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CD30 ligand deficiency accelerates glioma progression by promoting the formation of tumor immune microenvironment

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ABSTRACT

CD30 ligand (CD30L, CD153), belonging to the tumor necrosis factor superfamily, has been reported to act as an immune regulator mainly in several autoimmune diseases and Hodgkin's lymphoma. However, little is known about its regulation in the glioma microenvironment. In this study, using a GL261 mouse glioma model, we showed that CD30L deficiency in the host accelerated glioma growth and reduced mouse survival, which might be associated with the accumulation of tumor-infiltrating immune cells, especially tumor-associated macrophages, myeloid-derived suppressor cells and $CD8^+$ $PD\text{-}1^+$ T cells. Moreover, CD30L deficiency resulted in distinct subsets of tumor-associated macrophages compared with those of wild-type mice. Furthermore, compared with those of wild-type mice, tumor-associated macrophages and microglia in CD30L-deficient mice adopted a more pro-tumorigenic phenotype within tumors. $CD8^+$ T cells in CD30L-deficient mice decreased the expression of ki-67. Therefore, these results suggest that CD30L deficiency promotes the exhaustion of $CD8^+$ T cells and the infiltration of tumor-associated macrophages and microglia. Our findings provide evidence for a new potential immunotherapy for glioma targeting CD30/CD30L signaling.

1. Introduction

Gliomas are the most common primary malignancies of the central nervous system (CNS), with poor prognosis and a high mortality rate [1]. The glioma microenvironment contains tumor cells and many different noncancerous cells, including astrocytes, pericytes, endothelial cells, and a range of immune cells. Immune cells mainly contain tumor-associated macrophages (TAMs), microglia, myeloid-derived suppressor cells (MDSCs), $PD1^+CD8^+$ T cells and so on, which play an important role in tumor progression and response to treatment [2,3].

TAMs in glioma derive mainly from circulating blood monocytes and resident microglia [4]. The glioma-infiltrating TAMs can be both pro-tumorigenic and pro-angiogenic [5,6]. According to analysis of patient glioma tissue, TAMs decrease the production of pro-inflammatory cytokines and downregulate the expression of key molecules involved in T cell costimulation, such as CD80, CD86, and CD40. Therefore, these cells have poor capability to activate T cells in glioma [7]. MDSCs are a heterogeneous group of cells that comprise myeloid progenitor cells and immature myeloid cells [8]. MDSCs increase the secretion of arginase I and NO in the serum of glioma patients [9–11]. NO suppresses the function of T cells through the inhibition of JAK3 and STAT5 function and the promotion of apoptosis in T cells [11–13]. In addition, the upregulation of arginase I in MDSCs promotes the catabolism of L-arginine. L-Arginine promotes the proliferation of $CD8^+$ T cells [14]. Therefore, MDSCs can suppress anti-tumor responses

Abbreviations: CD30LKO, CD30L knock-out C57BL/6; CNS, central nervous system; CTL, cytotoxic $CD8^+$ T lymphocyte; DC, dendritic cell; G-MDSC, granulocytic MDSC; HRS, Hodgkin and Reed-Sternberg; IL-1β, interleukin-1β; MDSC, myeloid-derived suppressor cell; MFI, mean fluorescence intensity; MHC, II class II major histocompatibility complex; M-MDSC, monocytic MDSC; PAGE, polyacrylamide gel electrophoresis; PFA, paraformaldehyde; TAM, tumor-associated macrophage; TGF-β, transforming growth factor-β; TNFSF, tumor necrosis factor superfamily

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mediated by $CD8^+$ T cells. Glioma-infiltrating $CD8^+$ T cells derived from glioblastoma tissue express $CD8^+$ $CD25^-$, which indicates a lack of activation [2,7]. These glioma-infiltrating $CD8^+$ T cells upregulate the expression of PD-1 and become inhibited by immune checkpoint signaling pathways, which promote the progression of glioma [15]. In addition, TAMs secrete several cytokines, such as interleukin (IL)-1β, IL-6, IL-10 and transforming growth factor-β (TGF-β), that suppress $CD8^+$ T cell responses within tumors [16].

CD30 ligand (CD30L, CD153), the ligand for CD30, is a 26–40 kDa type II membrane-associated glycoprotein belonging to the tumor necrosis factor superfamily (TNFSF) [17]. CD30L is expressed on activated $CD8^+$ T cells, γδ T cells, macrophages, dendritic cells (DCs), and $CD4^+$ $CD3^-$ $CD11c^-$ accessory cells [18]. The expression of CD30L on $CD4^+$ and $CD8^+$ T cells can stimulate their proliferation and increase the secretion of cytokines such as IL-2, IL-4, IFN-γ and TNF-α [19]. Meanwhile, negative regulatory effects have been reported in the CD30/CD30L signaling pathway. The $CD30^+$ large-cell anaplastic lymphoma cell lines can have their proliferation attenuated cytolytic cell death induced by CD30L binding [20]. Therefore, we reasoned that targeting CD30L in the glioma microenvironment may alter the state of tumor-infiltrating immune cells and influence glioma progression.

To test this hypothesis, glioma expansion and survival time were examined by using wild-type (WT) and CD30LKO mice, which were intracranially injected with the GL261 glioma cell line. Our results demonstrated that deficiency of CD30L might promote the progression of glioma. The enhancement of glioma progression was associated with increased tumor-infiltrating TAMs, MDSCs and PD-1^+ $CD8^+$ T cells. We then found that TAMs and microglia in CD30LKO mice adopted a more pro-tumorigenic phenotype and altered the immune response to the glioma. These results supported the notion that manipulation of CD30L could influence the progression of glioma.

2. Materials and methods

2.1. Mice

Age- and sex-matched C57BL/6J (B6, as WT mice) male mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The generation and preliminary characteristics of CD30LKO mice were described previously [21], and eight or more generations backcrossed onto WT mice. All mice were maintained under specific pathogen-free conditions and were offered food and water ad libitum. All mice were used at 6–8 week of age. This study was approved by the Animal Welfare and Ethics Committee of China Medical University (IACUC No. 2018054). Experiments were performed under the control of the Guidelines for Animal Experiments.

2.2. Cell lines and tumor implantation

The murine GL261 glioma cells were bought by Chinese Academy of Sciences and is derived from a chemically-induced C57BL/6 murine astroglioma [22]. This cell line was stably transfected with EGFP (GL261-EGFP) (Shanghai Genechem Co.) and maintained in DMEM (Biological Industries) supplemented with 10% FBS (Gibco), penicillin and streptomycin (Solarbio). Gliomas were established in mice as previously described [23]. Briefly, WT or CD30LKO mice were anesthetized and immobilized in a stereotactic head frame, and 5×10^5 GL261-EGFP glioma cells in 3 μL PBS were stereotactically injected into the right striatum: 1 mm anterior, 2 mm lateral and 3 mm deep relative to the bregma, using a 5 μL Hamilton syringe.

2.3. Hematoxylin-eosin staining

Twenty-one days after GL261-EGFP injection, brains were isolated after cardiac perfusion. The coronal brain beneath the injection track was cut into two parts. Tissues were fixed in 4% paraformaldehyde (PFA). Then, the tissue was surrounded with OCT (Solarbio) and frozen in a −80 °C refrigerator. Coronal brain was sectioned (5 μm) using a Leica cryostat and stained with hematoxylin and eosin (Solarbio).

2.4. Tumor volume analysis

21 days after GL261-EGFP injection, brains were isolated after cardiac perfusion and fixed in 4% buffered formaldehyde for morphological evaluation. According to the previous article [24], we calculated the tumor volume. The authors assumed that tumors were similar to the shape of a sphere and the longest radius was in the site of the injection track. We cut the brain from the injection track into two parts and cut three series of sections from one part. The brain section is 10 μm. We calculated an average radius from these sections and used the spherical formula $4/3\pi r^3$ to obtain the tumor volume.

2.5. Immunofluorescent staining

Brain sections were washed with PBS and blocked with 10% normal serum blocking for 2 h. Then the sections were incubated with primary antibodies for anti-CD31 (1:200, Servicebio) for 16 h at 4 °C, and followed by PE conjugated secondary antibodies (Servicebio). After stained by DAPI (Solarbio), sections were examined with confocal microscope (Thermo).

2.6. Western blot analysis

Mice were perfused before dissection. The glioma tissues and morphologically normal tissue adjacent to the glioma tissues (designated para-carcinoma tissue) were harvested from GL261-EGFP-injection mice. The normal brain tissues were detached from normal mice. The brain tissues were lysed in 1 × RIPA (Beyotime), supplemented with PMSF (Beyotime). Protein concentration was tested by Nanodrop (Thermo) and 20 μg of protein was added to SDS-polyacrylamide gel electrophoresis (PAGE) after boiling for 6 min in 5 × SDS loading buffer (Beyontime). Proteins were electrophoresed at 80 V for 30 min and followed by 120 V for 1 h, then transferred to a polyvinylidene difluoride membrane (0.2 μm, Millipore) at 200 mA for 1 h. After blocked with 2% nonfat milk at room temperature for 1 h, the membranes were incubated with antibodies. Primary antibody of CD30L (1:1000, Thermo) and β-actin (1:2000, Proteintech) was incubated overnight at 4 °C. After incubating with horseradish peroxidase-conjugated secondary antibody (1:10,000, Proteintech), proteins were visualized using an enhanced chemiluminescence reagent (Wanleibio).

2.7. Flow cytometry

Lymphocytes were isolated from GL261-EGFP-injected brain as previously described [24]. Mice were anesthetized and perfused with PBS. Brains were mechanically homogenized, and digested in 2 mg/mL collagenase II (Sigma) and 100 ng/mL Dnase I (Sigma) at 37°C for 45 min. Digestion was terminated by adding 2 volumes of RPMI medium containing 10% FBS. Cells were passed through a 70 μm cell strainer, and separated using a discontinuous Percoll (GE Healthcare) gradient (30%/70%). Surface staining was performed with fluorescein conjugated antibodies in PBS after pretreating with Fc block (24G2). For intracellular staining of Ki-67, we followed the protocol of manufacturer in the presence of Transcription Factor Buffer Set (BD Pharmingen). For intracellular cytokine staining, cells were stimulated with 25 ng/mL of PMA (Sigma) and 1 μg/mL of ionomycin (Sigma) for 5 h at 37 °C, and 10 μg/mL of brefeldin A (Sigma) was added for the last 4 h of culture. After culture, cells were harvested and surface stained, and then intracellular staining was performed according to the manufacturer's instructions (BD Pharmingen). Antibodies used in the study include: anti-CD45.2-APC-Cy7 (Biolegend), anti-CD11b-BV421 (Biolegend), anti-KI-67-PE (Biolegend), anti-CD8-BV785 (Biolegend), anti-

PD-1-PE (Biolegend), anti-Ly6C-BV421 (Biolegend), anti-Ly6G-PE (Biolegend), anti-I-A/I-E-V500 (BD Pharmingen), anti-CD11b-APC (Biolegend), anti-CD206-PE (Biolegend), anti-F4/80-APC (Biolegend), anti-CD80-PE (Biolegend), anti-IFN-γ-PE (Biolegend), anti-TNF-α-BV421 (Biolegend), anti-CD8-FITC (Biolegend) and anti-PD-L1-APC (Biolegend). All data were collected on a BD FACSCelesta flow cytometer and analyzed by FlowJo 10 software (BD Pharmingen).

2.8. Isolation and activation of glioma-infiltrating $CD8^+$ T cells

Total $CD8^+$ T cells isolated from GL261-EGFP-injected brain by positive selection using anti-mouse CD8 microbeads (Miltenyi Biotec) were activated by anti-CD3 (Biolegend) and anti-CD28 (Biolegend) at 2 μg/mL on precoated plates based on previous studies [25]. After 16 h, $CD8^+$ T cells were harvested from plates and analyzed by flow cytometry.

2.9. Depletion of $CD8^+$ T cells

WT and CD30LKO mice were administered 200 μg rat-anti-CD8 antibodies (Bio-X-Cell) i.p. on days 7, 5 and 3, followed by Gl261-EGFP cells injection on day 0.

2.10. In vivo treatment of mice with mCD30-Ig

The mCD30-Ig were obtained as previously described [26]. At 7 and 12 days after GL261-EGFP tumor injection, 200 μg of mCD30-Ig was injected via i.v.

2.11. Statistical analysis

Survival was analyzed by Log-rank test. Statistical significance was calculated by *t*-test. All data were analyzed using GraphPad Prism 7 and differences with *P*-values of < 0.05 were considered statistically significant.

3. Results

3.1. CD30L deficiency results in promotion of glioma progression

To explore the role of CD30L in the formation of the glioma microenvironment, GL261-EGFP cells were injected into the brains of WT or CD30LKO mice. To examine the level of CD30L expression in brain tissue of normal and GL261-bearing WT mice, we tested brain tissue by western blot on day 21 post injection. As shown in Fig. 1a, the expression of CD30L was higher in para-carcinoma tissue and tumor tissue of GL261-bearing WT mice than in normal tissue of WT mice, indicating that CD30L may have an effect on glioma progression. The assessment of survival and tumor size of GL261-bearing mice showed that CD30LKO mice had a shorter survival curve and larger tumor size than those of WT mice, suggesting that tumor progression was accelerated in CD30LKO mice. On day 21 post injection, CD30LKO mice had larger tumors with significantly higher vessel density than those observed in WT mice (Figs. 1b and 2). To determine whether CD30 signaling via CD30L is involved in the formation of the glioma microenvironment, we injected mCD30-Ig into GL261-EGFP-implantated WT mice via i.v. The results showed that WT mice receiving mCD30-Ig had a shorter survival curve and larger tumor size than WT mice (Fig. 1b–d). These results demonstrate that GL261-bearing WT mice upregulate the expression of CD30L in the brain and that the lack of CD30L in the tumor microenvironment promotes the growth of glioma.

3.2. CD30L deficiency results in the accumulation of host immune cells in glioma

To identify the contributor in the glioma microenvironment promoting glioma progression in CD30LKO mice, we isolated and tested the glioma-infiltrating immune cells, which have been shown to be related to the prognosis of patients with high-grade astrocytoma [27], by flow cytometry 21 days after GL261-EGFP injection. Approaches to distinguish glioma-infiltrating immune cells have traditionally relied on the use of CD45 and CD11b antibodies to separate resident microglia ($CD45^{low}$ $CD11b^+$) from TAMs ($CD45^{hi}$ $CD11b^+$) and lymphocytes ($CD45^{hi}$ $CD11b^-$) of hematopoietic origin ($CD45^{hi}$) [28]. As shown in Fig. 3a, the number of total cells was relatively higher in CD30LKO mice than in WT mice. Furthermore, the frequency and absolute number of TAMs and microglia significantly increased in CD30LKO mice relative to that in WT mice (Fig. 3b). Meanwhile, the cell number of lymphocytes was strongly upregulated in CD30LKO mice compared to that in WT mice (Fig. 3b). Therefore, we speculate that glioma-infiltrating immune cells within the brain may contribute to the promotion of glioma progression in CD30LKO mice.

3.3. CD30L deficiency alters the distribution of different TAMs and MDSC subsets in glioma

TAMs in glioma derive from infiltrating bone marrow-derived monocytes and resident microglia [29], so TAMs are a mixed population. To gain insights into the compartments of TAMs in GL261-EGFP-bearing mice, we analyzed TAM subsets by flow cytometry 21 days after tumor implantation. After reaching the tumor, monocytes undergo a specific maturation pathway traceable by changes in the expression of surface markers, especially the loss of Ly6C and the acquisition of class II major histocompatibility complex (MHC II) molecules [30]. A recent study showed that $Ly6C^{hi}$ MHC II^- cells are $Ly6C^{hi}$ monocytes and $Ly6C^-$ MHC II^+ cells are TAMs. Moreover, $Ly6C^{lo}$ MHC II^- cells are eosinophils and neutrophils [31]. The population of TAMs in glioma was heterogeneous and encompassed four subsets, which could be distinguished based on differential expression of MHC II and Ly6C (Fig. 4a). The frequencies and absolute numbers of $Ly6C^{hi}$ MHC II^- cells (gate 1: Fig. 4a), $Ly6C^{lo}$ MHC II^- cells (gate 2: Fig. 4a) and $Ly6C^+$ MHC II^+ cells (gate 4: Fig. 4a) in TAMs of CD30LKO mice significantly increased compared with those in WT mice. Compared with that of WT mice, the frequency of $Ly6C^-$ MHC II^+ cells (gate 3: Fig. 4a) in TAMs of CD30LKO mice significantly decreased, yet the number of $Ly6C^-$ MHC II^+ cells (gate 3: Fig. 4a) was relatively higher in TAMs of CD30LKO mice than those of WT mice.

TAMs are considered to be facilitators of tumor progression because of their immunosuppressive properties. Among these cells are those termed MDSCs [32]. In mice, MDSCs broadly express both CD11b and Gr-1 surface markers, and they can be further subdivided into $Ly6G^+$ $Ly6C^{lo}$ granulocytic MDSCs (G-MDSCs) and $Ly6G^-$ $Ly6C^{hi}$ monocytic MDSCs (M-MDSCs) [33]. We analyzed subtypes of MDSCs within tumors by flow cytometry 21 days after GL261-EGFP injection. As shown in Fig. 4b, compared with WT mice, the frequency and cell number of M-MDSCs and G-MDSCs in CD30LKO mice increased markedly. These results indicate that CD30L deficiency promotes the infiltration of TAM and MDSC subsets.

3.4. CD30L deficiency alters the phenotype of TAMs and microglia in glioma

To further characterize the population of TAMs and microglia, we assessed the expression of active and inhibitory molecules in the TAMs and microglia at 21 days after tumor implantation. We used flow cytometry to analyze the phenotype of TAMs and microglia. TAMs and microglia can express the general macrophage marker CD206, a marker for alternatively activated macrophages, which contributes to immunosuppression [34]. PD-L1 is expressed in infiltrating TAMs and activated microglia, which could account for T cell dysfunction and apoptosis [10,35]. Ki-67 is the most widely used marker for proliferation [36]. Flow cytometric analysis showed that the inhibitory receptor

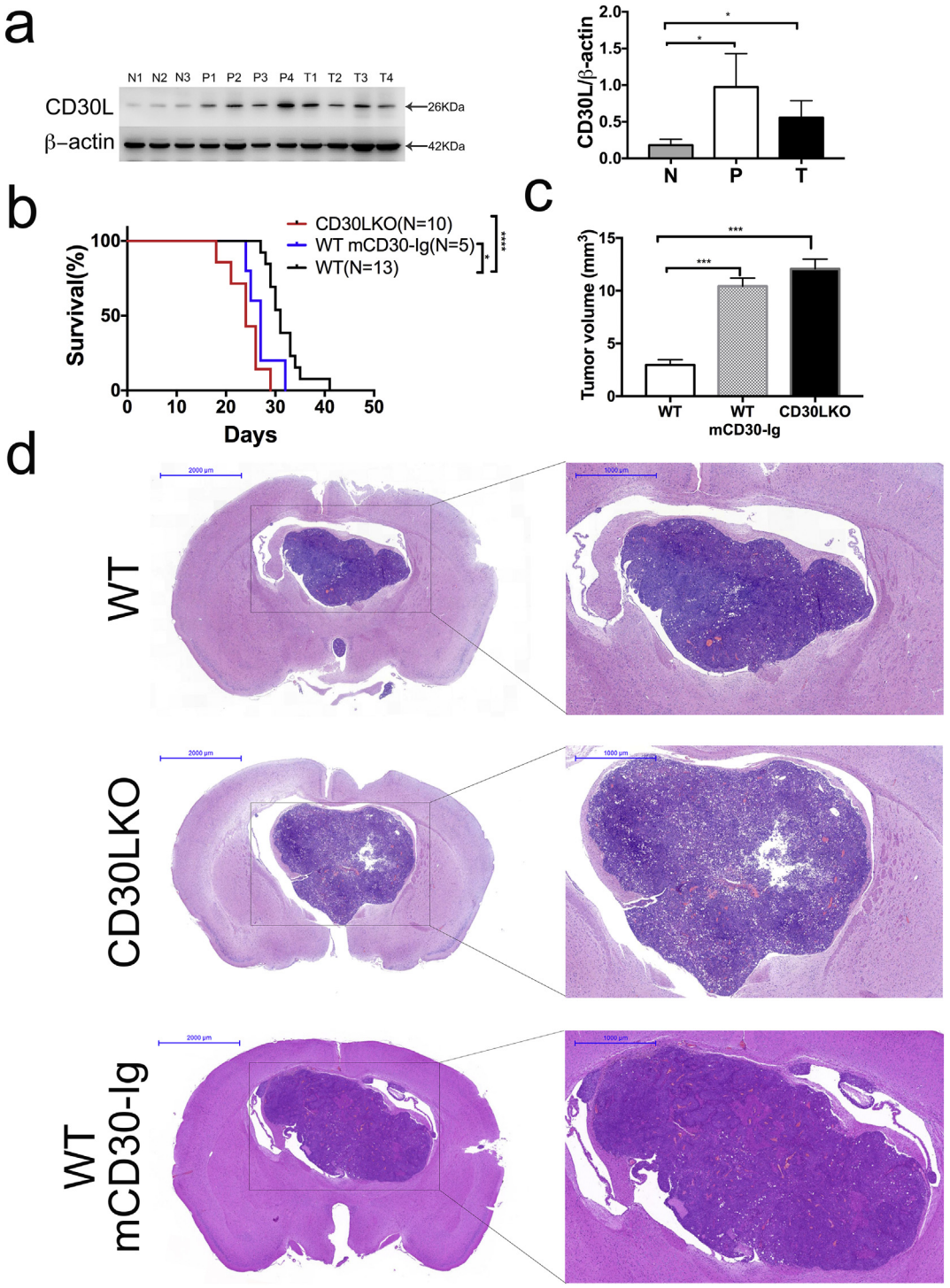

Fig. 1. CD30L-depleted mice accelerate glioma growth. WT and CD30LKO were injected with 5×10^5 GL261-EGFP glioma cells. (a) The expression of CD30L protein was detected by western blot in normal tissue (N), para-carcinoma tissue (P) and tumor tissue (T). The gray level of CD30L was expressed in normal tissue (N), para-carcinoma tissue (P) and tumor tissue (T). (b) The survival duration of the mice was monitored and is shown in a Log-rank test survival curve (*, $P < 0.05$; ****, $P < 0.0001$). (c) Tumor volume was calculated at day 21 post injection (***, $P < 0.001$). (d) Representative H&E stained slices from GL261-bearing WT, CD30LKO and WT with mCD30-Ig mice at day 21 post injection. Magnification, ×1, ×2.

PD-L1 and proliferation marker ki-67 were significantly upregulated on TAMs and microglia in CD30LKO mice when compared with those in WT mice (Fig. 5a and c). Moreover, the number of $CD206^+$ TAMs and $CD206^+$ microglia was relatively higher in CD30LKO mice than in WT mice (Fig. 5b). Therefore, we speculated that TAMs and microglia might express molecules associated with immunosuppression in glioma.

In addition, we evaluated the expression of MHC II and CD80, which are costimulatory ligands and important for supporting immune cell responses [37]. Additionally, we examined the expression of F4/80, a mature phagocytic cell marker [24]. The MFI and frequency of F4/80 and CD80 in TAMs and microglia were significantly downregulated in CD30LKO mice relative to those in WT mice. In addition, we find that the MFI of MHC II was significantly downregulated on microglia in CD30LKO mice when compared with that in WT mice (Fig. 5d–f). These

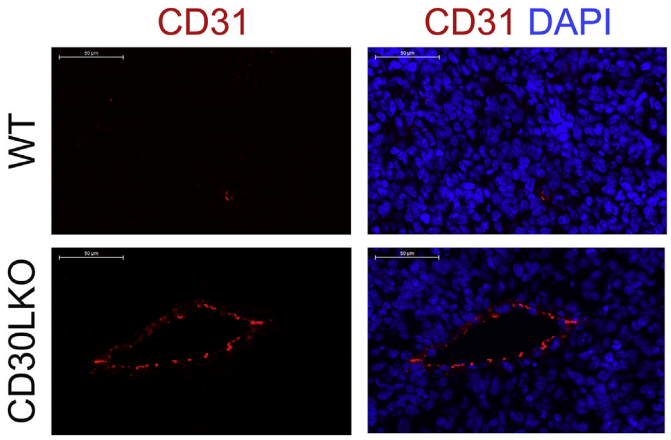

Fig. 2. Representative images of CD31 (red) staining with DAPI (blue) on brain sections of WT and CD30LKO mice at day 21 post glioma injection. Magnification, ×40. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

results might conclude that the lower expression of CD80, F4/80 and MHC II in TAMs and microglia in CD30LKO mice reflects their less active phenotype than those in WT mice. Overall, CD30L deficiency may cause TAMs and microglia to be more active in supporting invasion and immunosuppression.

3.5. CD30L deficiency may lead to the upregulation of PD-1 in $CD8^+$ T cells in glioma

We then attempted to measure the immune characteristics of glioma-infiltrating $CD8^+$ T cells, which account for a majority part of $CD45^+$ $CD11b^-$ cells. Since $CD8^+$ T cells play an important role in anti-tumor immunity, we tested the role of $CD8^+$ T cells using anti-CD8 depletion antibodies in glioma. Mice received three doses of anti-CD8 antibody 7, 5 and 3 days prior to GL261-EGFP implantation. Then, we assessed the survival curve and tumor size of CD8-depleted WT and CD30LKO mice. Fig. 6a and b shows that CD8-depleted CD30LKO mice had shorter survival curves and larger tumor sizes than those in CD8-depleted WT mice, suggesting that not only $CD8^+$ T cells but also TAMs can contribute to tumor progression. We checked the expression of inhibitory and proliferation molecules on glioma-infiltrating $CD8^+$ T cells by flow analysis at day 21 after tumor injection. As shown in Fig. 7a, the frequency and absolute number of $CD8^+$ $PD\text{-}1^+$ T cells significantly increased in CD30LKO mice relative to those in WT mice. Moreover, the frequency but not the cell number of Ki-67^+ $CD8^+$ T cells was relatively lower in CD30LKO mice than that in WT mice (Fig. 7b). Because most of glioma-infiltrating $CD8^+$ T cells expressed

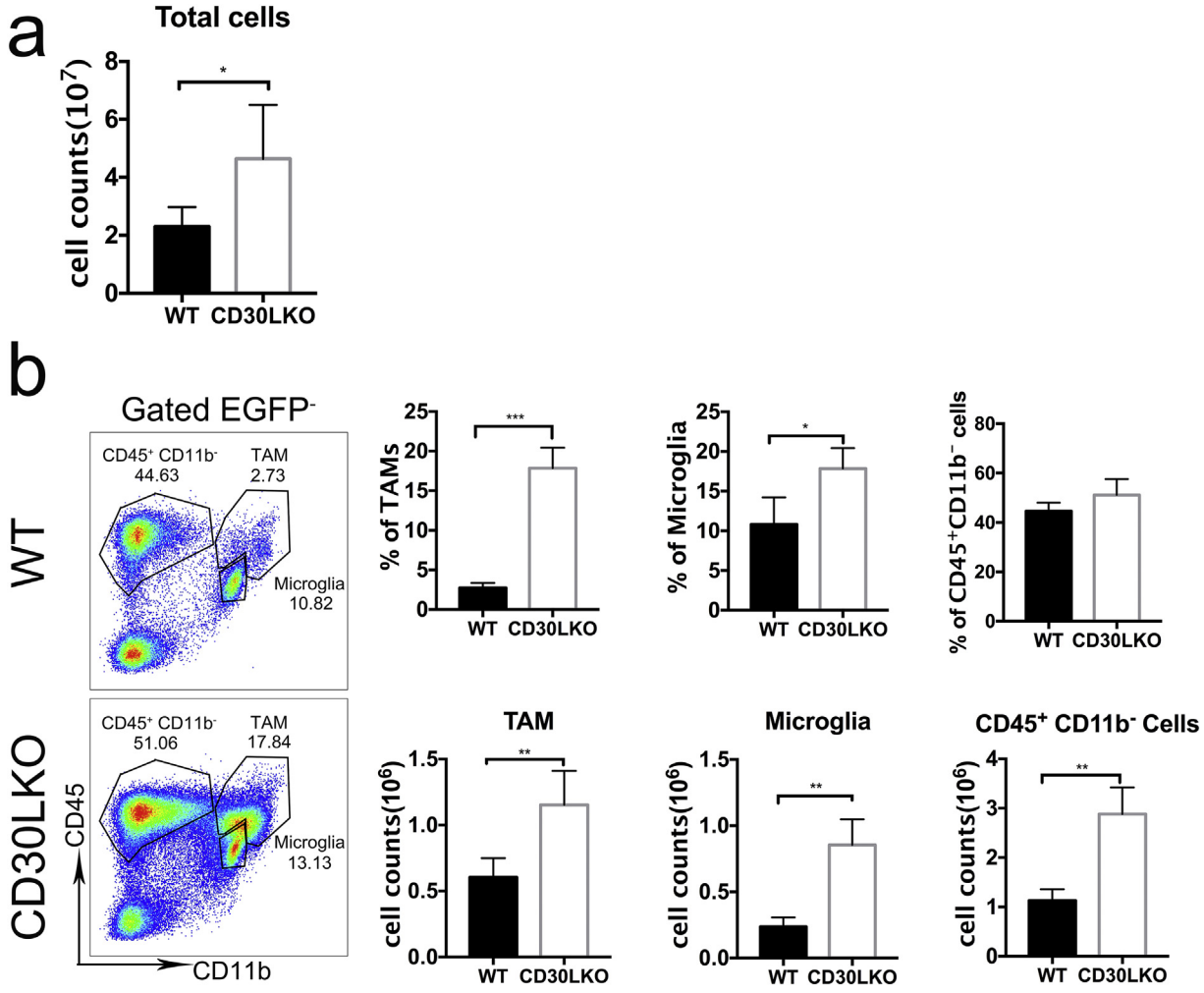

Fig. 3. CD30L deficiency promotes the infiltration of immune cells in the brains of glioma-bearing mice. (a) The absolution number of infiltrating immune cells from whole brain tissue of WT and CD30LKO mice was determined by flow cytometry 21 days after implantation. (b) Infiltrating immune cells were gated on $EGFP^-$ cells and further analyzed for TAMs, microglia and $CD45^+$ $CD11b^-$ cells. The frequencies and absolute numbers of TAMs, microglia and $CD45^+$ $CD11b^-$ cells are shown. Data shown are the mean ± SEM of representative data from 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

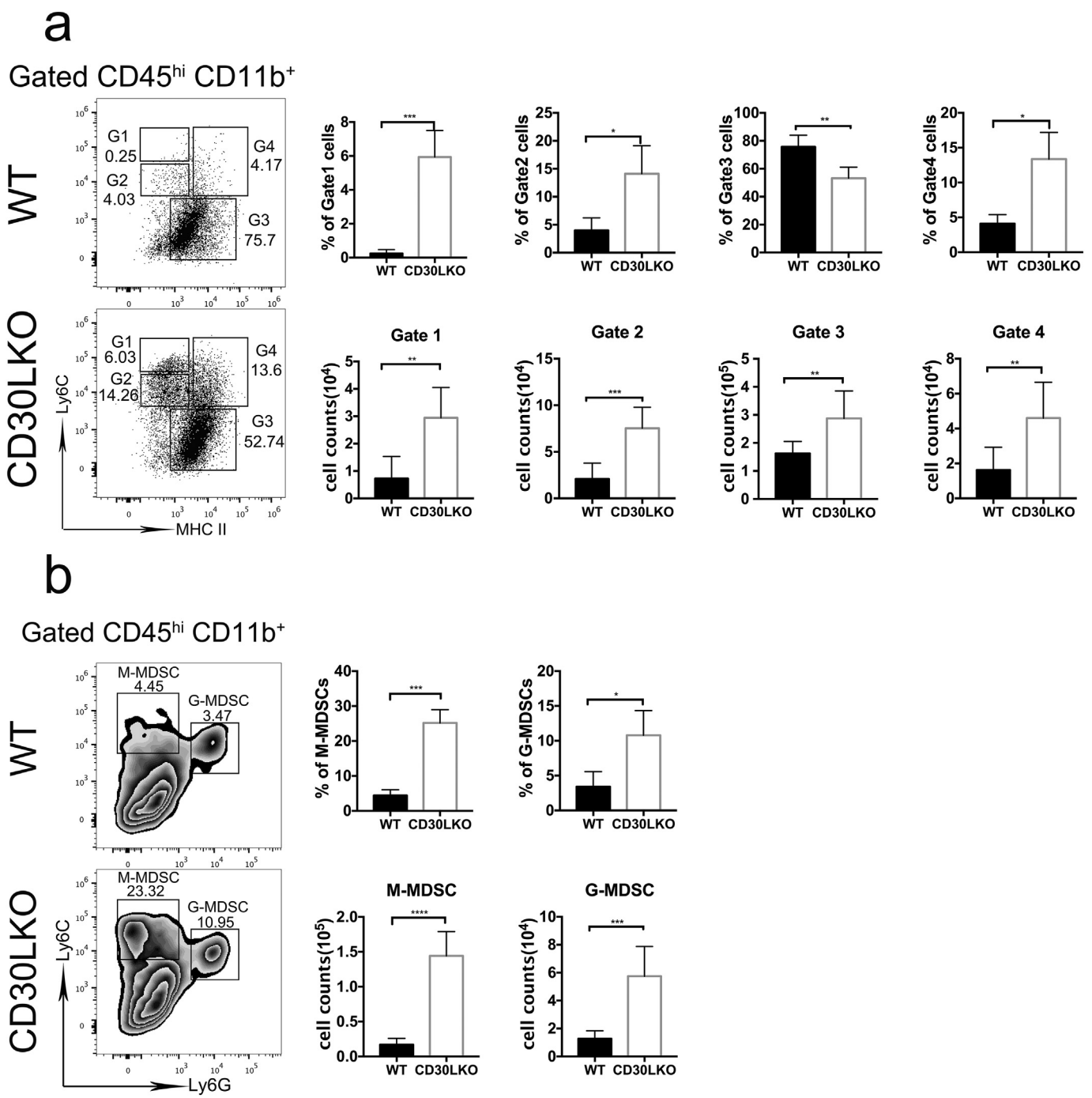

Fig. 4. Characterization of different TAM and MDSC subsets in glioma between WT and CD30LKO mice. The subsets of TAMs and MDSCs were gated on $CD45^{hi}$ $CD11b^{+}$ cells by flow cytometry 21 days after injection. (a) Frequencies and absolution numbers of different TAM subsets of in glioma are shown. (b) Frequencies and absolution numbers of MDSC subsets in TAMs are shown. Data shown are the mean ± SEM of representative data from 4 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

PD-1, intracellular IFN-γ and TNF-α, markers of T cell activation, were hardly detected by flow cytometry. We assessed glioma-infiltrating $CD8^{+}$ T cells after 16 h of ex vivo stimulation with anti-CD3/CD28 antibody (Fig. 7c). CD30LKO glioma-infiltrating $CD8^{+}$ T cells had decreased IFN-γ and TNF-α levels. Thus, compared with those of WT mice, CD30LKO glioma-infiltrating $CD8^{+}$ T cells exhibited a lower activity and anti-tumor phenotype. These results indicate that CD30L deficiency significantly augments the immunosuppressive status of $CD8^{+}$ T cells.

4. Discussion

In this article, we showed that CD30L deficiency facilitated glioma progression. Accordingly, CD30L deficiency promoted tumor growth and significantly shortened the survival time of glioma-bearing mice, suggesting that the expression level of CD30L in the glioma microenvironment plays a key role in tumor progression. The mechanism by which CD30L deficiency accelerates glioma progression may involve enhancement of the immunosuppressive immune responses mediated by TAMs, MDSCs, microglia or PD-1^{+} $CD8^{+}$ T cells in the tumor microenvironment.

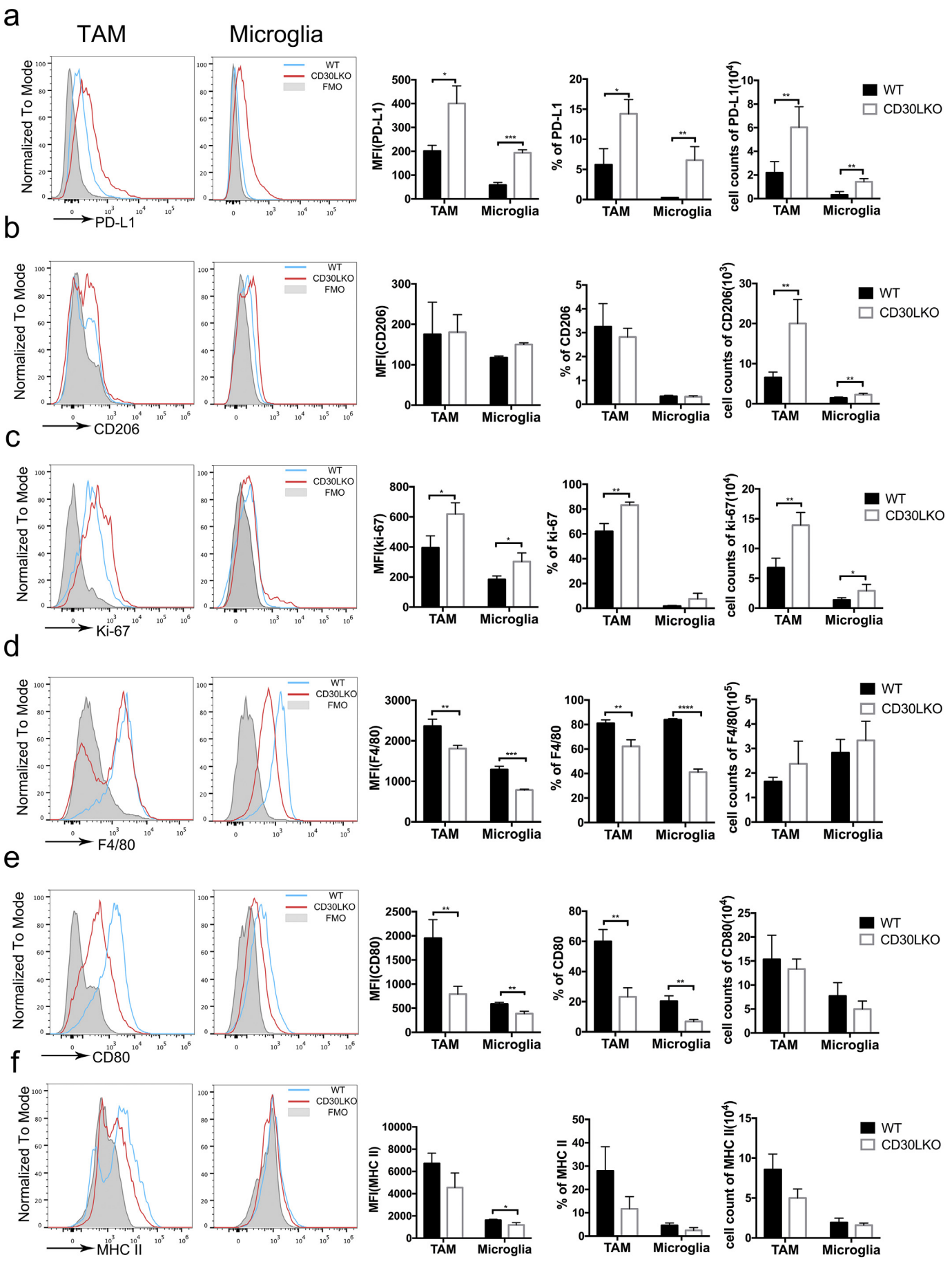

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Fig. 5. CD30L deficiency promotes a more pro-tumorigenic phenotype of TAMs and microglia. The expression of PD-L1, CD206, ki-67, F4/80, CD80 and MHC II on TAMs and microglia was evaluated by flow cytometry 21 days after implantation. The mean fluorescence intensity (MFI), percentage and absolute numbers of PD-L1 (a), CD206 (b), ki-67 (c), F4/80 (d), CD80 (e) and MHC II (f) of WT (red) and CD30LKO (blue) mice are shown. Data shown are the mean ± SEM of representative data from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

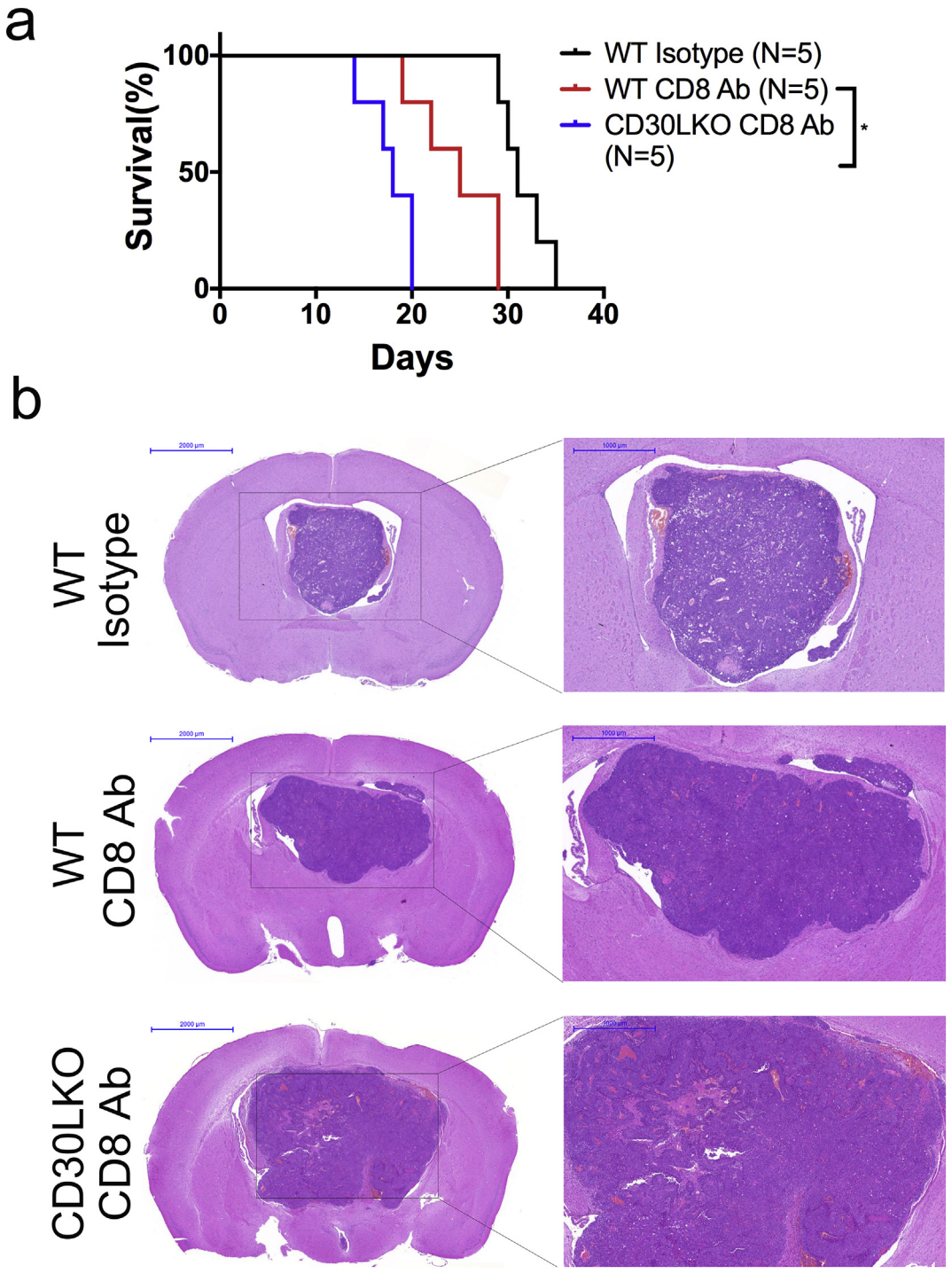

Fig. 6. The role of $CD8^+$ T cells in antitumor immunity against glioma. GL261-bearing WT and CD30LKO mice were depleted of $CD8^+$ T cells. The survival curve and H&E staining of these mice was assessed. (a) The survival duration of mice was monitored, and the Log-rank test survival curve is shown (*, $P < 0.05$). (b) Representative H&E stained slices from GL261-bearing WT and CD30LKO mice with $CD8^+$ T cell depletion at day 21 post injection are shown. Magnification, ×1, ×2.

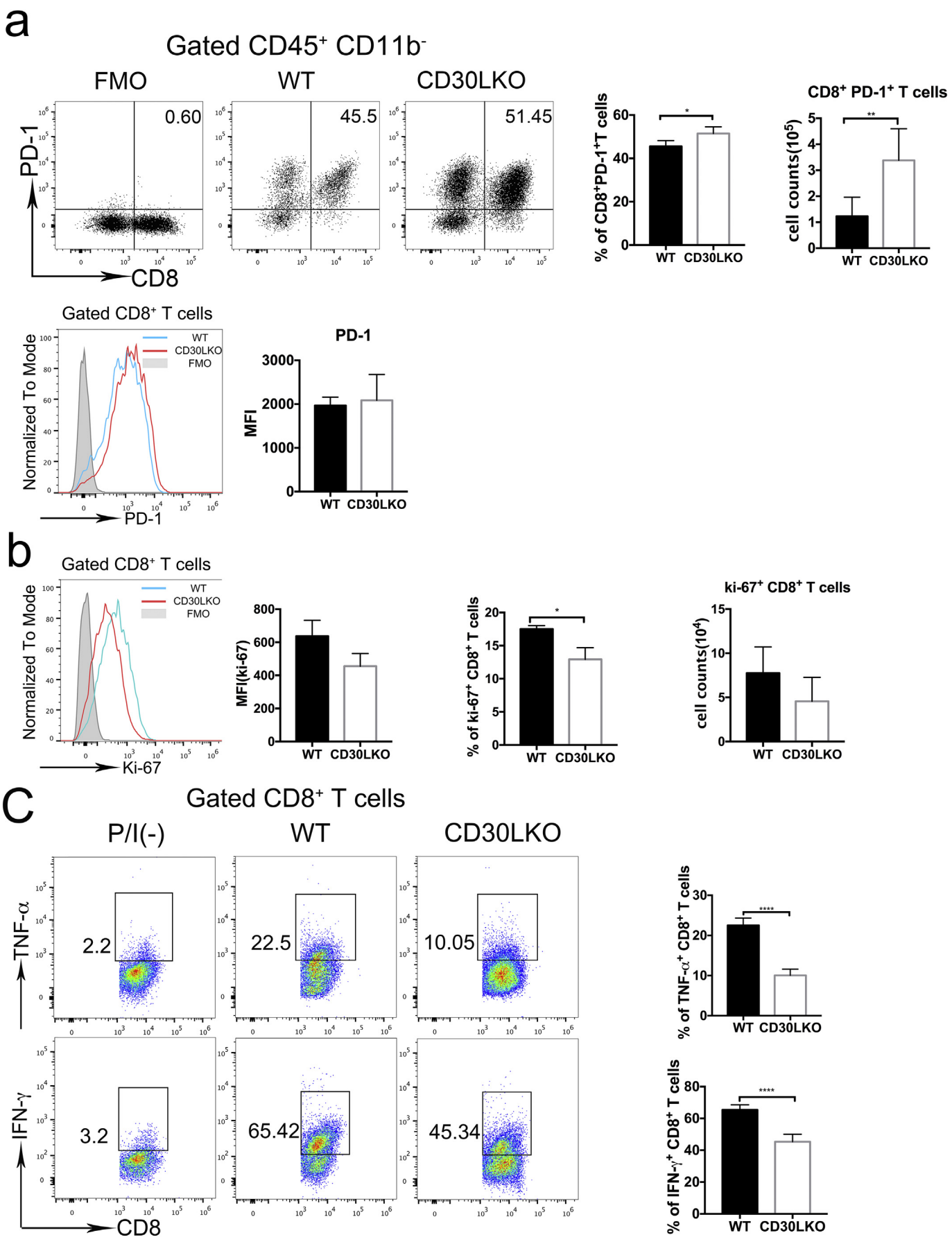

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Fig. 7. CD30L deficiency promotes the exhaustion of tumor-infiltrating $CD8^+$ T cells. Expression of inhibitory molecules PD-1 (a) and proliferation marker ki-67 (b) of glioma-infiltrating $CD8^+$ T cells are shown by flow cytometry 21 days after implantation. The expression of PD-1 and ki-67 on $CD8^+$ T cells is shown as the mean fluorescence intensity (MFI), percentage and absolution numbers. (c) Sixteen hours after stimulation of isolated glioma-infiltrating $CD8^+$ T cells with anti-CD3 and anti-CD28 antibodies, IFN-γ^+ and TNF-α^+ cells were quantified as a percentage of $CD8^+$ T cells. P/I, phorbol myristate acetate/ionomycin. Data shown are the mean ± SEM of representative data from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

CD30 was first discovered in Hodgkin and Reed-Sternberg (HRS) cells [38]. CD30Lcan promote the growth of Hodgkin's lymphoma by providing survival signals for $CD30^+$ HRS cells [39] and increasing their proliferation [40]. However, our data presented conflicting results, showing the inhibitory role of CD30L in glioma progression. We speculated that CD30L might be associated with the effect of CD30L on tumor-infiltrating lymphocytes and myeloid cells, as CD30L is expressed in immune cells and regulates the immune system [19].

In this study, WT and CD30L-deficient mice were injected with GL261 cells, allowing us to specifically examine the role of CD30L in the glioma microenvironment. Our data showed that deletion of CD30L in the host increased the tumor size and shortened the survival of glioma-bearing mice, suggesting that the glioma growth was relative to CD30L in the tumor microenvironment, rather than to CD30L in the tumor cells. Therefore, unlike the tumor-promoting role in Hodgkin's lymphoma, CD30L shows a potential anti-tumor role in the glioma microenvironment.

Studies have shown that bone marrow-derived monocytes are the predominant population of infiltrating TAMs in glioma [41]. Our findings showed that in a murine glioma model, the deletion of CD30L might increase the cell number of monocytes, which increased the source of TAMs. Moreover, the total numbers of TAMs and microglia were markedly increased in the brains of GL261-bearing CD30LKO mice compared with those of WT mice because the proliferation marker ki-67 of TAMs and microglia increased in CD30LKO mice compared with that in WT mice. Therefore, CD30L deficiency in the glioma model can promote the proliferation of TAMs and microglia. Considering that TAMs and microglia in the glioma model strongly expressed CD206 in CD30LKO mice compared with those in WT mice, these cells might correspond to the M2 phenotype in CD30LKO mice [42]. Previous reports showed that most TAMs are M2-like macrophages [43], and they can facilitate the tumor progression because of their immunosuppressive properties and their promotion of angiogenesis [44]. Further analysis suggested that TAMs and microglia in glioma expressed a lower active phenotype of CD80, MHC II and F4/80, a mature phagocytic marker [24], in CD30LKO mice than that in WT mice. Hussain et al. [7] also found that TAMs decrease T cell costimulation molecules, leading to their poor ability to induce T cell responses in glioma. Therefore, the antigen presentation and phagocytosis of TAMs and microglia in GL261-bearing CD30LKO mice are reduced.

In this study, compared with those in WT mice, the cell number of TAMs and MDSCs in GL261-bearing CD30LKO mice significantly increased together. Previous data supported an association between TAMs and MDSCs [45]. Meanwhile, the association could be explained as follows. First, it is possible that TAMs are the source of MDSCs. The glioma microenvironment could cause normal monocytes to an MDSC-like phenotype [46]. Second, immunomodulatory factors produced by increased TAMs could recruit MDSCs [47].

MDSCs in mice can be subdivided into M-MDSCs and G-MDSCs [48]. Our studies indicated that GL261-bearing CD30LKO mice increased the percentage and cell number of M-MDSCs or G-MDSCs compared with those in WT mice. G-MDSCs secrete large amounts of ROS [49]. Because ROS are active for a short time, G-MDSCs need a cell-to-cell connection with $CD8^+$ T cells [50]. Direct connection of MDSCs and $CD8^+$ T cells leads to the nitration of the T cell receptor and the CD8 molecule, which inhibit the activation of T cells [51]. M-MDSCs can increase the production of Arg1 and promote the apoptosis of $CD8^+$ T cells [49]. Meanwhile, the increased level of arginase produced by M-MDSCs promotes the secretion of ROS that subsequently inhibited the proliferation of $CD8^+$ T cells [52]. The increasing number of MDSCs can play a tumor-promoting role in CD30LKO mice.

Glioma is characterized by immunosuppression in the tumor microenvironment which consists mainly of macrophages, microglia and lymphocytes [53]. Most T cells in the glioma microenvironment are exhausted. They can upregulate inhibitory receptors and decrease the production of effector cytokines, leading to tumor immune evasion [54]. In the study, a decrease in cytokine production and an increase in inhibitory molecules of glioma-infiltrating $CD8^+$ T cells derived from GL261-bearing CD30LKO mice were found when compared with those of WT mice, indicating that the $CD8^+$ T cell effector response was weakened in CD30LKO mice. PD-1 is an indicator of exhaustion [55], and the high PD-1 expression of T cells indicates the loss of anti-tumor abilities [54]. To provide evidence for a connection between TAMs and $CD8^+$ T cells, we analyzed PD-L1 expression on TAMs. We found that PD-L1 expression was significantly upregulated in TAMs and microglia of CD30LKO mice compared with those of WT mice. A previous study showed that the IFN-γ produced by $CD8^+$ T cells in the tumor microenvironment not only activates tumor killing but also induces PD-L1 expression between macrophages and tumor cells. Then, PD-1^+ $CD8^+$ T cells interacting with PD-$L1^+$ cells in the tumor microenvironment deliver an inhibitory signal to attenuate the proliferation and effector functions of $CD8^+$ T cells [56]. Moreover, the decreased proliferation ability of $CD8^+$ T cells in CD30LKO mice was confirmed, because of the downregulation of the proliferation marker ki-67 in CD30LKO mice. Thus, the exhausted $CD8^+$ T cells in CD30LKO mice can facilitate the glioma progression. Using an anti-CD8 depletion antibody, we observed the contribution of $CD8^+$ T cells to slowing tumor growth. However, there was a prolonged survival curve in CD8-depleted WT mice compared with that of CD8-depleted CD30LKO mice, indicating that the crucial roles of controlling glioma growth were played by $CD8^+$ T cells and TAMs.

A limitation of this study is that the role of CD30L in clinical analysis needs to be further measured in the future. In this study, we creatively showed that CD30L can inhibit the growth of glioma. Moreover, exploring CD30L can provide a new immunotherapeutic approach.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval and ethical standards

All applicable international and national guidelines for the care of animals were followed. All animal experimental protocols were approved by the Animal Welfare and Ethics Committee of China Medical University (IACUC No.2018054).

Animal source

WT C57BL/6 (B6) mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

Cell line authentication

GL261 cells were obtained from Chinese Academy of Sciences.

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