Agonistic CD40 therapy induces tertiary lymphoid structures but impairs responses to checkpoint blockade in glioma

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Gliomas are brain tumors characterized by an immunosuppressive microenvironment. Immunostimulatory agonistic CD40 antibodies (αCD40) are in clinical development for solid tumors, but are yet to be evaluated for glioma. Here, we demonstrate that systemic delivery of αCD40 in preclinical glioma models induces the formation of tertiary lymphoid structures (TLS) in proximity of meningeal tissue. In treatment-naïve glioma patients, the presence of TLS correlates with increased T cell infiltration. However, systemic delivery of αCD40 induces hypofunctional T cells and impairs the response to immune checkpoint inhibitors in pre-clinical glioma models. This is associated with a systemic induction of suppressive CD11b+ B cells post-αCD40 treatment, which accumulate in the tumor microenvironment. Our work unveils the pleiotropic effects of αCD40 therapy in glioma and reveals that immunotherapies can modulate TLS formation in the brain, opening up for future opportunities to regulate the immune response.
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-treating fields, the outcome for GBM patients remains poor with a median survival of less than 24 months.1 While checkpoint inhibitors (CPIs) targeting PD-1 and CTLA-4 have seen clinical success in several human solid tumors and experimental murine glioma models, their efficacy has proven limited in GBM patients. This is not surprising since brain immune responses are adapted to a sensitive and immunospecialized microenvironment. Therapeutic approaches designed specifically for the brain tumor microenvironment are therefore 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 response. CD40 is expressed on several antigen presenting cells (APCs) and agonistic CD40 antibodies (aCD40) have broad immunostimulatory effects. Indeed, aCD40 can polarize macrophages towards a tumor-suppressive profile and enhance antigen presentation by dendritic cells (DCs)6,7. Moreover, CD40 activation of B cells regulates activation, antibody production, germinal center formation and antigen presentation.8,10 aCD40 antibodies are currently in clinical development for numerous solid tumors.11 However, there is conflicting evidence regarding their efficacy in glioma since outcomes have varied depending on experimental model and combinatorial treatment regimens.12–14 A thorough understanding of how aCD40 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 lymphoxygen (LT) is instrumental in driving TLS formation.17,18 The engagement of the LTαβ heterotrimer (or the homologous ligand TNFSF14) with the lymphoxygen β receptor (LTβR) expressed on stromal cells induces chemokines associated with leukocyte recruitment, the formation of FDC networks and of germinal centers in B cell follicles.20 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 prognosis.22–26 B cells are the dominant component of TLS and B cell-depleting antibodies recently gained clinical approval for MS patients as they ameliorate disease severity,27 while in melanoma depletion of B cells reduces CD8+ T cell infiltration.28 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 naive T cells.29 Notably, the presence of TLS in glioma has not been described to date.

In this study, we demonstrate that systemic exposure to aCD40 in glioma-bearing mice leads to reduced CD8+ T cell cytotoxicity and impairs the response to CPIs. This is associated with the expansion of a suppressive CD11b+ B cell population. However, aCD40 stimulation of B cells also enhances the formation of TLS in the brain of glioma-bearing mice. Interestingly, TLS are present in human glioma and correlate with increased intratumoral T cells in GBM, suggesting an association between TLS and regulation of immune responses in glioma patients. Our study demonstrates the presence of TLS in human glioma and reveals the multifaceted effects of aCD40 in murine glioma models, which have important therapeutic implications.

Results

aCD40 enhanced TLS formation in murine glioma models. To investigate the effects of agonistic CD40 antibodies (aCD40) on the tumor microenvironment, we intravenously administered aCD40 or the corresponding rlgG2a isotype control 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 (Supplementary Fig. 1a and Supplementary Movie 1). We defined TLS as compact clusters of CD45+ cells with a dense core of B220+B cells. aCD40 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 (Supplementary Fig. 1a, b). TLS did not form in the brain of untreated tumor-free mice or after mock injection of tumor cells followed by aCD40 therapy (Supplementary Fig. 1c). In summary, TLS were observed in brains of glioma-bearing mice and aCD40 treatment enhanced their formation.

aCD40-induced TLS resembled lymphoid tissues. 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 Supplementary Fig. 1j). A staining for the antigen binding fragment (Fab) portion of mouse IgG revealed that most B220+B cells were IgG+ antibody-producing cells and that TLS contained few IgG+B220low−/− plasma cells (Supplementary Fig. 1d), suggesting that antigen selection may have occurred within these structures. The TLS contained F4/80+ macrophages (Supplementary Fig. 1e), CD11c+ DCs (Fig. 1i and Supplementary 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 that these cells phagocytosed GFP-positive tumor cell contents (Supplementary Fig. 1f). T regulatory cells (Tregs) were also observed in the TLS (Supplementary Fig. 1g).

TLS formed around CD31+ vessels that varied in size (Fig. 1l, n and Supplementary Fig. 1n–o) and were surrounded by a distinct network of collagen IV (Fig. 1n and Supplementary Fig. 1n) and fibronectin (Fig. 1o and Supplementary Fig. 1o). The majority of B cells inside these structures stained positive for CD62L (Fig. 1m), a selectin that mediates infiltration of naïve leukocytes into lymphoid tissues.

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 2 weeks after the last administration of aCD40, indicating that continuous treatment was not required for TLS persistence (Supplementary Fig. 1h, i).

To characterize gene expression signatures associated with TLS,30 we laser capture micro-dissected TLS from aCD40-treated GL261 tumors and isolated RNA from the collected tissue (Supplementary Fig. 1j). Genes for lymphoxygen β (Ltb), C–C motif chemokine ligand 13 (Ccl13), 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 level (Supplementary Fig. 1k).
altogether, αCD40 induced the formation of tertiary lymphoid structures (TLS) 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 and stained positive for mouse IgG, which is indicative of antibody production, B cell follicular organization, and germinal center formation.

B cells were required for αCD40-induced TLS formation. To investigate the mechanism through which αCD40 induced TLS in vivo, we stained tumor sections for the rat-derived αCD40 antibody. Cells that stained positive for αCD40 were observed in both TLS and tumor area (Fig. 2b, c and Supplementary Fig. 2a). The therapeutic antibody co-localized with B220+ B cells only in the TLS of αCD40-treated mice (Fig. 2a, b), suggesting that αCD40 mainly stimulated B cells in αCD40-induced TLS. A few CD11b+ cells in the TLS also stained positive for αCD40 (Fig. 2c).

To understand whether αCD40 induced the production of TLS-associated cytokines in B cells, we isolated CD19+ cells from mouse spleen and stimulated them in vitro with αCD40. After 48 h of stimulation, B cells aggregated in clusters that became progressively larger over time (Fig. 2d). αCD40 increased the expression of Ltα and Tnfsf14 in B cells 48 h and 72 h after stimulation.
Fig. 2 B cells expressed LTα upon αCD40 stimulation and were required for TLS formation. All panels except (d–g) show data from GL261 tumor-bearing mice treated with αLG2a or αCD40. a–c Immunofluorescent staining of therapeutic rat antibodies (αRat) in TLS co-stained for αB220 and αCD11b and CD11c. Images are representative of three mice. Arrows: cells positive for αRat. White square areas are magnified to the right in b, c. Scale bars: 50 μm. d Representative images of murine CD19+ splenic B cells stimulated in vitro with αLG2a or αCD40 antibodies at indicated time points. e–g Gene expression of Lta, Ltb, and Tnsf14 in B cells shown in d. n = 4 independent experiments. One-way ANOVA with Dunnett’s correction for multiple comparison. In e, f p (rLG2a 6 h vs. αCD40 48 h) = 0.0017, p (rLG2a 6 h vs. αCD40 72 h) = 0.005, p (αCD40 6 h vs. αCD40 48 h) = 0.0012, p (αCD40 48 h vs. αCD40 72 h) = 0.0035. In f p (αCD40 6 h vs. αCD40 72 h) = 0.0223. In g p (rLG2a 6 h vs. αCD40 48 h) = 0.0439, p (αCD40 6 h vs. αCD40 48 h) = 0.0015, p (αCD40 48 h vs. αCD40 72 h) = 0.0169. h–k Gene expression of Lta and Ltb in CD19+ B220+ B cells sorted from spleen and cranial lymph nodes. i, n rLG2a = 4 mice, n(αCD40) = 5 mice. j, k n = 7 mice/group. p = 0.0285, j p = 0.0452. Two-tailed t-test, *p < 0.05. l Representative plot of quantifications shown in m, n, m Quantification of CD19+ B220+ brain-infiltrating B cells as a percentage of CD45+ cells at day 20. n(αLG2a) = 8 mice; n(αCD40) = 7 mice. n Quantification of CD19+ B220+ brain-infiltrating B cells as a percentage of CD45+ cells at day 25, n = 8 mice/group, p = 0.0003. m, n Two-tailed t-test. o Quantification of CD19+ B220+ B cells as a percentage of CD45+ cells in the blood of tumor-bearing mice treated with αLG2a or αCD40 antibodies, with (+) or without (−) B cell depletion with an αCD20 antibody, n = 4 mice/group. All indicated p-values are p < 0.0001. One-way ANOVA with Tukey’s correction for multiple comparison. p Quantification of the number of dense CD45+ B220+ clusters per 80 μm-thick section in αCD40-treated tumor-bearing brains with (+) or without (−) B cell depletion with an αCD20 antibody, n(αCD40) = 8 mice, n(αCD20 + αCD40) = 5 mice. p = 0.0234. Two-tailed t-test. h–n Black circle indicates rat IgG2a (αLG2a), red square indicates agonistic CD40 antibodies (αCD40). o, p Black circle indicates αLG2a, black square indicates αLG2a + αCD20, red circle indicates αCD40, red square indicates αCD40 + αCD20. For all graphs in this figure, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Bars: mean ± SEM. arb. units = arbitrary units. Source data are provided as a Source Data file.

stimulation (Fig. 2e, g), while Ltb expression increased after 72 h (Fig. 2f). In line with this, B cells in the spleen and superficial cranial lymph nodes of αCD40-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 αCD40-treated mice compared to the αLG2a group (Fig. 2l–n). To determine whether αCD40 stimulation of B cells was required for TLS formation, we depleted B cells 3 days before the initiation of αCD40 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 αCD40 therapy or B cell depletion (Supplementary Fig. 2b, c). Collectively, these observations demonstrate that TLS formation was mediated by αCD40 stimulation of B cells.

TLS were associated with increased T cell infiltration in human glioma. While αCD40 enhanced TLS formation, TLS were also present in αLG2a-treated 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 pre-clinical glioma models, we screened patient samples that included meningeval tissue. We collected a cohort of 26 treatment-naïve patients with de-novo gliomas, 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 (Fig. 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 as “organized TLS”. CD35+ FDCs were present in both types of TLS.
Increased number of T cells in TLS was observed in organized TLS (Fig. 3). Both TLS types included Ki67+ cells (Fig. 3f, m) and formed around PNAd+ HEVs (Fig. 3g, n). TLS also had rare CD23+ follicular B cells (Supplementary Fig. 3a, c) and CD138+ plasma cells (Supplementary 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 (Supplementary 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.

**aCD40 treatment resulted in impaired T cell responses.** Consistent with what we observed in human GBM, quantification of intratumoral T cells in aCD40-treated mice revealed a trend to an increased number of T cells in TLS+ brains compared with TLS- brains (Fig. 4a and Supplementary Fig. 4a). However, aCD40 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. Thus, we characterized the T cell response in the tumor after aCD40 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 aCD40 were observed on cytotoxic T cells (Fig. 4g; MC01, MC02), in line with a proportional increase of CD8+ T cells (Fig. 4h). The percentage of effector CD8+ T cells (CD44+CD62L-) was higher in aCD40-treated mice (Fig. 4i), however a greater proportion of effector cells were CD127+KLRG1– (Fig. 4j), pointing to a short-lived effector phenotype. A similar T cell phenotype was observed in the spleen after aCD40 treatment (Supplementary Fig. 5a–f). Markers of proliferation, exhaustion and maturation on tumor-infiltrating CD8+ T cells indicated a decreased activation status upon aCD40 therapy (Fig. 4k). In line with this, aCD40-treated mice exhibited decreased percentages of CD69+ and CD107a+ cytotoxic T cells (Fig. 4l, m). A few model-specific responses to aCD40 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). aCD40 did not affect the proportion of Tregs (Supplementary Fig. 4b, c). Systemically, we observed a typical cytokine response to aCD40, characterized by increased serum levels of pro-inflammatory cytokines after intravenous administration (Supplementary Fig. 5g–p).

To assess T cell functionality after aCD40 treatment, we isolated splenocytes from GL261 glioma-bearing mice and re-stimulated them in vitro with concanavalin A (ConA) for 24 h (Fig. 4o). CD8+ splenocytes derived from aCD40-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 aCD40-treated mice, which also exhibited decreased activation and proliferation (Supplementary Fig. 6a–d), and displayed impaired cytotoxicity and killing capability upon co-culture with GL261 tumor cells (Supplementary Fig. 6e–h). In summary, aCD40 induced a pro-inflammatory cytokine response consistent with previous studies in other tumor types, but was associated with impaired T cell responses in the glioma microenvironment and in the spleen of glioma-bearing mice.

**aCD40 impaired the efficacy of immune checkpoint inhibitors.** We sought to understand if CPIs could rescue the aCD40-induced T cell hypofunction. GL261 glioma-bearing mice were administered four doses of aCD40 and/or anti-PD-1 blocking antibodies (aPD-1). aCD40 increased the proportion of brain-infiltrating CD8+ T cells alone or in combination with aPD-1, in comparison with rlgG2a and aPD-1 monotherapy (Fig. 5a). However, the percentage of CD69+Ki67+ cytotoxic T cells was reduced in both aCD40-treated groups compared with aPD-1 monotherapy (Fig. 5b). Accordingly, co-administration of aCD40 and aPD-1 resulted in decreased survival compared with aPD-1 monotherapy (Fig. 5c). While aCD40 alone increased the number of TLS compared to rlgG2a, the combination regimen did not enhance TLS number compared to rlgG2a or aPD1 monotherapy (Fig. 5d). However, a substantial proportion of TLS with a larger surface area were present in co-treated mice (Supplementary Fig. 7a, b).

The circulating levels of rat-IgG were reduced in the aCD40 groups (Supplementary Fig. 7c), which may result from target-mediated clearance and/or from anti-rat-IgG responses elicited from repeated exposure to rat-IgG. To exclude the possibility that drug clearance was the reason for the reduced survival in the combination group, we assessed a combination of aCD40 with a fully murine aCTLA-4 antibody and also evaluated a single dose of aCD40 followed by three doses of aPD-1 (Supplementary Fig. 7d–i). In both cases aCD40 therapy hampered the effect of the CPIs (Supplementary Fig. 7, d, f). Interestingly, aCD40 was still able to induce TLS formation when combined with aCTLA-4 as compared to aCTLA-4 monotherapy.

A HSNE analysis of CD8+ T cells (Fig. 5e–j) revealed that aPD-1 polarized the cytotoxic T cell response towards an active and proliferating state (Fig. 5i, j; MC02), while aCD40 resulted in impaired activation and/or proliferation (Fig. 5i, j; MC03, MC06, MC04, and MC01). Strikingly, co-administration of aCD40 and aPD-1 shifted the cytotoxic T cell response towards a low-activation, low-proliferation state (Fig. 5i, j; MC03, MC06). In summary, aCD40 induced a hypofunctional T cell state that inhibited the efficacy of CPIs.

**aCD40 activated brain-infiltrating DCs and myeloid cells.** To understand the cellular mechanisms involved in the aCD40-induced CD8+ T cell hypofunction, 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 aCD40 therapy (Supplementary Fig. 8a, b). aCD40 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 (Supplementary Fig. 8c). aCD40 did not alter the relative amount of IL-12+ DCs or myeloid cells (Supplementary Fig. 8d, e), but decreased the proportion of IL-10+ DCs (Supplementary Fig. 8f, g). Altogether, aCD40 promoted an activated phenotype of brain-infiltrating DCs and myeloid cells.

**aCD40 induced CD11b-expressing B cells.** Next, we investigated the phenotype of intratumoral B cells in aCD40-treated glioma-bearing mice. aCD40 therapy increased the expression of CD86, MHC-II, and IL-12 alone or in combination with aPD-1 (Fig. 6a, b), but also increased the proportion of CD5+CD14+ B cells (Supplementary Fig. 9a, b). B cells expressing CD5 and CD14 have previously been classified as regulatory B10 cells and can inhibit...
CD4+ T cell responses via secretion of IL-10. However, gene expression of immunosuppressive factors including IL10, TGFβ1, CCL22, and LGALS1 was not increased in B cells after αCD40 therapy (Supplementary 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 reduced T cell functionality.

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. A similar effect was observed in the spleen (Supplementary Fig. 10a). In the brain, CD11b+ B cells were rarely observed within the TLS (Fig. 6e) but were predominantly present in the tumor area (Fig. 6f) and had lower surface levels of CD11b compared to myeloid cells (Supplementary 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 studies. Notably, IL-10 levels were systemically high in CD11b+ B cells alone or in combination with αPD-1 (Fig. 6k).

**Fig. 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 μm. a, b Representative of 21 immature TLS. h, i Representative of 16 organized TLS. Stainings in c–g and j–n were performed on one representative immature TLS and one representative organized TLS. 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). n(Grade II + III, negative) = 7, n(Grade II + III, immature TLS) = 1, n(Grade II + III, organized TLS) = 2, n(GBM, negative) = 8, n(GBM, immature TLS) = 3, n(GBM, organized TLS) = 5. p Number of T cells infiltrating the tumor area in GBM patients negative for TLS (gray circle) versus GBM patients positive for TLS (orange triangle: immature TLS; red triangle: organized TLS). n = 7 patients/group. p = 0.0142. Two-tailed t-test. Bars: mean ± SEM.

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 μm. For all graphs in this figure, *p < 0.05. Source data are provided as a Source Data file.
Fig. 4 αCD40 therapy resulted in suppressed cytotoxic T cell responses in preclinical glioma models. 

a Quantification of CD8+ T cells in the tumor area of αCD40-treated GL261 glioma-bearing brains that were positive (red square) or negative (red circle) for TLS. n(TLS−)=7 mice, n(TLS+)=10 mice. Two-tailed t-test.

b, c Kaplan–Meier survival curve of GL261 (n=19 mice for rlg2a, n=20 mice for αCD40 group) and CT-2A (n=10 mice/group) tumor-bearing mice treated with αCD40 or rlg2a on days 10, 13, 16, and 19 (as indicated by arrows). Black line: rlg2a; Red line: αCD40. Log-rank test.

d-h 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 rlg2a or αCD40. n(rlg2a)=5 mice, n(αCD40)=7 mice. d HSNE analysis identified six meta-clusters (MC) of CD3+ T cells, expressing different levels of CD4 and CD8 (e).

f Spatial distribution of CD3+ T cells from GL261 and CT-2A tumor-bearing brains, in rlg2a-treated vs. αCD40-treated mice. g Frequency distribution of CD3+ T cells from each model and treatment group in each MC. h Data was obtained from GL261 and CT-2A tumor-bearing mice treated with rlg2a or αCD40. h CD8+ T cells as a percentage of CD45+ cells. n(GL261)=8 mice/group, n(CT-2A)=5 mice/group.  

p(G256)<0.0001, p(CT-2A) < 0.0001. i, n show data on CD8+ T cells. i CD4+CD62L− cells, n(GL261-rlg2a)=16/mice/group, n(GL261-αCD40)=7 mice. p(GL261)=0.0031, p(CT-2A)<0.0001. j CD127+KLRG1+ cells, n(GL261-rlg2a)=16/mice/group, n(GL261-αCD40)=7 mice. p(GL261)=0.003, p(CT-2A)=0.0004. l CD69+ cells, n(GL261-rlg2a)=7 mice, n(GL261-αCD40)=8 mice. n(CT-2A)=5 mice/group. p(GL261)=0.0024, p(CT-2A)=0.0005. m CD107a+ cells, n(GL261)=6 mice/group; n(CT-2A)=5 mice/group. p(GL261)=0.01, p(CT-2A)<0.0001. n PDL1+TIM3+LAG3+ cells (n(GL261-rlg2a)=5 mice, n(GL261-αCD40)=7 mice, n(CT-2A)=5 mice/group).  

p(GL261)<0.0462. h-j and l-m Two-tailed t-test. k Heat map showing the mean fluorescence intensity (MFI) of proliferation, exhaustion and memory markers on CD8+ T cells. n(GL261-rlg2a)=5 mice, n(GL261-αCD40)=7 mice, n(CT-2A)=5 mice/group.  

O Experimental layout used to obtain data shown in panels (p-r). In brief, GL261 glioma-bearing mice were treated with rlg2a or αCD40 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 24 h. p Percentage of CD8+ splenocytes in different generations (G), where cells in G0 did not proliferate and cells in G6 underwent six cycles of proliferation. An example of how generations are defined is shown in Supplementary Fig. 6a. p(G0)=0.0464, p(G6)=0.0069. Multiple t-test with Sidak–Bonferroni correction. q Mean fluorescence intensity (MFI) of CD69 on CD8+ splenocytes. p=0.0248. r CD107a+ T cells as a percentage of CD8+ splenocytes. p=0.0002. q, r Two-tailed t-test. p-r n=5 mice/group. For all graphs: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars: mean ± SEM. In all graphs except (a): black circle indicates rat IgG2a (rlg2a), red square indicates agonistic CD40 antibodies (αCD40). Source data are provided as a Source Data file.
**CD11b** B cells inhibited CD8+ T cell responses. Surface expression of CD11b on B cells inhibits CD4+ T cell responses, resulting in lower T cell proliferation and IFNγ production. 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 (Supplementary Fig. 11a, b). Blocking CD11b rescued activation (CD69), cytotoxicity (CD107a+ cells), and IFNγ production. To determine the mechanism of CD11b-mediated inhibition, we co-cultured these cells with splenocytes in the presence or absence of a CD11b-neutralizing antibody (Supplementary Fig. 11a, b).

**Fig. 5** αCD40-induced T cell hypofunction impaired the efficacy of checkpoint blockade. All panels show data from GL261 tumor-bearing mice treated with rIgG2a, αCD40, and/or αPD1. 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. One-way ANOVA with Tukey’s correction for multiple comparisons. a All indicated p-values are p < 0.0001. b P(R/IA2a vs. αCD40 +αPD1) = 0.0073, P(αCD40 vs. αPD1) = 0.0064, P(αPD1 vs. CD40+αPD1)<0.0001. c Kaplan–Meier survival curves of mice treated as indicated on days 10, 13, 16, and 19 (as shown by arrows). n = 10 mice/group. P(R/IA2a vs. αPD1) = 0.0008, P(R/IA2a vs. αCD40 +αPD1) = 0.0211, P(αCD40 vs. αPD1) = 0.0006, P(αPD1 vs. αCD40 +αPD1) = 0.0025, P(αCD40 vs. αCD40+αPD1) = 0.037. Log-rank test. d Number of dense CD45+ B220+ clusters per section identified in the indicated treatment groups. n (rIgG2a) = 8 mice, n (αCD40) = 10 mice, n (αPD1) = 11 mice, n(αPD1 + αCD40) = 17 mice. P(α/IA2a vs. αPD1) = 0.0139, P(αCD40 vs. αPD1) = 0.0101. One-way ANOVA with Tukey’s correction for multiple comparisons. e Spatial clustering of tumor-infiltrating CD3+ cells in GL261 tumors in different treatment groups, determined by Level-1 Hierarchical Stochastic Neighbor Embedding (HSNE) analysis of flow cytometry data. 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. a, b and e-j n (rIgG2a) = 5 mice, n (αCD40) & (αPD1 + αCD40) = 7 mice, n (αPD1) = 8 mice. For all graphs in this figure, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Bars: mean ± SEM. Black circle indicates rat IgG2a (rIgG2a), red square or diamond indicate agonistic CD40 antibodies (αCD40), blue triangle (pointing up) indicates PD1 blocking antibodies (αPD1), green triangle (pointing down) indicates αPD1 + αCD40. Source data are provided as a Source Data file.

**CD11b** B cells inhibited CD8+ T cell responses. Surface expression of CD11b on B cells inhibits CD4+ T cell responses, resulting in lower T cell proliferation and IFNγ production. 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 (Supplementary Fig. 11a, b). Blocking CD11b rescued activation (CD69), cytotoxicity (CD107a+ cells) and IFNγ production of CD8+ T cells stimulated with CD3/CD28 beads (Supplementary Fig. 11c–g).

Tumor-infiltrating CD11b+ B cells displayed higher levels of MHC-II compared to their CD11b− counterparts in the αCD40-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 αCD40-treated groups (Fig. 6i, j), which is in line with a CD11b-
mediated internalization of the TCR complex as previously reported. Moreover, similarly to CD8+ T cells (Fig. 5b), intratumoral CD4+ T cells showed lower activation and proliferation in aCD40-treated mice (Supplementary Fig. 10d, e), and depletion of B cells prior to in vivo administration of aCD40 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 aCD40-treated mice.

**Discussion**

Agonistic CD40 antibodies are under clinical development for multiple solid tumors and are being investigated in two Phase I clinical trials of CNS malignancies (NCT03389802; NCT04547777). In this study, systemic delivery of aCD40 impaired T cell responses, promoted 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 (TLI) cells, other cell types can express Lt to induce TLS. B cells can act as TLI cells in the gut and their transient activation via aCD40 antibodies enhanced TLS maturation in an artificial model of TLS induction. Our study reveals that aCD40 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 cells, this strongly suggests that aCD40 could have similar effects on TLS induction in glioma patients.

Similarly to other cancer types, the presence of TLS was associated with increased T cell infiltration in human GBM, which could be beneficial if the T cells are activated and primed against the tumor. However, a larger cohort is needed to further elucidate the role of TLS in glioma immunity, response to checkpoint blockade and patient survival. It is also important to determine how TLS formation in the brain is affected by other cancer therapies, as corticosteroid treatment and hypofractionated radiotherapy have been associated with reduced TLS formation in other cancer types.

In aCD40-treated glioma-bearing mice, TLS also correlated with increased numbers of intratumoral T cells. However, systemic aCD40 treatment led to an accumulation of CD11b+ B cells in the brain and spleen, which was associated with impaired cytotoxic T cell responses and reduced efficacy of CPI therapy. While it is clear that innate B cells can produce regulatory cytokines, 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) and B cell depletion increased the abundance of T cells in the tumor specifically in aCD40-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.

CD11b expression was not directly induced by aCD40 stimulation of B cells. Rather, it was associated with an increase in systemic IL-10 after aCD40 treatment which was observed also when the B cells were depleted, suggesting that these cells were not the main IL-10 producers. Thus, targeting CD40 specifically to B cells could help circumvent 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 aCD40 and IFNγ elicited anti-tumor immunity in glioma-bearing mice. Contrary to what we observed in glioma models, aCD40 therapy generally enhances immune response in peripheral tumor models. Therefore, the lack of therapeutic effect in glioma is likely due to the distinct immune regulation in the brain. For instance, T cells are sequestered in the bone marrow specifically in response to intracranial tumors, leading to T cell lymphopenia which considerably affects the response to immunotherapy. Moreover, B cells are particularly important for antigen presentation and T cell-mediated anti-tumor immunity in the brain, thus the acquisition of a suppressive CD11b+ B cell phenotype may explain the detrimental effect of aCD40 on T cell responses specifically in brain tumors.

CD11b+ B cells were rarely present within aCD40-induced TLS, therefore they are not likely to mediate immunosuppression within these structures. However, we observed T regulatory cells (Tregs) in aCD40-induced TLS. Since the presence of Tregs in TLS has been associated with suppressed anti-tumor immune responses and tumor progression, the role of aCD40-induced TLS in glioma has to be further investigated.

Our study demonstrates that systemic aCD40 therapy results in reduced cytotoxic T cell responses and decreases the efficacy of CPIs in preclinical glioma models. A potential limitation of this study is that the preclinical glioma models used have a high mutational burden, and are more responsive to immune checkpoint blockade than most de novo human gliomas. Nevertheless, the induction of regulatory B cells and of TLS by aCD40 is likely to have a similar impact on the immune response in human glioma, making this relevant information for clinical trials currently investigating aCD40 therapy in patients with primary CNS tumors. Our work also reveals that TLS are present in glioma patients and that immunotherapies can modulate these structures in murine glioma models. The importance of TLS in response to CPIs is not known, and needs to be further investigated. In our study, aPD1 therapy, but not aCTLA-4, hampered the ability of aCD40 to induce TLS formation in the brain, consistent with the role of PD-1 in regulating B cell survival in germinal centers. The finding that TLS in the brain can be manipulated thermally opens up possibilities for triggering or suppressing immune responses, which has broader implications for brain malignancies and autoimmune diseases of the central nervous system.

**Methods**

**Cell lines.** The GL261 (gift from Dr. Geza Safaryn, NRIRR, Budapest, Hungary) and CT-2A (gift from Dr. T. Seyfried, Boston College, Boston, MA, USA) cell lines were transfected with lentiviruses to express GFP and luciferase. 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, Carlsbad, CA, USA) and the mice were observed until full recovery from anesthesia on a heated pad. Mice were exposed to 12 h light on/light off cycles. For injection of tumor cells, mice (at least 7 weeks of age) were anesthetized with 2.5% isoflurane and immobilized in a stereotactic frame on a heated animal holding platform. A hole was drilled in the skull at ~1 mm anteroposterior and +1.5 mm mediolateral stereotactic coordinates from the bregma. GL261 cells (2 × 10^6) were delivered in 2 µl 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 ≥0.5 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 CI/14, C26/15, N164/15, and 5.8.18-19249/2019), 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 perfusion fixation with 10% (vol/vol) phosphate-buffered saline (PBS) (Thermo Fisher Scientific) and 10 ml of 4% (wt/vol) parafomaldehyde (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 µg/dose), rat-anti-mouse PD-1 (clone: RMF1-14, Cat# BE0146, 200 µg/dose), mouse-anti-mouse CTLA-4 (clone: 9D9, Cat# BE0164, 100 µg/dose), rat-anti-mouse CD20 (clone: AIS812, Cat# BE0302, 200 µg/dose) antibodies (Abs) were administered intravenously in a final volume of 100 µl aCD40 Abs were administered either in
implantation. followed by repeated doses of control groups in each experiment (100 µg/dose during rIgG2a vs. alone or in combination with rIgG2a for 20 min on a low brake (brake resuspending the cells in a solution of 25% BSA (in PBS) and centrifuging at 650× in PBS.

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 Capture microdissection, and FACS were collected in RLT lysis buffer (Qiagen, 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, 2 mM l-glutamine, 10 mM HEPES, 20 µM β-mercaptoethanol, 1 mM sodium-pyruvate, 100 U/ml penicillin-streptomycin (all purchased from Thermo Fisher Scientific, Waltham, MA, USA) and 100 U/ml IL-2 (Novartis, Basel, Switzerland). T cell assays were performed with cells isolated from GL261 glia-bearing mice. To achieve ex vivo stimulation of brain-infiltrating CD8+ T cells or splenocytes, cells were isolated on day 22-post tumor implantation (3 days after the last cd40 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 committed for 24 h. CD8+ TILs were stimulated for 24 and 72 h. 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.

流 cytometry and FACS. Cells were stained using a live-dead dye (Supplementary Tables 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 Tables 4 and 5). All antibodies were diluted from stock concentration according to the ratios reported in Supplementary Tables 4 and 5. For staining of FoxP3, the FOXP3 Fix/Perm Buffer Set (BioLegend) was used following the instructions of the manufacturer. For intracellular cytokine staining, the following intracellular fixation and permeabilization kit was used (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) (data was collected using BD FACSDiva 8.0.2) or CytoFLEX LS (Beckman Coulter, Brea, CA, USA) (data was collected using CytExpert 2.4); alternatively, the cells were sorted directly into RT Lysis buffer (Qagen) using FACS AriaIII (BD BioSciences). Data were analyzed using FlowJo version 10.5.3 (FlowJo LLC, Ashland, OR, USA) or Cytoscape version 2.2.1 65.67. Gating strategies used in this paper can be found in Supplementary Figs. 12 and 13.

HSNE analysis. HSNE analysis was performed using Cytoscape version 2.2.1.66. 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 Cytoscape version 2.2.1 65.67 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 used: CD3, CD4, CD8, CD62L, CD127, CXCR5, CD45, 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 IgG2a-treated and 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-Probes antibody Panel 1 Mouse Inflammation & Permeability 6-plex was used to determine 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 plate readouts, the data were analyzed using the Sector Imager 2400 (Meso Scale Discovery) and final protein concentrations were calculated by reference to 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 of post-tumor implantation from mice treated with rlgG2a, aCD40, aCD40-1, and aCD40 + aCD40-1 antibodies, using Microvette CB300 Capillary Blood Collection Tubes (Sarstedt, Nunmrecht, Germany). The IgG (Total) Rat UniCoated 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. Vibriostatic sections (80 μm-thick) were prepared from PFA-fixed brains. Vibratome sections 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 centrifugation, brains were collected and snap-frozen in isopentane. Cryosections (7 μm-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), together with DAPI nuclear stain (Thermo Fisher Scientific, Waltham, MA, 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.). Images were collected using a Zeiss Axioimager microscope (Zeiss, Oberkochen, Germany), and slides were counterstained with hematoxilin. 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.). Images were collected using a Zeiss Axioimager microscope (Zeiss, Oberkochen, Germany), and slides were counterstained with hematoxylin.

**Immunohistochemical staining of human samples.** After intracerebral perfusion, postmortem 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 μm-thick) were prepared from PFA-fixed brains. Vibratome sections 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 centrifugation, brains were collected and snap-frozen in isopentane. Cryosections (7 μm-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) 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.). Images were collected using a Zeiss Axioimager microscope (Zeiss, Oberkochen, Germany), and slides were counterstained with hematoxylin.
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Competing interests
S.M.M. is the founder and shareholder of Immunex AB and Virolabeled 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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