Agonistic CD40 antibody therapy induces tertiary lymphoid structures but impairs the response to immune checkpoint blockade in glioma

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24 **Abstract**

25 Gliomas are brain tumors characterized by immunosuppression. Immunostimulatory agonistic
 26 CD40 antibodies (α CD40) are in clinical development for solid tumors but are yet to be
 27 evaluated for glioma. Here, systemic delivery of α CD40 led to cytotoxic T cell dysfunction and
 28 impaired the response to immune checkpoint inhibitors in preclinical glioma models. This was
 29 associated with an accumulation of suppressive CD11b⁺ B cells. However, α CD40 also induced
 30 tertiary lymphoid structures (TLS). In human glioma, TLS correlated with increased T cell
 31 infiltration indicating enhanced immune responses. Our work unveils the pleiotropic effects
 32 of α CD40 therapy in glioma, which is of high clinical relevance.

Glioblastoma (GBM), or grade IV glioma, is the most common malignant primary brain tumor in adults. Despite multimodal treatment strategies with surgery, radiotherapy, chemotherapy and recently tumor-treatment fields, the outcome for GBM patients remains poor with a median survival of less than 24 months¹. While checkpoint inhibitors (CPIs) targeting PD-1 and CTLA-4 have seen clinical success in several human solid tumors² and experimental murine glioma models³, their efficacy has proven limited in GBM patients⁴. This is not surprising since brain immune responses are adapted to a sensitive and immunospecialized microenvironment. Thus, novel therapeutic approaches are urgently needed to mount an effective immune response against GBM.

Gliomas are highly infiltrated by bone marrow-derived macrophages and brain-resident microglia, which promote tumor growth and suppress the immune response⁵. CD40 is expressed on several antigen presenting cells (APCs) and agonistic CD40 antibodies (α CD40) have broad immunostimulatory effects. Indeed, α CD40 can polarize macrophages towards a tumor-suppressive profile and enhance antigen presentation by dendritic cells (DCs)⁶⁻⁸. Moreover, CD40 activation of B cells regulates activation, antibody production, germinal center formation and antigen presentation^{9,10}. α CD40 antibodies are currently in clinical development for numerous solid tumors¹¹. However, there is conflicting evidence regarding their efficacy in glioma since outcomes have varied depending on experimental model and combinatorial treatment regimen¹²⁻¹⁴. A thorough understanding of how α CD40 therapy impacts different compartments of the brain immune response is necessary to evaluate its potential for the treatment of glioma.

Tertiary lymphoid structures (TLS) are ectopic lymphoid aggregates that form at sites of chronic inflammation and resemble secondary lymphoid organs, including a B cell follicle surrounded by a T cell zone with mature DCs and a network of follicular dendritic cells (FDCs)^{15,16}. Ectopic expression of lymphotoxin (LT) is instrumental in driving TLS formation^{17,18}. The engagement of the LT α 1 β 2 heterotrimer (or the homologous ligand TNFSF14) with the lymphotoxin β receptor (LT β R) expressed on stromal cells induces chemokines associated with leukocyte recruitment¹⁹, the formation of FDC networks and of germinal centers in B cell follicles²⁰. Importantly, TLS affect disease progression. TLS are considered to aggravate the inflammatory response in autoimmune disease such as multiple sclerosis (MS)²¹, while in several types of cancer they are associated with improved response to immunotherapy and a favorable prognosis²²⁻²⁶. B cells are the dominant component of TLS and B cell-depleting antibodies recently gained clinical approval for MS patients as they ameliorate disease severity²⁷, while in melanoma depletion of B cells reduces CD8⁺ T cell infiltration²⁸. In the context of cancer, TLS are believed to provide an alternative to tumor-draining lymph nodes as a site of antigen presentation and activation of naïve T cells¹⁶. Notably, the presence of TLS in glioma has not been described to date.

We demonstrate that systemic exposure to α CD40 in glioma-bearing mice led to reduced CD8⁺ T cell cytotoxicity and impaired the response to CPIs. This was associated with the expansion of a suppressive CD11b⁺ B cell population. However, α CD40 stimulation of B cells enhanced the formation of TLS in the brain of glioma-bearing mice. Interestingly, TLS were present in human glioma and correlated with increased intratumoral T cells in GBM, suggesting an association between TLS and enhanced immune responses in glioma patients. Our study

reveals the multifaceted effects of α CD40 in glioma, which have important therapeutic implications.

Results

α CD40 enhanced TLS formation in the brain of glioma-bearing mice. To investigate the effects of agonistic CD40 antibodies (α CD40) on the tumor microenvironment, we intravenously administered α CD40 to C57BL/6 mice with syngeneic gliomas. Immunofluorescence staining of brain sections from glioma-bearing mice revealed the presence of immune cell clusters with a distinct core of B cells that were reminiscent of TLS (Extended Data Fig. 1a and Extended Data Movie 1). We defined TLS as compact clusters of CD45⁺ cells with a dense core of B220⁺ B cells. α CD40 was associated with increased numbers and total surface area of TLS in both GL261 (Fig. 1a-c) and CT-2A (Fig. 1d-f) glioma models. The TLS were consistently located close to the meninges (around the cortex or close to choroid plexuses) in proximity of the tumor tissue (Extended Data Fig. 1a-b). TLS did not form in the brain of untreated tumor-free mice or after mock injection of tumor cells followed by α CD40 therapy (Extended Data Fig. 1c-d). In summary, TLS were observed in brains of glioma-bearing mice and α CD40 treatment enhanced their formation.

α CD40-induced TLS had a characteristic cell composition and gene expression signature.

We further characterized TLS composition and maturity by staining for a set of well-established markers. The TLS included CD3⁺ T cells and were dominated by B cells, of which a large proportion expressed the follicular B cell marker CD23 (Fig. 1g). We rarely observed

proliferating B cells inside the TLS, but a substantial proportion of CD3⁺ cells were Ki67⁺, indicating T cell functionality (Fig. 1h and Extended Data Fig. 1l). The TLS contained F4/80⁺ macrophages (Extended Data Fig. 1g), CD11c⁺ DCs (Fig. 1i and Extended Data Fig. 1m) and CD35⁺ or CD21⁺ follicular dendritic cells (FDCs), which formed intimate connections with the surrounding B cells (Fig. 1j-k). Moreover, the presence of rare CD11c⁺GFP⁺ DCs within the TLS indicated uptake of tumor-associated antigens (Extended Data Fig. 1e). T regulatory cells (Tregs) were observed in the TLS to a varying extent (Extended Data Fig. 1f).

TLS formed around CD31⁺ vessels that varied in size (Fig. 1l,n and Extended Data Fig. 1n-o) and were surrounded by a distinct network of collagen IV (Fig. 1n and Extended Data Fig. 1n) and fibronectin (Fig. 1o and Extended Data Fig. 1o). While the high endothelial venule markers PNA^d or MAdCAM-1 were not detected in the TLS vasculature (data not shown), the majority of B cells inside these structures stained positive for CD62L (Fig. 1m), a selectin that mediates infiltration of naïve leukocytes into lymphoid tissues²⁹.

The TLS varied in size, ranging from small and poorly organized clusters (Fig. 1n) to large aggregates with a follicle-like structure (Fig. 1i) where T cells were predominantly located outside the B cell zone, facing the tumor tissue. TLS were present more than two weeks after the last administration of αCD40, indicating that continuous treatment was not required for TLS persistence (Extended Data Fig. 1h-i).

To characterize gene expression signatures associated with TLS³⁰, we laser capture micro-dissected TLS from αCD40-treated GL261 tumors and isolated RNA from the collected tissue (Extended Data Fig. 1j). Genes for lymphotoxin β (*Ltb*), C-X-C motif chemokine ligand 13 (*Cxcl13*), and C-C motif chemokine ligand 19 (*Ccl19*) were highly expressed in the TLS compared

with the tumor or healthy tissue dissected from the same brain (Fig. 1p-r), while the gene for C-
C motif chemokine ligand 21 (*Ccl21*) was expressed at a similar levels (Extended Data Fig. 1k).
Altogether, α CD40 induced the formation of tertiary lymphoid structures that contained a B cell
core, T cell zones, CD11c⁺ DCs and CD35⁺/CD21⁺ FDCs. While B cells rarely proliferated within
the TLS, a large proportion expressed the follicular B cell marker CD23, which is indicative of B
cell follicular organization and early-stage germinal center formation. The sizes and degree of
organization of these structures were variable, but were generally increased by α CD40 therapy.

B cells expressed *Lta* upon α CD40 stimulation and were required for TLS formation. To
investigate the mechanism through which α CD40 induced TLS *in vivo*, we stained tumor
sections for the rat-derived α CD40 antibody. Cells positive for α CD40 were observed in both TLS
and tumor area (Fig. 2b-c and Extended Data Fig. 2a). The therapeutic antibody co-localized
with B220⁺ B cells only in the TLS of α CD40-treated mice (Fig. 2a-b), suggesting that α CD40
mainly stimulated B cells in α CD40-induced TLS. A few CD11b⁺ cells in the TLS also stained
positive for α CD40 (Fig. 2c).

To understand whether α CD40 induced the production of TLS-associated cytokines in B cells,
we isolated CD19⁺ cells from mouse spleen and stimulated them *in vitro* with α CD40. After 48
hours of stimulation, B cells aggregated in clusters that became progressively bigger with time
(Fig. 2d). α CD40 increased the expression of *Lta* and *Tnfsf14* in B cells 48 hours and 72 hours
after stimulation (Fig. 2e, g), while *Ltb* expression increased after 72 hours (Fig. 2 f). In line with
this, B cells in the spleen and superficial cranial lymph nodes of α CD40-treated glioma-bearing
mice had increased *Lta* expression (Fig. 2h,j), while *Ltb* was constitutively expressed in B cells at

both locations (Fig. 2i,k). The proportion of B cells in the brain was similar across treatment groups on day 20 post tumor implantation, while it was higher on day 25 in α CD40-treated mice compared to the rlgG2a group (Fig. 2l-n). To determine whether α CD40 stimulation of B cells was required for TLS formation, we depleted B cells three days before the initiation of α CD40 therapy (Fig. 2o). B cell depletion effectively inhibited the formation of TLS (Fig. 2p). In contrast, the formation of T cell aggregates characterized by a core of CD3⁺ T cells and a network of CD11c⁺ cells was not affected by α CD40 therapy or B cell depletion (Extended Data Fig. 2b-c). Collectively, these observations demonstrate that TLS formation was mediated by α CD40 stimulation of B cells.

TLS are present in human glioma and are associated with increased T cell infiltration.

Small TLS were occasionally observed in untreated glioma-bearing mice (Fig. 1a-f). To determine the clinical relevance of our findings, we investigated whether similar structures were present in patients with glioma. As TLS were consistently located close to the meninges in preclinical glioma models, we screened patient samples that included meningeal tissue. We collected a cohort of 26 patients, which included 6 grade II gliomas, 4 grade III gliomas and 16 grade IV glioblastomas (Supplementary Table 1).

We identified CD45⁺CD20⁺CD3⁺ aggregates resembling TLS, which varied in their level of organization (Fig. 3a-n). Some clusters lacked a follicle-like organization (Figure 3a-d), thus we defined them as “immature TLS”. Some aggregates instead had a clear CD20⁺ B cell core (Fig 3h-k), which we defined “organized TLS”. CD35⁺ FDCs were present in both types of TLS (Fig. 3e,l). Occasionally, a clear CD35⁺ FDC network was observed in organized TLS (Fig. 3l). Both TLS types

included Ki67⁺ cells (Fig. 3f,m) and formed around PNA⁺ HEVs (Fig. 3g,n). TLS also had rare CD23⁺ follicular B cells (Extended Data Fig. 3a,c) and CD138⁺ plasma cells (Extended Data Fig. 3b,d).

Immature and/or organized TLS were identified in patients with grade II/grade III glioma (3/10) and in glioblastoma patients (8/16) (Fig. 3o and Supplementary Table 1). TLS were most frequently found in close proximity to meningeal tissue, but were also observed in the white matter (close to the tumor bulk) or directly within the tumor tissue (Extended Data Fig. 3e-f). Importantly, the presence of TLS in GBM patients was associated with an increased abundance of tumor-infiltrating T cells (Fig. 3p-q). In summary, TLS were present in human glioma of various grades and were associated with an increased abundance of intratumoral T cells in GBM patients.

αCD40 treatment resulted in impaired T cell responses in glioma-bearing mice. Consistent with what we observed in human GBM, quantification of intratumoral T cells in αCD40-treated mice revealed a trend to an increased number of T cells in TLS⁺ brains compared with TLS⁻ brains (Fig. 4a and Extended Data Fig. 4a). However, αCD40 did not improve survival in either the GL261 or the CT-2A models (Fig. 4b-c).

CD40 stimulation is known to mediate anti-tumor immunity by inducing a CD8⁺ T cell response via DC activation^{6-8,31}. Thus, we characterized the T cell response in the tumor after αCD40 therapy using flow cytometry (Supplementary Table 4). Hierarchical stochastic neighbor embedding (HSNE) analysis indicated that CD3⁺ T cells from GL261 and CT-2A models clustered according to the treatment regime rather than the tumor model (Fig. 4d-g). The most

pronounced effects of α CD40 were observed on cytotoxic T cells (Fig. 4g; MC01, MC02), in line with a proportional increase of CD8⁺ T cells among all brain-infiltrating immune cells (Fig. 4h). The percentage of effector CD8⁺ T cells (CD44⁺CD62L⁻) was higher in α CD40-treated mice (Fig. 4i), however a greater proportion of effector cells were CD127⁻KLRG1⁺ (Fig. 4j), pointing to a short-lived effector phenotype³². A similar T cell phenotype was observed in the spleen after α CD40 treatment (Extended Data Fig. 5a-f). Markers of proliferation, exhaustion and maturation on tumor-infiltrating CD8⁺ T cells indicated a decreased activation status upon α CD40 therapy (Fig. 4k). In line with this, α CD40-treated mice exhibited decreased percentages of CD69⁺ and CD107a⁺ cytotoxic T cells (Fig. 4l,m). A few model-specific responses to α CD40 were apparent, as the percentage of PD-1⁺TIM3⁺LAG3⁺ cytotoxic T cells increased in the GL261 model but showed a downward trend in the CT-2A model (Fig. 4n). α CD40 did not affect the proportion of Tregs (Extended Data Fig. 4b-c). Systemically, we observed a typical cytokine response to α CD40, characterized by increased serum levels of pro-inflammatory cytokines after intravenous administration (Extended Data Fig. 5g-p).

To assess T cell functionality after α CD40 treatment, we isolated splenocytes from GL261 glioma-bearing mice and re-stimulated them *in vitro* with concanavalin A (ConA) for 24 hours (Fig. 4o). CD8⁺ splenocytes derived from α CD40-treated mice showed decreased proliferation, lower CD69 and a reduced percentage of CD107a⁺ cells (Fig. 4p-r). Similar results were observed for tumor-infiltrating CD8⁺ T cells isolated from α CD40-treated mice, which also exhibited decreased activation and proliferation (Extended Data Fig. 6a-d), and displayed impaired cytotoxicity and killing capability upon co-culture with GL261 tumor cells (Extended Data Fig. 6e-h). In summary, α CD40 induced a pro-inflammatory cytokine response consistent with

previous studies in other tumor types³³ but was associated with impaired T cell responses in the glioma microenvironment and in the spleen of glioma-bearing mice.

αCD40 impaired the efficacy of immune checkpoint inhibitors in murine glioma. We sought to understand if CPIs could rescue the αCD40-induced dysfunctional T cell phenotype. GL261 glioma-bearing mice were administered four doses of αCD40 and/or anti-PD-1 blocking antibodies (αPD-1). αCD40 increased the proportion of brain-infiltrating CD8⁺ T cells alone or in combination with αPD-1, in comparison with rIgG2a and αPD-1 monotherapy (Fig. 5a). However, the percentage of CD69⁺Ki67⁺ cytotoxic T cells was reduced in both αCD40-treated groups compared with αPD-1 monotherapy (Fig. 5b). Accordingly, co-administration of αCD40 and αPD-1 resulted in decreased survival compared with αPD-1 monotherapy (Fig. 5c). αPD-1 co-administration impaired αCD40-mediated TLS induction (Fig. 5d), however a substantial proportion of TLS with a larger surface area were still present in co-treated mice (Extended Data Fig. 7a-b).

The circulating levels of rat-IgG were reduced in the αCD40 groups (Extended Data Fig. 7c), which may result from target-mediated clearance and/or from anti-rat-IgG responses elicited from repeated exposure to rat-IgG³¹. To exclude the possibility that drug clearance was the reason for the reduced survival in the combination group, we assessed a combination of αCD40 with a fully murine αCTLA-4 antibody and also evaluated a single dose of αCD40 followed by three doses of αPD-1 (Extended Data Fig. 7d-i). In both cases αCD40 therapy hampered the effect of the CPIs (Extended Data Fig. 7d,f).

A HSNE analysis of CD8⁺ T cells (Fig. 5e-j) revealed that αPD-1 polarized the cytotoxic T cell response towards an active and proliferating state (Fig. 5i,j; MC02), while αCD40 resulted in impaired activation and/or proliferation (Fig. 5i,j; MC03, MC06, MC04, MC01). Strikingly, co-administration of αCD40 and αPD-1 shifted the cytotoxic T cell response towards a low-activation, low-proliferation state (Fig. 5i,j; MC03, MC06). In summary, αCD40 induced a dysfunctional T cell state that inhibited the efficacy of CPIs.

αCD40 activated brain-infiltrating dendritic cells and myeloid cells. To understand the cellular mechanisms involved in the αCD40-induced CD8⁺ T cell dysfunction, we performed FACS analysis of tumor-infiltrating immune cells that express the CD40 molecule (Supplementary Table 4). The proportion of brain-infiltrating DCs and myeloid cells decreased after αCD40 therapy (Extended Data Fig. 8a-b). αCD40 did not enhance the production of immunosuppressive molecules such as arginase, IL-10 and PD-L1 and increased the expression of the activation marker CD86 (Extended Data Fig. 8c). αCD40 did not alter the relative amount of IL-12⁺ DCs or myeloid cells (Extended Data Fig. 8d-e) but decreased the proportion of IL-10⁺ DCs (Extended Data Fig. 8f,g). Altogether, αCD40 promoted an activated phenotype of brain-infiltrating DCs and myeloid cells.

Systemic delivery of αCD40 induced an accumulation of CD11b-expressing B cells.

Next, we investigated the phenotype of intratumoral B cells in αCD40-treated glioma-bearing mice. αCD40 therapy increased the expression of CD86, MHC-II and IL-12 alone or in combination with αPD-1 (Fig. 6a-b), but also increased the proportion of CD5⁺CD1d⁺ B cells

(Extended Data Fig. 9a-b). B cells expressing CD5 and CD1d have previously been classified as regulatory B10 cells and can inhibit CD4⁺ T cell responses via secretion of IL-10^{34,35}. However, gene expression of immunosuppressive factors including *Il10*, *Tgfb1*, *Ccl22* and *Lgals1* was not increased in B cells after αCD40 therapy (Extended Data Fig. 9c-j). In addition, production of IL-10 was increased in mice treated with αCD40 alone but not in combination with αPD-1 (Fig. 6c). Thus, it is not likely that regulatory B10 cells were the main mediators of the αCD40-induced T cell dysfunction.

αCD40 resulted in a striking increase of CD11b⁺ B cells alone or in combination with αPD-1 (Fig. 6d), which have been linked to suppressed CD4⁺ T cell responses^{36,37}. A similar effect was observed in the spleen (Extended Data Fig. 10a). In the brain, CD11b⁺ B cells were rarely observed within the TLS (Fig. 6e), were predominantly present in the tumor area (Fig. 6f) and had lower surface levels of CD11b compared to myeloid cells (Extended Data Fig. 10b).

To understand whether CD11b upregulation was a direct effect of αCD40 stimulation of B cells or secondary to a systemic release of cytokines, we stimulated murine splenic B cells *in vitro*. αCD40 stimulation did not induce CD11b upregulation on B cells *in vitro*, while exposure to IL-10 did (Fig. 6g), consistent with previous studies³⁷. Notably, IL-10 levels were systemically elevated in αCD40-treated mice, with or without B cell depletion (Extended Data Fig. 10c). In conclusion, systemic delivery of αCD40 resulted in the expansion of CD11b-expressing B cells in the brain and spleen of glioma-bearing mice, which was associated with a systemic increase of IL-10.

CD11b⁺ B cells inhibit CD8⁺ T cell responses. Surface expression of CD11b on B cells inhibits CD4⁺ T cell responses, resulting in lower T cell proliferation and IFN γ production³⁷. To investigate whether CD11b on B cells could suppress CD8⁺ T cell responses, we induced CD11b expression on B cells *in vitro* and co-cultured these cells with splenocytes in the presence or absence of a CD11b-neutralizing antibody (Extended Fig 11a,b). Blocking CD11b rescued activation (CD69), cytotoxicity (CD107a and IFN γ) and proliferation of CD8⁺ T cells stimulated with CD3/CD28 beads (Extended Data Fig. 11c-g).

Tumor-infiltrating CD11b⁺ B cells displayed higher levels of MHC-II compared to their CD11b⁻ counterparts in the α CD40-treated groups (Fig. 6h), indicating an increased capability to interact with CD4⁺ T cells. Notably, the surface levels of CD3 were decreased on both CD4⁺ and CD8⁺ tumor-infiltrating T cells in α CD40-treated groups (Fig 6i-j), which is in line with a CD11b-mediated internalization of the TCR complex as previously reported³⁷. Moreover, similarly to CD8⁺ T cells (Fig. 5b), intratumoral CD4⁺ T cells showed lower activation and proliferation in α CD40-treated mice (Extended Data Fig. 10d-e) and depletion of B cells prior to *in vivo* administration of α CD40 increased the overall abundance of intratumoral T cells (Fig. 6k). Altogether, these data suggest that CD11b⁺ B cells underlie the suppression of T cell responses observed in α CD40-treated mice.

Discussion

Agonistic CD40 antibodies are under clinical development for multiple solid tumors¹¹ and are being investigated in two Phase I clinical trials of CNS malignancies [NCT03389802; NCT04547777]. In this study, systemic delivery of α CD40 promoted dysfunctional T cell

responses and the expansion of suppressive CD11b⁺ B cells, but also enhanced the formation of TLS in the brain.

The mechanisms involved in TLS formation during pathological conditions can vary. Besides lymphoid tissue inducer (LTi) cells, other cell types can express *Lt* to induce TLS^{38,39}. B cells can act as LTi cells in the gut³⁸ and their transient activation via α CD40 antibodies enhanced TLS maturation in an artificial model of TLS induction⁴⁰. Our study reveals that α CD40 stimulation of B cells promotes TLS formation in glioma-bearing mice by upregulating *Lta*. Notably, we identified TLS with varying levels of organization in the brain of patients with lower grade gliomas and GBM. Together with the fact that CD40 activation can induce *Lta* expression in human B cells⁴¹, this strongly suggests that α CD40 could have similar effects on TLS induction in glioma patients.

Similarly to other cancer types⁴², we found higher numbers of intratumoral T cells in human GBMs positive for TLS. Although the role of TLS in glioma requires evaluation in a larger cohort, this suggests an association between the presence of TLS and enhanced immune responses in GBM patients. In α CD40-treated glioma-bearing mice, TLS were also associated with increased T cell infiltration in the tumor. However, α CD40 therapy did not result in increased survival and was rather associated with dysfunctional cytotoxic T cell responses, which efficiently impaired the efficacy of CPIs. Notably, this correlated with an accumulation of CD11b⁺ B cells in the brain and spleen of α CD40-treated mice. While it is clear that innate type B cells can produce regulatory cytokines⁴³, Liu *et al.* found that CD11b⁺ B cells can suppress CD4⁺ T cells through cell-to-cell interaction in a CD11b-dependent manner, inducing internalization of the T cell receptor (TCR)³⁷. In line with this, intratumoral T cells in α CD40-

treated mice had decreased surface levels of CD3 (suggesting internalization of the TCR complex) and B cell depletion increased the abundance of T cells in the tumor specifically in α CD40-treated mice. Moreover, blocking CD11b on B cells *in vitro* rescued CD8⁺ T cell responses, suggesting that CD11b⁺ B cells are capable of suppressing cytotoxic T cells.

Interestingly, CD11b expression was not directly induced by α CD40 stimulation of B cells. Rather, it was associated with an increase in systemic IL-10 after α CD40 treatment which was observed also when the B cells were depleted, suggesting that they were not the main IL-10 producers. Thus, targeting α CD40 specifically to B cells could help circumventing the upregulation of CD11b while still inducing B cell activation and expression of *Lta*. In line with this, a recent study reported that systemic administration of 4-1BBL⁺ B cells activated *in vitro* with α CD40 and IFN γ elicited anti-tumor immunity in glioma-bearing mice⁴⁴. B cells are important for antigen presentation and T cell-mediated anti-tumor immunity in the brain⁴⁵, thus the acquisition of a suppressive CD11b⁺ B cell phenotype may explain the detrimental effect of α CD40 on T cell responses specifically in brain tumors.

CD11b⁺ B cells were rarely present within α CD40-induced TLS, therefore they are not likely to mediate immunosuppression within these structures. However, we observed T regulatory cells (Tregs) in α CD40-induced TLS. Since the presence of Tregs in TLS has been associated with suppressed anti-tumor immune responses⁴⁶ and tumor progression⁴⁷, the role of α CD40-induced TLS in glioma has to be further investigated.

In summary, our study demonstrates that systemic α CD40 therapy results in dysfunctional cytotoxic T cell responses and reduces the efficacy of CPIs in preclinical glioma models, which is relevant information for clinical trials currently investigating α CD40 in patients with primary

CNS tumors. Our work also reveals for the first time that TLS are present in glioma patients and that immunotherapies can modulate these structures in murine glioma models. The finding that TLS in the brain can be manipulated therapeutically opens up new possibilities for triggering or suppressing immune responses, which has broader implications for brain malignancies and autoimmune diseases of the central nervous system.

Online methods

Cell lines. The GL261 (gift from Dr. Geza Safrany, NRIRR, Budapest, Hungary) and CT-2A (gift from T. Seyfried, Boston College, Boston, MA, USA) cell lines were transfected with lentiviruses to express GFP and luciferase, as described previously⁴⁸. GL261 cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, CA, USA) with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Life Technologies). CT-2A cells were cultured in RPMI-1640 (Life Technologies) supplemented with 10% (vol/vol) heat-inactivated FBS. All cell lines were cultured at 37°C and 5% CO₂ in a humidified cell incubator. The cell lines were not authenticated after purchase but routinely tested negative for mycoplasma contamination using the MycoAlert Detection Kit (Lonza, Basel, Switzerland).

Orthotopic murine glioma models. GL261 or CT-2A cells were orthotopically injected in mouse brains to obtain models of glioma with high mutational burden. Six to ten-week-old female C57BL/6 mice were purchased from Taconic M&B (Bomholt, Denmark) or Janvier Labs (Le Genest-Saint-Isle, France) and housed in Sealsafe Plus GM500 cages (Tecniplast, Buguggiate, VA, Italy). For injection of tumor cells, mice (at least 7 weeks of age) were anesthetized with

2.5% isoflurane and immobilized in a stereotaxic frame on a heated surface. A midline incision was made on the scalp and a hole was drilled in the skull at -1 mm anteroposterior and +1.5 mm mediolateral stereotactic coordinates from the bregma. GL261 cells (2×10^4) or CT-2A cells (5×10^4) were delivered in 2 μ l of Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific, Waltham, MA, USA) at a depth of 2.7 mm. The incision was closed using Vetbond tissue glue (3M, St. Paul, MN, USA) and the mice were observed until full recovery from anesthesia on a heated surface. For survival studies, mice were monitored daily and sacrificed at the appearance of tumor-induced symptoms, such as hunched posture, lethargy, persistent recumbency, and weight loss, resulting in a score of ≥ 0.6 according to the Uppsala University (Uppsala, Sweden) scoring system for animal welfare. All animal experiments were approved by the Uppsala County regional ethics committee (permits C1/14, C26/15, N164/15 and 19429/2019), and were performed according to the guidelines for animal experimentation and welfare of Uppsala University. At the survival end-point, mice were sacrificed via cervical dislocation or anesthetized for intracardiac perfusion with 10ml of phosphate-buffered saline (PBS) (Thermo Fisher Scientific) and 10ml of 4% (wt/vol) paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO, USA). At the experimental end-point (day 20-25), mice were sacrificed via cervical dislocation.

***In vivo* antibody therapies.** Cages were randomly assigned to different treatment groups. Agonistic rat-anti-mouse CD40 (clone: FGK4.5, Cat# BE0016, 100 μ g/dose), rat-anti-mouse PD-1 (clone: RMP1-14, Cat# BE0146, 200 μ g/dose), mouse-anti-mouse CTLA-4 (clone 9D9, Cat# BE0164, 100 μ g/dose), rat-anti-mouse CD20 (clone AISB12, Cat# BE0302, 200 μ g/dose)

antibodies (Abs) were administered intravenously in a final volume of 100 μ l. α CD40 Abs were administered either in repeated doses (on day 10, 13, 16 and 19 after tumor implantation) or in a single dose regimen (on day 9 after tumor implantation). α PD-1 antibodies were administered (a) alone, (b) in combination with α CD40 antibodies in repeated doses (on day 10, 13, 16 and 19 after tumor implantation) or (c) in a sequential treatment regimen (on day 10, 13 and 16 after tumor implantation, following the administration of a single dose of α CD40 Abs on day 9). α CTLA-4 Abs were administered alone or in combination with α CD40 Abs on day 10, 13, 16 and 19 after tumor implantation. α CD20 antibodies were administered on day 7 after tumor implantation, followed by repeated doses of α CD40 as specified above. The isotype control rat IgG2a (clone: 2A3, Cat# BE0089) was administered intravenously to the control groups in each experiment (100 μ g/dose during rIgG2a vs α CD40 experiments; 200 μ g/dose during combination experiments with repeated treatment regimen; 100 μ g/dose on day 9 followed by 200 μ g/dose on days 10, 13 and 16 during the experiment with sequential treatment regimen). All antibodies for *in vivo* studies were purchased from BioXCell, Lebanon, NH, USA.

Isolation of immune cells from tumor-bearing mice. Single cell suspensions of tumor-bearing brains were obtained by enzymatic dissociation of the whole brain minus the cerebellum using a gentleMACS Octo Dissociator and the Tumor Dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Myelin depletion was achieved by either using Myelin Removal Beads II (Miltenyi Biotec) or by resuspending the cells in a solution of 25% BSA (in PBS) and centrifuging at 2600 RPM for 20min on a low brake (brake =2) to separate the myelin ring from the cell pellet. CD45⁺ immune cells and CD8⁺ T cells were enriched using either Mouse CD45

MicroBeads (Miltenyi Biotec) or Mouse CD8 (TIL) MicroBeads (Miltenyi Biotec), respectively. Spleens were mechanically dissociated and cranial lymph nodes were digested by using 2.0 Wunsch U/ml of liberase TL (Roche, Basel, Switzerland) for 20 min at 37°C. Lymph nodes and spleens were subsequently passed through a 70µm strainer (Corning, Sigma-Aldrich, St. Louis, MO, USA) in PBS to obtain a single cell suspension. After isolation, cells were used for gene expression analysis, flow cytometry, FACS or *ex vivo* assays. For the *ex vivo* stimulation experiment, the isolated CD45⁺ cells were instead cultured with PMA (50 ng/ml), ionomycin (500 ng/ml), and Brefeldin A (1 µg/ml) for 5h (Leukocyte Activation Cocktail, with BD GolgiPlug, BD Biosciences, San Jose, CA, USA).

***Ex vivo* T cell functionality assays.** All T cell functionality assays were performed in 96-well plates in T cell medium: RPMI 1640 (Life Technologies, Carlsbad, CA, USA) added with 10% FBS, 2mM L-glutamine, 10 mM HEPES, 20µm β-mercaptoethanol, 1 mM sodium-pyruvate, 100 U/ml penicillin-streptomycin (all purchased from Thermo Fisher Scientific, Waltham, MA, USA) and 100 IU/ml IL-2 (Novartis, Basel, Switzerland). T cell assays were performed with cells isolated from GL261 glioma-bearing mice. To achieve *ex vivo* stimulation of brain-infiltrating CD8⁺ T cells or splenocytes, cells were isolated on day 22 post tumor implantation (three days after the last αCD40 treatment on day 19) and cultured in T cell medium added with 2 µg/ml of concanavalin A (Sigma-Aldrich, St. Louis, MO, USA). Splenocytes were stimulated for 24h. CD8⁺ TILs were stimulated for 24h and 72h. Before plating, cells were stained using the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific) following the manufacturer's instructions. At each

time-point, cells were collected and stained to assess activation status (CD69) and proliferation status (cell trace violet) by flow cytometry.

To assess T cell functionality *ex vivo*, brain-infiltrating CD45⁺ immune cells were isolated on day 22 post tumor implantation (three days after the last α CD40 treatment on day 19) and were co-cultured with GL261 cells expressing luciferase at a 7 : 1 ratio (immune cells : tumor cells). Cells were co-cultured for 24h and 72h in T cell medium. At each time-point, cells were collected and stained to assess IFN γ production and degranulation (CD107a) of CD8⁺ T cells by flow cytometry. Viability of GL261 cells at 72h was assessed using the ONE-Glo™ Luciferase Assay System (Promega, Madison, WI, USA) following the instructions of the manufacturer.

Isolation of splenic B cells from wt mice. Primary mouse B cells were isolated from the spleen of 8- to 12-week-old C57BL/6 mice (males or females, bred in-house). Spleens were mechanically dissociated and passed through a 70 μ m cell strainer (Corning, Sigma-Aldrich, St. Louis, MO, USA) to obtain a single-cell suspension in sterile PBS. Splenic B cells were isolated by positive selection using anti-CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, and were confirmed $\geq$ 98% positive for B220 by flow cytometry.

In vitro stimulation of splenic B cells. To investigate gene expression after α CD40 stimulation, murine splenic B cells were plated at 2.5×10^6 /ml in 24-well plates in B cell medium: RPMI 1640 + 10% FBS (Life Technologies, Carlsbad, CA, USA) + penicillin-streptomycin (100 U/ml, Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated with 10 μ g/ml of α CD40 (clone:

FGK4.5) (BioXCell, Lebanon, NH, USA) or rat IgG2a (clone: 2A3, BioXCell) for 6h, 24h and 48h and collected for gene expression analysis.

To investigate whether CD40 stimulation or IL-10 affected CD11b expression, murine splenic B cells were plated at 2.0×10^6 cells/ml in B cell medium + 5mM of Mg^{2+} in 96-well plates. Cells were incubated for 48h with (a) medium alone, (b) 2 μ g/ml of lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA), (c) 2 μ g/ml of LPS + 10 μ g/ml of rat IgG2a (clone: 2A3, BioXCell), (d) 2 μ g/ml of LPS + 10 μ g/ml of α CD40 (clone: FGK4.5, BioXCell), (e) 2 μ g/ml of LPS + 50 ng/ml of IL-10 (Recombinant mouse IL-10, Biolegend, San Diego, CA, USA). After 48h, cells were collected and stained to assess CD11b surface expression by flow cytometry.

CD11b inhibition assay. To induce CD11b expression on B cells *in vitro*, murine B cells were isolated from wt spleens and incubated with LPS for 48h as indicated above. The remaining splenocytes were cultured for 48h in T cell medium to promote T cell expansion. After 48h, T cells were stained using the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, MA, USA) and incubated with MACSiBeads α CD3/CD28 (Miltenyi Biotec, Bergisch Gladbach, Germany) following the instructions of the manufacturers. The percentage of CD11b⁺ B cells was assessed by flow cytometry and B cells and T cells were co-cultured at a T cell : CD11b⁺ B cell ratio of 1 : 1. Cells were co-cultured for 72h in the presence of an α CD11b neutralizing antibody (clone M1/70, 10 μ g/ml, Biolegend, San Diego, CA, USA) or a control rIgG2b k antibody (clone RTK4530, 10 μ g/ml, Biolegend). After 72h, cells were collected and stained to assess expression levels of CD69, CD107a, IFN γ and proliferation of CD8⁺ T cells by flow cytometry.

RNA isolation, cDNA synthesis and qPCR. Samples from cell culture, laser capture microdissection, and FACS were collected in RLT lysis buffer (Qiagen, Hilden, Germany). RNA was isolated using the RNeasy Plus Mini or RNeasy Micro kits (Qiagen), according to the manufacturer's instructions. Reverse-transcription into cDNA was performed using the SuperScript III kit (Life Technologies, Carlsbad, CA, USA). Since the number of sorted B cells was low, cDNA was pre-amplified using the SsoAdvanced™ PreAmp Supermix (Biorad, Hercules, CA, USA). qPCR was performed using 2× SYBR Green PCR Master Mix (Life Technologies) in MicroAmp® Optical 96-well Reaction Plates (Applied Biosystem, Foster City, CA, USA) with 0.25 mM sense and antisense primers per well (final reaction volume 20µl). Primer sequences can be found in Supplementary Table 2. Plates were run in a QuantStudio3 Real-Time PCR machine (Applied Biosystem). Relative gene expression compared to that of *Hprt* was calculated using the Δ CT method⁴⁹.

Flow cytometry and FACS. Cells were stained using a live-dead dye (Supplementary Table 4 and 5) following the instructions of the manufacturer. Unspecific Fc receptor binding in all single-cell suspensions was blocked by using anti-mouse CD16/CD32 antibody (clone 93, Biolegend, San Diego, CA, USA). Cells were stained for the markers of interest using fluorochrome-conjugated antibodies (Supplementary Table 4 and 5). All antibodies were diluted 1:100 from stock concentration. For staining of FoxP3, the FOXP3 Fix/Perm Buffer Set (BioLegend) was used following the instructions of the manufacturer. For intracellular cytokine staining, the eBioscience™ Invitrogen™ Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher

Scientific, Waltham, MA, USA) was used following the instructions of the manufacturer. Samples were run on FACSCanto II, LSR Fortessa (BD BioSciences, San Jose, CA, USA) or CytoFLEX LX (Beckman Coulter, Brea, CA, USA); alternatively, the cells were sorted directly into RLT lysis buffer (Qiagen) using FACS ArialII (BD BioSciences). Data were analyzed using FlowJo version 10.5.3 (FlowJo LLC, Ashland, OR, USA) or Cytosplore version 2.2.1^{50,51}. An example of the gating strategy used in FlowJo can be found in Extended Data Fig. 12.

HSNE analysis. HSNE analysis was performed using Cytosplore version 2.2.1^{50,51}. Data obtained from a T cell multicolor FACS panel (17 colors, Supplementary Table 4) were initially analyzed by using FlowJo version 10.5.3 (FlowJo LLC, Ashland, OR, USA) to select CD45⁺CD3⁺ live cells. The data were then uploaded to Cytosplore version 2.2.1^{50,51} and a Hierarchical Stochastic Neighbor Embedding (HSNE) analysis was performed on non-transformed data (number of scales = 5) to identify clusters of T cells with different phenotypes. The following active markers were selected: CD4, CD8, CD69, Ki67, PD-1, TIM3, LAG3, KLRG1, CD44, CD62L, CD127, CXCR5, CD25, and FOXP3, for level-1 analysis. Clustering was performed to identify populations of CD4⁺ and CD8⁺ T cells among all CD3⁺ T cells. The meta-cluster in which CD8⁺ were highly represented was submitted to level-2 analysis, to study the cytotoxic T cell response.

Cytokine analysis. Serum was collected on days 13, 19, and 25 post tumor implantation from rlgG2a- and α CD40-treated mice in Microvette CB300 Capillary Blood Collection Tubes (Sarstedt, Nümbrecht, Germany). Serum samples were analyzed by using a customized U-PLEX plate (Meso Scale Discovery, Rockville, MD, USA) to measure the absolute concentration of the

following cytokines and chemokines: IL-6, IL-10, IL-12p70, TNF- α , IFN- γ , and CXCL10. The V-PLEX Th17 Panel 1 Mouse kit (Meso Scale Discovery) was used to determine serum concentrations of IL-16 and IL-23. The assays were performed following the protocol provided by the manufacturer. Briefly, the U-PLEX plate was coated with capture antibodies directed against the above-mentioned targets. For both U-PLEX and V-PLEX plates, the capture antibodies were incubated with the serum samples and sulfo-tag labeled detection antibodies were used to detect the target proteins. The plates were analyzed using a Sector™ Imager 2400 (Meso Scale Discovery) and final protein concentrations were calculated by the DISCOVERY WORKBENCH software version 4.0 (Meso Scale Discovery) using a standard curve.

Quantification of rat IgG in serum. Serum samples were collected on day 19 post tumor implantation from mice treated with rIgG2a, α CD40, α PD-1 and α CD40+ α PD-1 antibodies, using Microvette CB300 Capillary Blood Collection Tubes (Sarstedt, Nümbrecht, Germany). The IgG (Total) Rat Uncoated ELISA Kit with Plates kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the amount of rat antibodies in serum samples, following the instructions provided by the manufacturer.

Immunofluorescent staining of mouse samples. After intracardiac perfusion, brains were collected, fixed overnight in 4% (wt/vol) paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO, USA) and cryoprotected in 30% (wt/vol) sucrose overnight. Vibratome sections (80 μ m-thick) were prepared from PFA-fixed brains. Vibratome slides were permeabilized in PBS containing 0.1% Triton-X100, followed by blocking in PBS containing 3% (wt/vol) bovine serum albumin

and 3% FBS (vol/vol). After cervical dislocation, brains were collected and snap-frozen in isopentane. Cryosections (7µm-thick) were prepared from snap-frozen brain tissue and fixed in ice-cold acetone (Sigma-Aldrich) for 10 min. Frozen slides were blocked in 3% (wt/vol) bovine serum albumin in PBS for 1 h. Sections were stained using primary antibodies directed against the proteins of interest (Supplementary Table 3). Nuclear staining was performed with Hoechst 33342 (Sigma-Aldrich). The slides were mounted using Fluoromount-G (Southern Biotechnology, Birmingham, AL, USA). Images were acquired using an inverted fluorescence confocal microscope (Leica SP8, Leica Microsystems, Wetzlar, Germany) or with a Zeiss Axioimager microscope (Zeiss, Oberkochen, Germany). ImageJ version 1.51⁵² (NIH, Bethesda, MD, USA) or CellProfiler⁵³ version 3.1.9 (Broad Institute, Cambridge, MA, USA) were used for staining analysis and quantifications. All immunofluorescence images showing TLS composition are representative of three or more structures analyzed. Quantification of T cell numbers in the tumor area was performed on vibratome sections from brains of glioma-bearing mice. A Leica SP28 confocal microscope (Leica Microsystems) was used to take 6 random images for each tumor sample at 25x magnification, of which 3 were taken in the tumor core and 3 included the tumor rim. T cells were counted using CellProfiler.

Laser capture microdissection. Cryosections (10µm-thick) were placed on RNase-free POL membrane frame slides (Leica Microsystems, Wetzlar, Germany) and fixed for 2 min in ice-cold acetone (Sigma-Aldrich, St. Louis, MO, USA). The sections were stained directly with conjugated antibodies against B220 and CD45 (Supplementary Table 3), together with DAPI nuclear stain (Thermo Fisher Scientific, Waltham, MA, USA) for 1 min. Slides were rinsed with diethyl

dicarbonate-treated PBS and dried before laser capture microdissection. TLS, tumor tissue and healthy brain tissue were microdissected using a Leica LMD6000 B microscope (Leica Microsystems) and collected in the cap of an RNase-free 0.5 ml tube (Thermo Fisher Scientific, Waltham, MA, USA) in RLT lysis buffer (Qiagen, Hilden, Germany).

Tumor material from glioma patients. A cohort of 26 human glioma samples was assembled, which included cases of grade II glioma, grade III glioma and grade IV glioblastoma as indicated in Supplementary Table 1. All samples were collected during surgery.

11 samples were *en bloc* resected tumors from patients with suspected low-grade glioma or glioblastoma. In brief, preoperative T2-FLAIR MRI sequences were recorded to delineate the tumor border. Subsequently, microsurgical *en bloc* resections were performed of the entire tumor volume, as recorded by T2-FLAIR MRI sequences, including a margin of 1–2 cm outside the radiological border, and extending into the normal-appearing brain tissue⁵⁴. The institutional review board at the Uppsala University Hospital (Uppsala, Sweden) approved the study (DNR2010/05).

13 samples of supratentorial glioblastomas were identified in the database of Department of Surgical Pathology, Uppsala University Hospital. Samples from stereotactic biopsy specimens, small biopsies or samples that did not present with viable meningeal coverings were excluded, leaving 13 subjects within the cohort. The study was authorized by the regional Ethics Committee of Uppsala, Sweden (DNR2015/089).

2 samples were collected during supramarginal glioblastoma resection where biobanking of tissue was approved by the Ethics Committee of Western Sweden (EPN/DNR: 559-12).

Assessment of histological samples and WHO classification was performed by neuropathologists S.L. and T.O.B.

Immunohistochemical staining of human samples. For all samples, tissue was fixed in formalin for at least 2 days. For large biopsies, representative 3mm-thick blocks were embedded in paraffin. For *en bloc* resected tissue, samples were cut into 6-8mm-thick blocks covering the entire tumor volume. This was followed by an additional formalin fixation for 24 hours followed by paraffin-embedding. Sequential paraffin-embedded 4μm-thick sections were stained using a Dako Autostainer Plus (DakoCytomation, Glostrup, Denmark) and Dako EnVision FLEX detection system (DakoCytomation). Slides were pretreated in target retrieval solution (S2367, Dako) or 1x citrate (S2031, Dako) in a pressure cooker and subsequently incubated with primary antibodies against IDH1-R132H (Dianova), CD45 (M0701, Dako), CD20 (IR604, Dako), CD8 (IR623, Dako), CD4 (M7310, Dako), CD35 (M0846, Dako), CD138 (M7228, Dako), CD23 (DAK-CD23, Dako), PNA^d (MECA-79, BioLegend, San Diego, CA, USA) and Ki67 (MIB-1, Dako) for 30 minutes at room temperature. Signal was developed using the MACH3 Mouse HRP Polymer detection kit (M3M530L, Biocare Medical, Pacheco, CA, USA) and slides were counterstained with hematoxylin. Stained samples were assessed for TLS presence by L.v.H, A.V, M.R and A.D in collaboration with a neuropathologist (S.L). Image analysis was performed using QuPath version 0.1.2⁵⁵ and T cells in the tumor were counted using the “positive cell detection” algorithm.

Statistical analysis. All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). Kaplan-Meier curves were analyzed by using the log-rank test. For all other

analyses, two-tailed *t*-test or one-way ANOVA with Tukey's correction for multiple comparison were used to determine statistically significant differences between two or more groups, respectively. For the cytokine analysis at multiple timepoints, multiple *t*-test analysis with Sidak-Bonferroni correction was performed. The significance threshold was set at $p \leq 0.05$. The *p*-value is indicated as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

L.v.H., A.V., M.R., and A.D. conceived the project and designed the experiments. L.v.H., A.V., M.R., K.V., T.v.d.W., M.G., H.H and I.P. performed the experiments. L.v.H., A.V., and M.R. analyzed the data and designed the figures. J.L. provided support for the laser capture microdissection experiments. A.S., S.L., M.Z., A.S.J. and T.O.B. provided the human glioma material. S.L. and T.O.B. assessed the clinical samples. M.E., M.H.U., M.C.I.K. and S.M.M. provided advice regarding experimental procedures and data interpretation. A.D. supervised the project. L.v.H., A.V., M.R., and A.D. interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

S.M.M. is the founder and shareholder of Immuneed AB and Vivologica AB and is the Chief Development Officer and shareholder of Ultimovacs ASA/AB. None of the mentioned companies have taken part in the study nor do they have a financial gain of the specific subject matter described herein. The other authors declare no competing interests.

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Main Figure legends

Figure 1: α CD40 induced formation of tertiary lymphoid structures (TLS) in the brain of GL261 and CT-2A glioma-bearing mice. Quantification of (a,d) the number and of (b,e) the total CD45⁺ surface area of dense CD45⁺B220⁺ clusters per 80 μ m-thick section, in the brain of GL261 and CT-2A tumor-bearing mice. n=9-10mice/group. *t*-test, **p*<0.05. Values: mean. (c,f) Representative immunofluorescent stainings of the quantified CD45⁺B220⁺ clusters in the GL261 and CT-2A models. Scale bars: 50 μ m. (g-o) Immunofluorescent stainings of α CD40-induced TLS in the GL261 model showing TLS composition and organization. All images were taken from samples collected at the survival endpoint, between day 23 and day 35 post tumor implantation (4 to 16 days after the last α CD40 treatment). Arrows in (h) indicate Ki67⁺ T cells. Arrows in (j) indicate dendrites of a CD35⁺ FDC interacting with surrounding B cells. Arrows in (k) indicate a CD21⁺ FDC interacting with surrounding B cells. Scale bars: 50 μ m. (p-r) Gene expression of TLS-inducing cytokines in laser capture micro-dissected CD45⁺B220⁺ clusters, compared with laser capture micro-dissected tumor tissue and normal brain tissue. n_(of LMD areas)=4-6/group. ANOVA with Tukey's multiple comparison correction, **p*<0.05, ***p*<0.01, ****p*<0.001. Values: mean.

Figure 2: B cells expressed LT α upon α CD40 stimulation and were required for TLS formation. All panels except (d-g) show data from GL261 tumor-bearing mice treated with rlgG2a or α CD40 antibodies. (a-c) Immunofluorescent staining of therapeutic rat antibodies (α Rat) in TLS co-stained for (a-b) B220 and (c) CD11b and CD11c. Arrows: cells positive for α Rat. White square areas are magnified to the right in (b,c). Scale bars: 50 μ m. (d) Representative images of murine CD19⁺ splenic B cells stimulated *in vitro* with rlgG2a or α CD40 antibodies at indicated time points. (e-g) Gene expression of *Lta*, *Ltb* and *Tnfsf14* in B cells shown in panel (d). Values: mean $\pm$ SD (n=4). ANOVA with Dunnett's correction for multiple comparison. **p*<0.05, ***p*<0.01. (h-k) Gene expression of *Lta* and *Ltb* in CD19⁺B220⁺ B cells sorted from (h-i) spleen (n=4-5mice/group) and (j,k) cranial lymph nodes (n=7mice/group). *t*-test, **p*<0.05. Values: mean. (l) Representative plot and (m-n) quantification of CD19⁺B220⁺ B cells as a percentage of CD45⁺ cells in the brain. n=7-8mice/group. *t*-test, ****p*<0.001. (o) Quantification of CD19⁺B220⁺ B cells as a percentage of CD45⁺ cells in the blood of tumor-bearing mice treated with rlgG2a (black) or α CD40 (red) antibodies, with (+) or without (-) B cell depletion with an α CD20 antibody. ANOVA with Tukey's correction for multiple comparison, *****p*<0.0001. n=4mice/group. (p) Quantification of the number of dense CD45⁺B220⁺ clusters per 80 μ m-thick section in α CD40-treated tumor-bearing brains with (+) or without (-) B cell depletion with an α CD20 antibody. n=5-8mice/group. *t*-test, **p*<0.05. Values in (m-p): mean.

Figure 3: Tertiary lymphoid structures were present in the brain of glioma patients and were associated with increased T cell abundance. Immunohistochemical stainings of human glioma sections showing the composition of (a-g) immature TLS characterized by a loose B cell core and (h-n) organized TLS characterized by a compact core of B cells. Black square areas in (e-g) and (m) are magnified to the right of each image. Scale bars: 50 μ m. (o) Number of grade II/grade III glioma patients and glioblastoma (GBM) patients included in our cohort that stained negative for TLS (gray), positive for immature TLS (orange) or positive for organized TLS (red). (p) Number of T cells infiltrating the tumor area in GBM patients negative for TLS (gray) versus GBM patients positive for TLS (orange: immature TLS; red: organized TLS). (q) Representative images of T cell infiltration in GBMs that were negative for TLS, positive for immature TLS or positive for organized TLS. Scale bars: 50 μ m.

Figure 4. α CD40 therapy resulted in suppressed cytotoxic T cell responses in preclinical glioma models. (a) Quantification of CD3⁺ T cells in the tumor area of α CD40-treated GL261 glioma-bearing brains that were positive or negative for TLS. n=7-10mice/group. *t*-test. Values: mean. (b-c) Kaplan-Meier survival curve of GL261 (n=19-20mice/group) and CT-2A (n=10mice/group) tumor-bearing mice treated with α CD40 or rlgG2a antibodies on days

10, 13, 16 and 19 (as indicated by arrows). Black: rlgG2a; Red: α CD40. **(d-g)** HSNE analysis of multicolor flow cytometry data showing spatial clustering of tumor-infiltrating CD3⁺ cells in GL261 and CT-2A tumor-bearing mice treated with rlgG2a or α CD40 antibodies. n=5-7mice/group **(d)** HSNE analysis identified 6 meta-clusters (MC) of CD3⁺ T cells, exhibiting different expression levels of CD4 and CD8 **(e)**. **(f)** HSNE plot showing the spatial distribution of CD3⁺ T cells from GL261 and CT-2A tumor-bearing brains, in rlgG2a-treated versus α CD40-treated mice. **(g)** Frequency distribution of CD3⁺ T cells from each indicated model and treatment group in each MC. Cells from GL261 and CT-2A tumors clustered uniformly within the MCs according to treatment regime. **(h-n)** All panels show data relative to the T cell response in the brain of GL261 and CT-2A tumor-bearing mice treated with rlgG2a or α CD40 antibodies. n=5-8mice/group. **(h)** CD8⁺ T cells as a percentage of CD45⁺ cells. **(i)** CD44⁺CD62L⁻, **(l)** CD69⁺, **(m)** CD107a⁺ and **(n)** PD1⁺TIM3³LAG3⁺ cells as a percentage of CD8⁺ T cells. **(j)** CD127⁺KLRG1⁺ cells as a percentage of CD44⁺CD8⁺ T cells. **(k)** Heat map showing the mean fluorescence intensity (MFI) of proliferation, exhaustion and memory markers on CD8⁺ T cells. n=5-7/group. Statistics in (h-n) were performed with *t*-test, **p*<0.05. ***p*<0.01. ****p*<0.001. *****p*<0.0001. Values: mean. **(o)** Schematic illustration of the experimental layout used to obtained data shown in panels (p-r). In brief, GL261 glioma-bearing mice were treated with rlgG2a or α CD40 antibodies on days 10, 13, 16 and 19 post-tumor implantation. On day 22 splenocytes were isolated, stained with cell trace violet (CTV) and re-stimulated *in vitro* with concanavalin A (ConA) for 24h. **(p)** Bar graph showing the percentage of CD8⁺ splenocytes in different generations (G), where cells in G0 did not proliferate and cells in G6 underwent 6 cycles of proliferation. An example of how generations were defined is shown in Extended Data Fig. 6a. **(q)** Mean fluorescence intensity of CD69 on CD8⁺ splenocytes. **(r)** CD107a⁺ T cells as a percentage of CD8⁺ splenocytes. n=5mice/group. *t*-test; **p*<0.05, ****p*<0.001.

Figure 5: α CD40-induced T cell dysfunctionality impaired the efficacy of checkpoint blockade. All panels show data from GL261 tumor-bearing mice treated with rlgG2a, α CD40 and/or PD1 blocking antibodies. **(a-b)** Quantification of **(a)** CD8⁺ T cells as a percentage of CD45⁺ cells and **(b)** CD69⁺Ki67⁺ CD8 T cells in the brain in the indicated treatment groups. n=5-8mice/group. ANOVA with Tukey's multiple comparison correction, ***p*<0.01, ****p*<0.001. **(c)** Kaplan-Meier survival curves of mice (n=10mice/group) treated as indicated on days 10, 13, 16, and 19 (as shown by arrows). Log-rank test; **p*<0.05, ***p*<0.01, ****p*<0.001. **(d)** Number of dense CD45⁺B220⁺ clusters per section identified in the indicated treatment groups. n=8-17mice/group. **(e)** Spatial clustering of tumor-infiltrating CD3⁺ cells in GL261 tumors in different treatment groups, determined by Level-1 HSNE analysis of flow cytometry data. n=5-8mice/group. **(f)** Expression of CD4 and CD8 across the HSNE plot shown in (e). **(g)** Three meta-clusters (MC) of CD3⁺ T cells were identified by Level-1 HSNE analysis. Two MC were classified as mainly CD4⁺ or CD8⁺. The latter was submitted to Level-2 HSNE analysis, revealing spatial clustering of tumor-infiltrating CD8⁺ cells in GL261 tumors in different treatment groups. **(h)** MCs of T cells identified in the HSNE plot in (g). **(i)** Heat map showing the expression levels of proliferation, exhaustion, and memory markers on T cells in each MC. **(j)** Distribution of T cells from the indicated treatment group across each MC.

Figure 6: Systemic delivery of α CD40 was associated with a CD11b⁺ regulatory phenotype of B cells. All panels besides panel (g) show data from GL261 tumor-bearing mice. **(a)** Heatmap showing the expression levels of activation and immunosuppression markers on B cells in the brain, in the indicated treatment groups. n=4-7mice/group. **(b-c)** Quantification of **(b)** IL-12⁺ and **(c)** IL-10⁺ cells as a percentage of B cells in the brain, in the indicated treatment groups. **(d)** CD11b⁺ cells as a percentage of B cells in the brain, in the indicated treatment groups. **(e-f)** Immunofluorescent stainings showing CD11b⁺ B cells **(e)** in the TLS and **(f)** in the tumor area. Scale bars: 50 μ m. **(g)** Quantification of CD11b⁺ cells as a percentage of wt murine splenic B cells after *in vitro* stimulation with the indicated agents (n=3-7mice/group). **(h)** Mean fluorescence intensity (MFI) of MHC-II on CD11b⁺ vs CD11b⁻ B cells in the brain, in the indicated treatment groups. n=4-7mice/group. Multiple *t*-test with Sidak-Bonferroni correction. ***p*<0.01, ****p*<0.001, *****p*<0.0001. **(i-j)** MFI of CD3 on **(i)** CD8⁺ and **(j)** CD4⁺ T cells in the indicated treatment

845 groups. **(k)** Quantification of the number of CD3⁺ T cells in the tumor area in rIgG2a- or αCD40-treated mice with or
 846 without B cell depletion. n=5-17 mice/group. If not indicated otherwise, statistics in this figure were performed with
 847 either *t*-test or ANOVA with Tukey's multiple correction. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0,0001. Values:
 848 mean.

Main Figures

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6