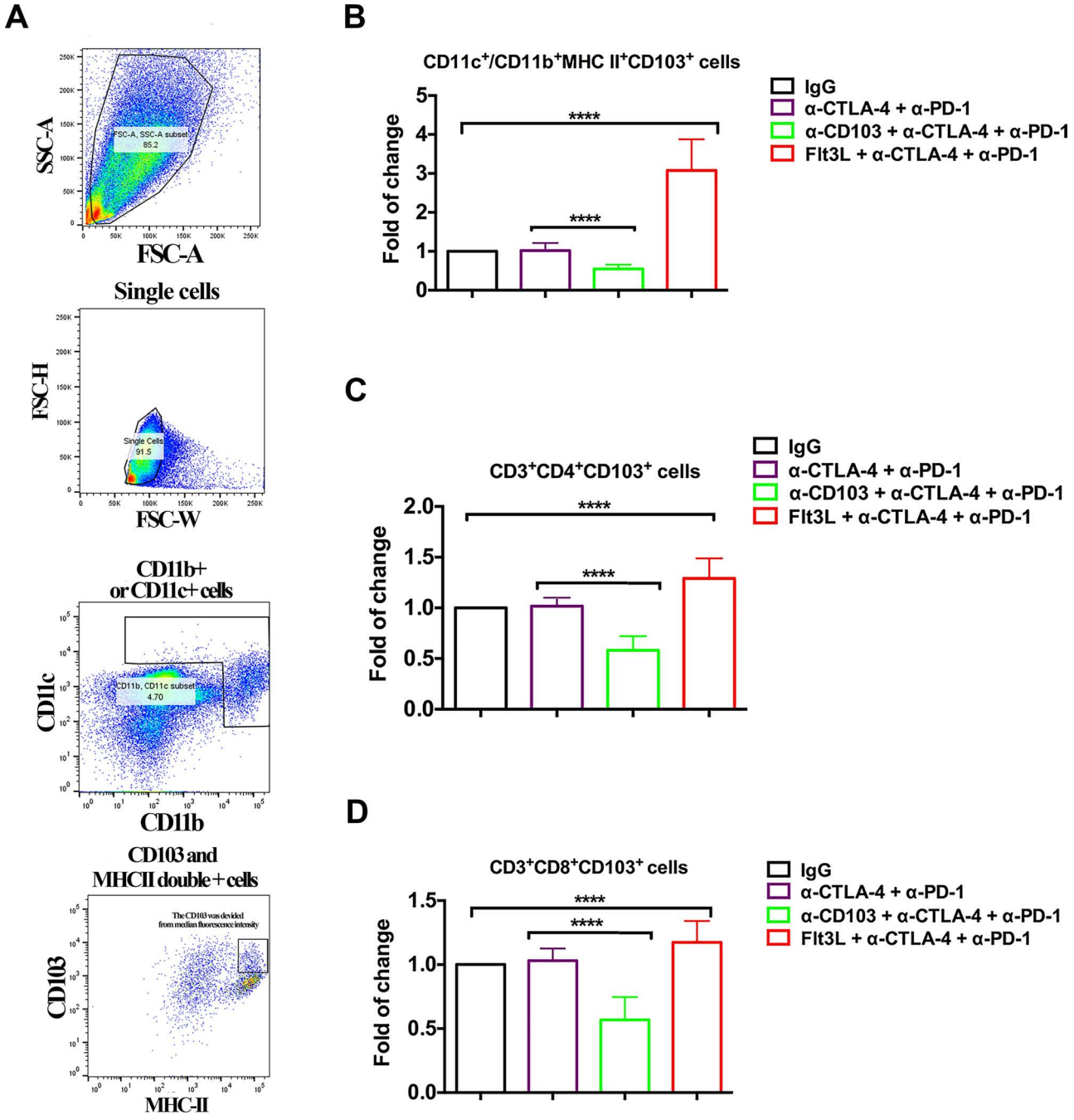

Figure 3. Alteration of tumor-infiltrating immune cell profile in the glioblastoma mouse model after Flt3L and immune checkpoint blockade treatment. The tumor tissue of the glioblastoma mouse models was collected and subjected to measuring the tumor-infiltrating immune cell number by flow cytometry analysis. (A) Representative pictures showing gating strategies of flow cytometry analysis for $CD103^+$ dendritic cells. (B–D) Bar plots showing the number of $CD11c^+/CD11b^+MHCII^+CD103^+$ dendritic cells, $CD3^+CD4^+CD103^+$ T cells, and $CD3^+CD8^+CD103^+$ T cells, respectively. All three immune cells were increased in the glioblastoma tumor tissue of mice treated with Flt3L + anti-CTLA-4 Abs + anti-PD-1 Abs and were significantly decreased by the anti-CD103 Abs + anti-CTLA-4 Abs + anti-PD-1 Abs treatment. Among the three kinds of immune cells, $CD11c^+/CD11b^+MHCII^+CD103^+$ dendritic cells were increased most, about three times that of the control group, but the other two kinds of immune cells did not change that much, although statistical significance was observed. The anti-CD103Abs + anti-CTLA-4 Abs + anti-PD-1 Abs is significantly different from the control group. $^{****}p < 0.0001$.

murine glioblastoma tumor model response, we further checked CD8 T-cell infiltration and expression of inhibitory immune checkpoints in the tumor tissue. We found different levels of $CD8^+$ cells within the glioblastoma tissues (Fig. 4A–C), with the highest mean number in the tumor tissues coming from the mice treated with Flt3L + anti-CTLA-4 Abs + anti-PD-1 Abs and the lowest mean number coming from the mice treated with anti-CD103 Abs + immune checkpoint blockade therapy (Fig. 4D).

Expression of PD-1 and CTLA-4 was usually found in T cells, including CD4 T cells and CD8 T cells. By flow cytometry analysis, we found that immune checkpoint blockades could reduce the expression level of PD-1 and CTLA-4 in tumor-infiltrating T cells (Fig. 5A–D). Adding Flt3L to the immune checkpoint blockade further inhibited PD-1 and CTLA-4 expression (Fig. 5A–D). However, when we added anti-CD103 Abs, the efficacy of immune checkpoint blockades on inhibiting immune checkpoint expression was totally lost (Fig. 5A–D). PD-L1 and PD-L2, major ligands of PD-1, were highly expressed in the Flt3L and immune checkpoint blockade combinational treatment group, indicating that a strong antitumor immune response happened (Fig. 5E and F). These data suggested that expanding $CD103^+$ DCs synergized with immune checkpoint blockades via increasing tumor-infiltrating CD8 T cells and regulating immune checkpoint expression.

Flt3L and Immune Checkpoint Blockade Combination Significantly Enhanced Overall Antitumor Immune Response

As our data indicated that Flt3L could synergize with immune checkpoint blockades via efficiently expanding $CD103^+$ DCs, tumor-infiltrating CD8 T cells, and regulating immune checkpoint expression, we further measured overall antitumor immunity in the murine glioblastoma tissues. In terms of the overall antitumor immunity, we focused on the level of IL-2, IFN-γ, TNF-α, and granzyme B in tumor tissues. As expected, levels of these proinflammatory factors were significantly increased by the Flt3L and immune checkpoint blockade combination in murine glioblastoma tissues (Fig. 6).

DISCUSSION

Glioblastoma, a type of malignant tumor found in the brain, is the most common type of glioma[23]. Glioblastoma is characterized by a high proliferation of tumor cells, intense angiogenesis, and a threatening location in the brain[24]. Because of its aggressive nature, the prognosis

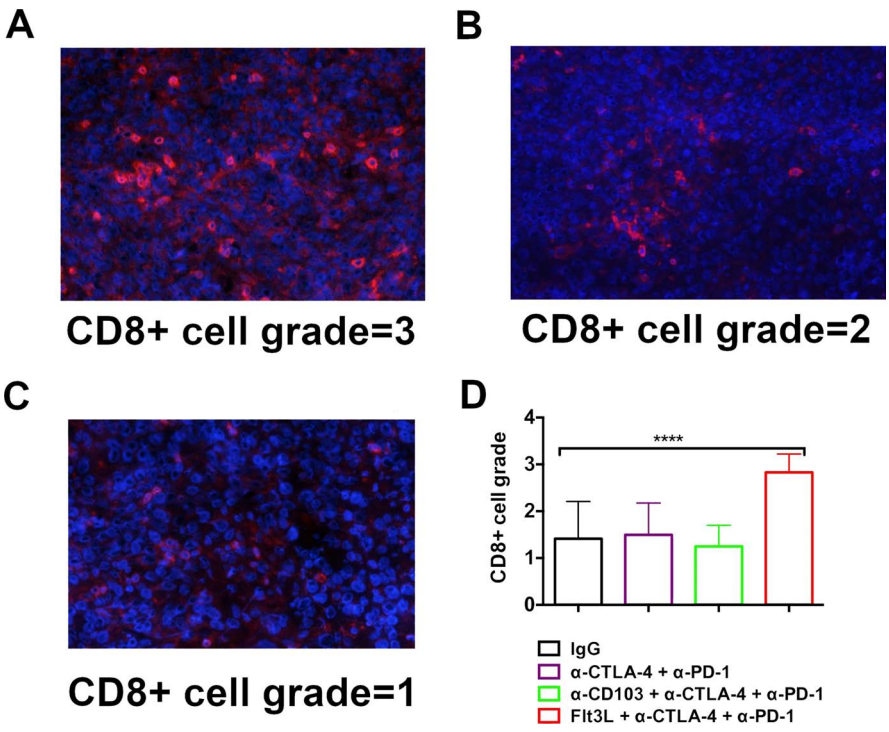

Figure 4. CD8 T cells in tumor tissues of glioblastoma mouse model after Flt3L and immune checkpoint blockade treatment. (A–C) Representative pictures showing the tumor tissue of glioblastoma mouse models with high (grade 3), medium (grade 2), and low (grade 1) $CD8^+$ cell numbers. (D) The mean grades of $CD8^+$ cells in the tumor tissue of the glioblastoma mouse model. Mice treated with Flt3L + anti-CTLA-4 Abs + anti+PD-1 Abs had the highest mean grade of $CD8^+$ cells, whereas mice treated with anti-CD103 Abs + anti-CTLA-4 Abs + anti+PD-1 Abs had the lowest level of $CD8^+$ cells. ****$p < 0.0001$.

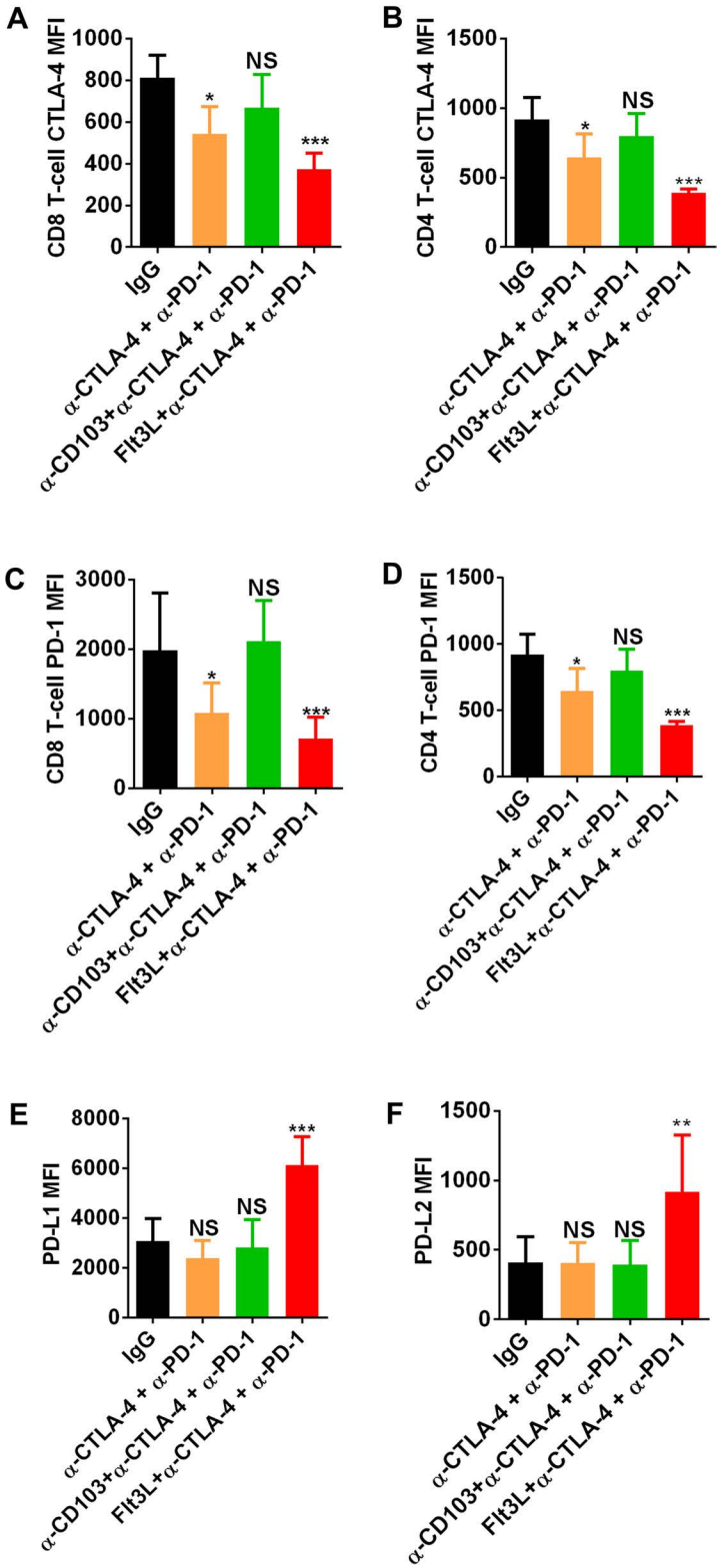

Figure 5. Expression of immune checkpoints after Flt3L and immune checkpoint blockade treatment. (A, B) Expression of CTLA-4 on CD8 and CD4 T cells was inhibited by immune checkpoint blockades. Flt3L further inhibits CTLA-4 expression in addition to immune checkpoint blockades. (C, D) Expression of PD-1 on CD8 and CD4 T cells was inhibited by immune checkpoint blockades. Flt3L further inhibit PD-1 expression in addition to immune checkpoint blockades. (E, F) Flt3L and immune checkpoint blockade combinational treatment induced expression of PD-L1 and PD-L2, indicating a responding phenotype. *$p<0.05$, *** $p<0.001$.

of glioblastoma is very poor even with the standard chemotherapy[6]. Therefore, finding novel therapies that have mechanisms to eliminate tumors that differ from traditional chemotherapy is critical for glioblastoma. Immunotherapy is a joint name for therapies that target immune cells in order to eliminate tumors[16,25]. Recently, immune checkpoint blockades have shown exciting anti-tumor effects in melanoma, lung cancer, and renal cancer patients[25–28]. However, the effect of immune checkpoint blockades in glioblastoma is still unclear. In the present

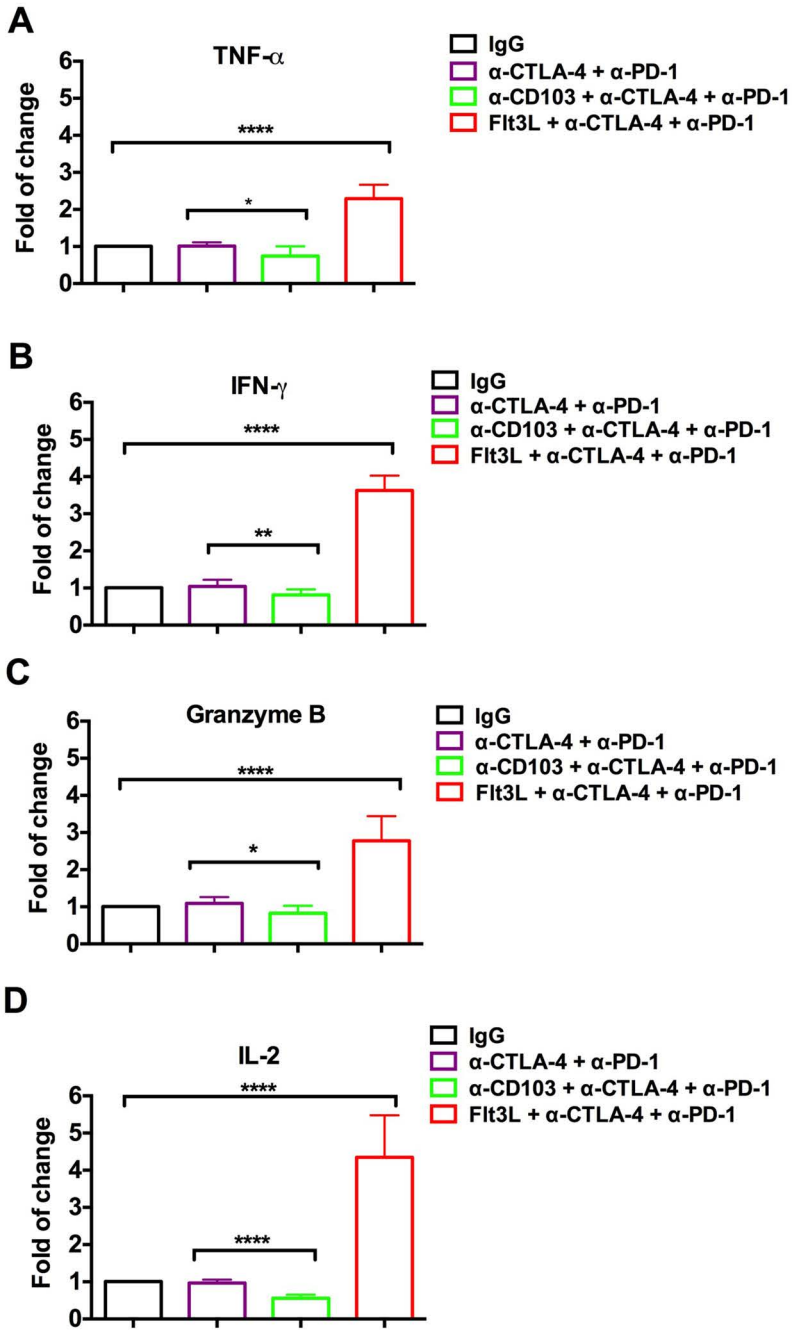

Figure 6. Antitumor cytokine expression after Flt3L and immune checkpoint blockade treatment. Tumor tissue from the glioblastoma mouse models was collected, and their cytokine levels were measured by ELISA. (A–D) Significantly increased TNF-α, IFN-γ, granzyme B, and IL-2 were observed within the tumor tissue from mice treated by Flt3L + anti-CTLA-4 Abs + anti-PD-1 Abs. Immune checkpoint blockade alone (anti-CTLA-4 Abs + anti-PD-1) did not alter the level of these cytokines apparently, whereas anti-CD103 Abs + anti-CTLA-4 Abs + anti-PD-1 treatment decreased the expression of these cytokines. ****$p < 0.0001$.

study, we aimed to clarify this issue in glioblastoma preclinical models.

Although immune checkpoint blockades showed significant clinical benefits in certain cancer patients, more patients were resistant due to the immune-exclusive nature of the tumors[29,30]. In our preclinical model of glioblastoma, we found that preclinical tumors are highly resistant to immune checkpoint blockades, even with a combination of two drugs in a high dose. Because we found that a high number of $CD103^+$ cells was correlated with better survival, we further regulated the number of $CD103^+$ cells to enhance the efficacy of immune checkpoints in glioblastoma preclinical tumors. Interestingly, by expanding the $CD103^+$ cell population with its growth factor, the tumors showed a significantly enhanced response to immune checkpoint blockades. In contrast, when $CD103^+$ cells were depleted, tumors were not only unresponsive to immune checkpoint blockades but also more aggressive. Our data showed the same trends as in previous reports that expanding the $CD103^+$ cell population led to a better outcome by enhancing antitumor immunity.

$CD8^+$ T cells are the major cytotoxic cells that cause tumor cell death[31]. When combined with $CD103^+$ growth factor, tumors showed more $CD8^+$ T-cell infiltration after treatment with immune checkpoint blockades. Other cytotoxic mediators such as TNF-α, granzyme B, and IFN-γ were also increased when $CD103^+$ cell growth factor was combined with immune checkpoint blockades. The high expression of PD-L1 by feedback mechanisms was considered to be a marker of immune checkpoint blockade response[20,32]. Here we found that tumors treated with $CD103^+$ cell growth factor showed a higher PD-L1 expression after immune checkpoint blockade treatment. Taken together, these data suggest that the efficacy of immune checkpoint blockades was promoted by targeting $CD103^+$ cells with its growth factor Flt3L in the glioblastoma preclinical model.

CD103 was found to be expressed in DCs and T cells[33,34]. To further narrow down the synergizing role of the immune checkpoint blockade in the $CD103^+$ cell growth factor, we measured the number of $CD103^+$ DCs and $CD103^+$ T cells. Interestingly, our data indicated that $CD103^+$ DCs are more sensitive to the growth factor and expanded significantly. These data suggested that the expansion of the $CD103^+$ DC population may be one of the predominant and initiating factors that modulate immune intensity in glioblastoma.

Taken together, the data in our study indicate that the $CD103^+$ cell growth factor Flt3L could synergize with immune checkpoint blockades in the murine glioblastoma model. Mechanistically, Flt3L efficiently expanded $CD103^+$ DCs, thereby leading to more CD8 T-cell infiltration, an immune-responsive immune checkpoint expression prolife, and a stronger antitumor immune response in murine glioblastoma. Further studies investigating the combination of $CD103^+$ cell growth factor Flt3L with immune checkpoint blockades in more glioblastoma models and early phase clinical trials are therefore highly needed.

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