

## ELTD1 as a multi-focal target for malignant gliomas: preclinical studies

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### Abstract

**Background.** Glioblastoma (GBM) is the most aggressive malignant primary brain tumor in adults. These high-grade gliomas undergo unregulated vascular angiogenesis, migration and cell proliferation allowing the tumor cells to evade cell-cycle checkpoints and apoptotic pathways. The Epidermal growth factor, latrophilin, and seven transmembrane domain-containing 1 on chromosome 1 (ELTD1) is an angiogenic biomarker that is highly expressed in malignant gliomas. Novel treatments targeting ELTD1 with monovalent monoclonal (mAb) and single chain variable fragment (scFv) antibodies were effective in increasing animal survival, decreasing tumor volume and normalizing the vasculature. Due to the success of our antibody treatments on angiogenesis, this study sought to determine if our anti-ELTD1 treatments affected other aspects of tumorigenesis (cell proliferation, migration, and apoptosis) in a G55 glioma xenograft preclinical mouse model.

**Methods.** Tumor tissue from untreated, mAb and scFv anti-ELTD1 treated animals was used to quantify the positivity levels of human mitochondrial antibody, c-MET and Ki-67 for cellular proliferation, migratory markers CD44v6, TRPM8, and BMP2, and cleaved caspase 3 to assess apoptotic activity.

**Results.** This approach demonstrated that our anti-ELTD1 treatments directly affected and decreased the human tumor cells within the tumor region. Additionally, there was a significant decrease in both cellular proliferation and migration due to anti-ELTD1 therapy. Lastly, anti-ELTD1 treatments successfully increased apoptotic activity within the tumor region.

**Conclusion.** Our data suggest that anti-ELTD1 therapies would be effective against malignant gliomas by having a multi-focal effect and targeting all four aspects of tumorigenesis.

### Key Points

- Anti-ELTD1 therapy directly affects and decreases human tumor cells.
- Anti-ELTD1 effectively decreases cellular proliferation and migration.
- Anti-ELTD1 therapy increases apoptotic activity.

The most common malignant glioma is glioblastoma (GBM, grade IV) and occurs in 5–7 cases per 100,000 persons per year.<sup>1</sup> Current standard of care includes maximal surgical

resection followed by concurrent treatments of radiotherapy and chemotherapy (e.g. temozolomide and bevacizumab).<sup>2</sup> However, patient survival remains low with an average survival

## Importance of the Study

Glioblastomas (GBM) are extremely aggressive and patients rarely survive 5 years postdiagnosis with standard treatment. Current chemotherapeutic agents used in clinics have historically been singular pathway therapies, which has allowed GBM cancer cells to become resistant to the common therapies. Previous studies targeting ELTD1 with varying antibodies established the therapy as an anti-angiogenic treatment against malignant gliomas. This study focuses on investigating whether anti-ELTD1 therapy also affects other

aspects of tumorigenesis such as cellular proliferation, migration, and apoptosis. This study showed that anti-ELTD1 therapies, directly target and reduce human cancer cells, and most importantly successfully reduced cellular proliferation and migration and increased apoptosis. This study is one of the first to demonstrate that targeting ELTD1 directly targets various aspects of tumorigenesis. This study increases the therapeutic potential of anti-ELTD1 over singular pathway drugs.

of 15 months postdiagnosis and a 5.8% 5-year survival.<sup>3,4</sup> Primary GBMs represent 90% of all cases and occur in patients (mean age of 65) without a prior history of a precursor malignant lesion.<sup>5</sup> On the other hand, GBMs that develop from a low-grade glioma, most commonly develops from an astrocytoma or oligodendroglioma, are considered secondary GBM.<sup>6</sup> Although tumor development differs, both primary and secondary GBMs have similar clinical presentations and are characterized by unregulated vascular angiogenesis, and uncontrolled migration and cell proliferation allowing the tumor cells to evade cell-cycle checkpoints and apoptotic pathways.<sup>7</sup> Due to high mortality and recurrence rates, there is crucial need to develop new therapies for GBM.

Historically, the epidermal growth factor, latrophilin and seven transmembrane domain containing protein 1 on chromosome 1 (ELTD1), also known as ADGRL4, is known as a biomarker of angiogenesis. ELTD1 was first discovered to be highly expressed in rat pup cardiomyocytes and smooth muscle cells.<sup>8</sup> More recently however, ELTD1 has been linked to both the progression and development of malignant gliomas and studies have shown that ELTD1 is expressed on both tumor and endothelial cells.<sup>9–11</sup> Two main angiogenic pathways also regulate ELTD1 expression. In normal vasculature, VEGF signaling is known to increase ELTD1 expression, while the DLL4/Notch pathway has an opposite effect and instead decreases the expression.<sup>12</sup> Novel treatments targeting ELTD1 with various antibodies have shown to be effective in increasing survival, decreasing tumor volumes, as well as normalizing the vasculature within the tumor region in GL261 syngeneic, and human G55 xenograft, mouse models.<sup>11,13,14</sup> Additionally, a recent molecular targeted MRI study by our group showed that molecular probes attached onto single chain variable fragment (scFv) ELTD1 antibodies had future diagnostic potential by reaching and exposing extremely diffuse tumor regions that would have otherwise been undetectable with conventional MRI.<sup>14</sup>

So, the next question becomes, what makes the anti-ELTD1 treatment effective, if all of the past therapies have failed? To be able to answer, we first need to look at the current chemotherapies. Temozolomide (TMZ) is the most common chemotherapeutic agent used to combat

GBMs that was approved for use in the United States in the early 2000s. This drug is an oral DNA alkylating agent that can cross the blood-brain barrier due to its small size and lipophilic properties.<sup>15</sup> TMZ is responsible for DNA methylation at the N7 and O6 site of guanine in guanine rich regions and O3 site of adenine that induce single and double stranded DNA breaks leading to cytotoxicity within the tumor cells.<sup>15</sup> O6-methylguanine DNA methyltransferase (MGMT) is known as a DNA “suicide” repair enzyme that repairs the damaged guanine nucleotides from TMZ activity and instead promotes tumorigenesis.<sup>15</sup> Unfortunately, approximately 50% of GBM patients treated with TMZ do not respond to the therapy due to high MGMT expression levels.<sup>16</sup> Additionally, Liu *et al.* has demonstrated that glioma stem cells can also have high expression levels of MGMT and therefore become resistant to TMZ.<sup>17</sup>

In 2009, the Food and Drug Administration (FDA) fast-tracked the approval of bevacizumab after promising phase II studies for primary and recurrent GBM.<sup>18</sup> GBMs are characterized by unregulated vascular angiogenesis driven by the vascular endothelial growth factor (VEGF) and Notch. Bevacizumab was created to combat angiogenesis in GBMs by binding onto VEGF-A, which inhibits its interaction with VEGF receptors 1 and 2 (VEGFR1, VEGFR2) on the surface of endothelial cells.<sup>19</sup> Although initial preclinical studies were promising, in clinics bevacizumab did not significantly increase patient survival.<sup>18,20</sup> Instead, several preclinical and clinical reports have suggested that GBM cells may acquire resistance to anti-angiogenic therapies by various mechanisms, such as increasing the recruitment of myeloid cells that drive tumor growth, promoting a migratory phenotype to ensure proper oxygen delivery, and up-regulation of pro-angiogenic molecules.<sup>21–24</sup>

The issue regarding GBM therapies is that TMZ and bevacizumab each target one pathway. This singular pathway approach allows the cancer cells bypass cell death through other mechanisms, and become resistant to the common treatments. Therefore, we aimed to investigate if our monovalent monoclonal (mAb, which we previously referred to as mAb) or single chain variable fragment (scFv, fragment) anti-ELTD1 treatments just targeted angiogenesis, as seen in our previous studies, or if they also affected

other aspects of tumorigenesis, such as apoptosis, cellular proliferation or migration.

## Methods

### G55 Xenograft Model and Treatment

All animal studies were conducted with the approval (protocol 17-48) of the Oklahoma Medical Research Foundation (OMRF) Institutional Animal Care Use Committee (IACUC) policies, which follow NIH guidelines. Human G55 xenograft cells were intracerebrally implanted in two-month old male Athymic nude mice (Hsd:Athymic Nude-Foxn1nu mice; Harlan Inc., Indianapolis, IN) as previously described.<sup>14,25</sup> Mice were divided into three groups: mmAb and scFv against ELTD1, or untreated ( $n = 4-6/\text{group}$ ). Once tumors reached 6–7 mm<sup>3</sup> (determined via MRI), mice were treated with 2 mg/kg of either mmAb or scFv against ELTD1 every 3–4 days (treated M/Th, T/F, W/Sat) via tail-vein catheters, until sacrifice, or were left as untreated controls. All mice were euthanized when tumors reached  $\geq 150$  mm<sup>3</sup> prior to tumor-induced death.

### Immunohistochemistry and Standard Staining

Five-micron thick histological sections, embedded in paraffin and mounted on HistoBondPlus slides (Statlab Medical Products, Lewisville, TX) were rehydrated and washed in Tris Buffered Saline (TBS). Rabbit antibodies were used for c-Met (cat# sc-10, 1:50, 4  $\mu\text{g/ml}$ , Santa Cruz Biotechnology, Santa Cruz, CA), Caspase-3 (cat# sc-7148, 1:50, 4  $\mu\text{g/ml}$ , Santa Cruz Biotechnology, Santa Cruz, CA), Ki-67 (cat# NB600-1209, 1:200, 2  $\mu\text{g/ml}$ , Novus Biologicals, Littleton, CO), CD44v6 (cat# orb13319, 1:300, 1.7  $\mu\text{g/ml}$ , biorbyt Ltd, San Francisco, CA), TRPM8 (cat# ab3243 1:500, 1  $\mu\text{g/ml}$ , abcam, Cambridge, MA), BMP2 (cat# ab14933, 1:200, 4  $\mu\text{g/ml}$ , Cambridge, MA). Mouse antibody for anti-Mitochondria was used to stain mitochondria for human cells. Slides were processed for Immunohistochemistry using Anti-Rabbit IgG ImmPRESS<sup>®</sup> Excel Amplified Polymer kit Peroxidase (cat# MP7601, Vector Labs, Burlingame, CA) or Anti-Mouse IgG ImmPRESS<sup>®</sup> Excel Amplified Polymer kit Peroxidase (cat# MP-7602, Vector Labs Inc., Burlingame, CA). Antigen retrieval (pH 6 Citrate Antigen Unmasking Solution (cat# H-3300, Vector Labs Inc., Burlingame CA) or pH 9 Tris based (cat# H3301, Vectors Labs, Burlingame CA)) was accomplished via twenty minutes in a steamer followed by 30 min cooling at room temperature for all antibodies. Sections were treated with a peroxidase blocking reagent (Bloxall, cat# SP-6000, Vector Laboratories, Inc, Burlingame, CA) to inhibit endogenous peroxidase activity, followed by 2.5% normal horse serum blocking reagent to inhibit nonspecific binding. Appropriate washes were in TBS. Antibodies were applied to each section and following incubation overnight at 4°C in a humidified chamber, sections were washed in TBS and reagents were applied according to the manufacturer's directions. Slides were incubated with NovaRed<sup>®</sup> (Vector Laboratories, Inc., Burlingame, CA) chromogen for visualization. Counterstaining was carried out

with Hematoxylin QS Nuclear Counterstain (Vector laboratories, Burlingame, CA). Appropriate positive and negative tissue controls were used.

All of the IHC slides were scanned using the Leica Aperio scanner (Leica Biosystems, Buffalo Grove, IL). To characterize IHC expression levels, five ROIs, captured digitally (20 $\times$ ), were identified in each case. Only areas containing tumor tissue were analyzed, excluding areas with necrosis and/or significant artifacts. The number of positive pixels was divided by the total number of pixels in the analyzed area. ROIs were analyzed and imaged using Aperio ImageScope (Leica Biosystems, Buffalo Grove, IL).

### Migration Chamber Assay

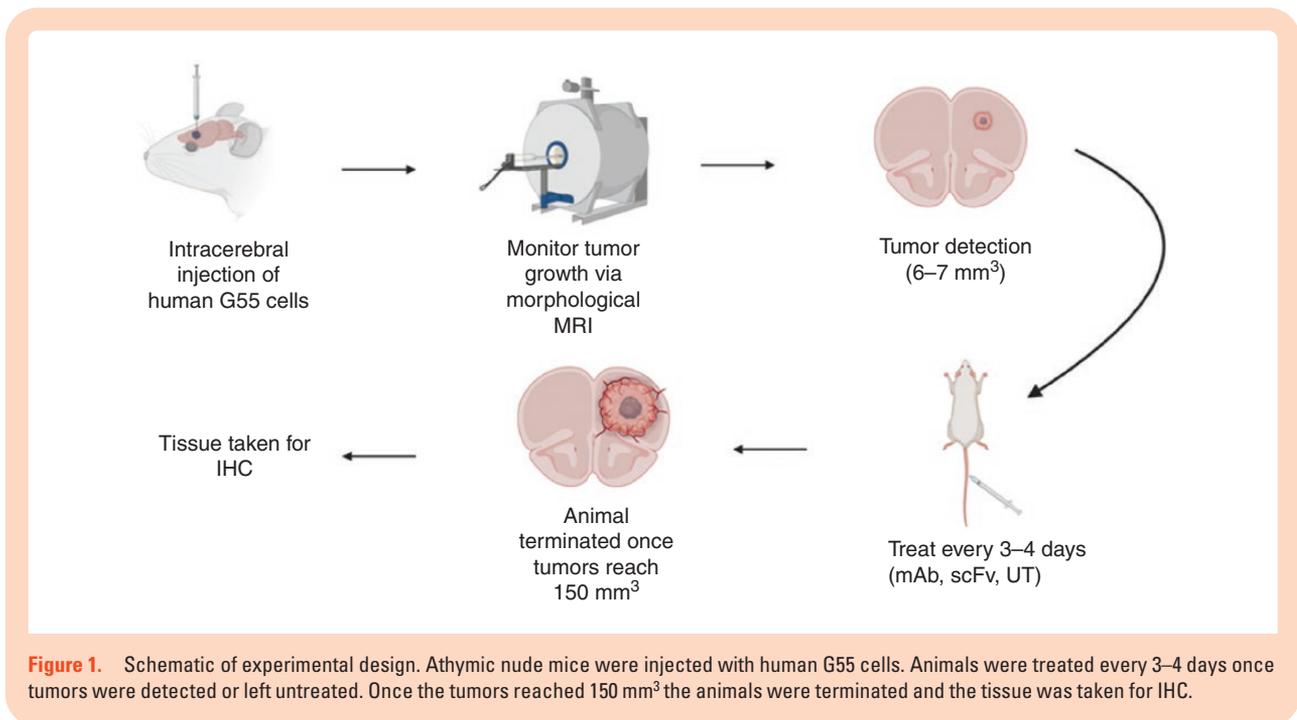
Migration analysis of GBM cells with 10 nM treatments of either mmAb or scFv anti-ELTD1 treatments or untreated as a control was carried out in six-well chambers with polydimethylsiloxane (PDMS) microchannels (5  $\times$  5  $\mu\text{m}^2$ ). 100  $\mu\text{l}$  of media containing  $2 \times 10^4$  G55 cells labeled with H2b-GFP were seeded near each PDMS device in the six-well chamber and allowed to settle for an hour in a CO<sub>2</sub> incubator. The cells in six-well chamber were then supplemented with fresh media with or without the treatments and the time was set as zero hour of migration. The cells were allowed to migrate in microchannels and were imaged at 16 h and 44 h using Perkin Elmer Operetta<sup>®</sup> high content imaging system [Cancer Functional Genomics Core Facility, OUHSC] under GFP filter in 10 $\times$  magnification. The images obtained were processed and the distance of migration by each cell was measured at 16 and 44 h timepoints using Columbus<sup>®</sup> software and the average cell migration velocity was calculated as  $\mu\text{m/h}$ .

### Statistical Analysis

Immunohistochemistry protein levels were analyzed and compared by one-way ANOVA with multiple comparisons (Tukey's). Data were represented as mean  $\pm$  SD, and  $P$ -values of either  $* < .05$ ,  $** < .01$ ,  $*** < .001$ ,  $**** < 0.0001$  were considered statistically significant.

## Results

GBM tumorigenesis consists of unregulated angiogenesis, increased cell proliferation and motility, and decreased apoptosis. Previous studies have shown that anti-ELTD1 therapies are successful in normalizing the tumor associated vasculature.<sup>13,14</sup> Therefore, we aimed to determine what other possible effects anti-ELTD1 treatment had on other aspects of tumorigenesis. Previous studies have shown no significant difference between nonspecific IgG control treatment and untreated animals, therefore we opted to use untreated animals as the control.<sup>11</sup> The tumor volumes and survival for each treatment group was previously published and the experimental strategy is summarized in Figure 1.<sup>14</sup> Briefly, the untreated animals had an average tumor volume of 133 mm<sup>3</sup> and 9 days survival post tumor detection. Monoclonal anti-ELTD1 treated animals had



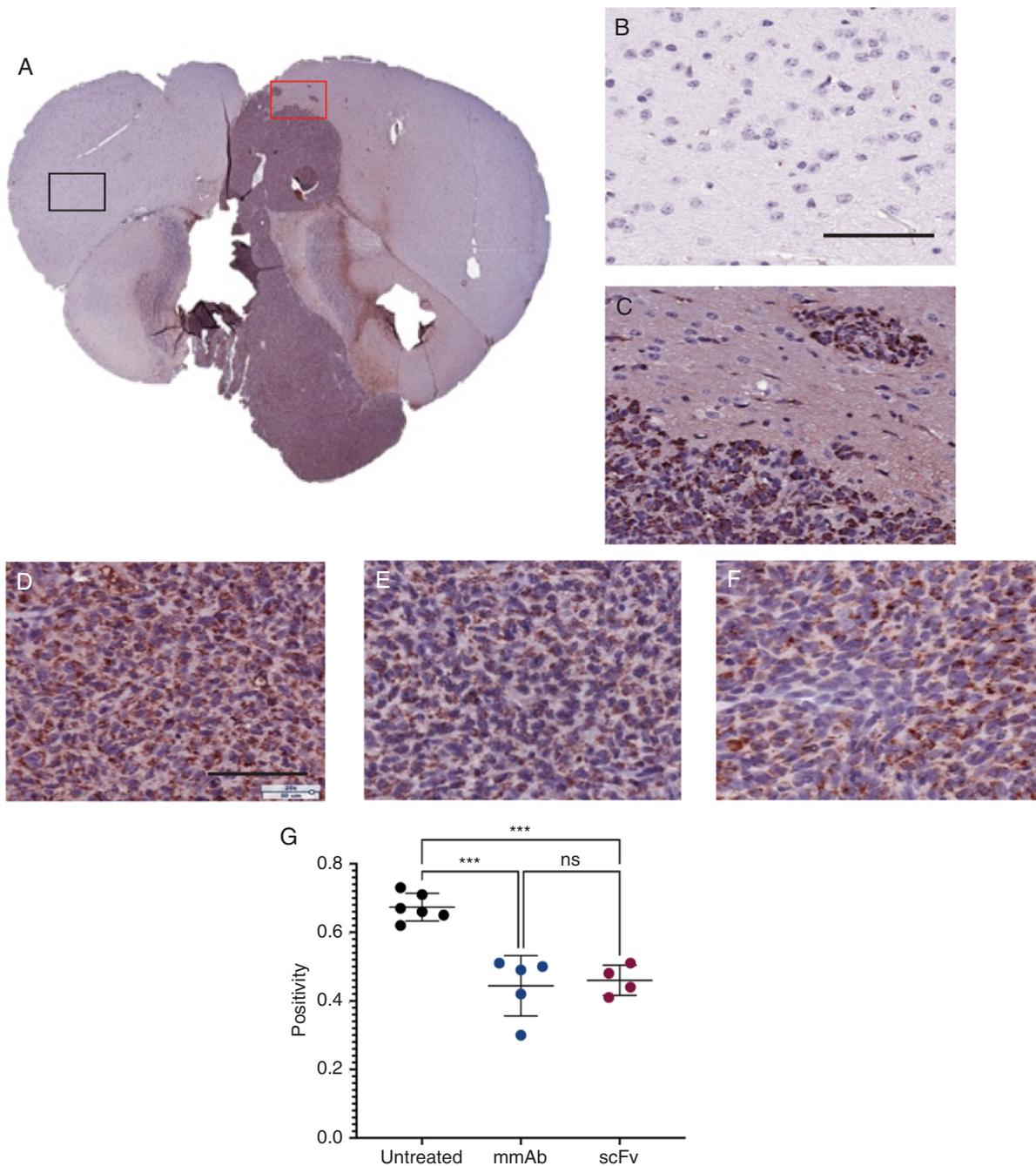
an average tumor volume at 9 days post tumor detection of 57 mm<sup>3</sup> and an average survival of 16 days while the fragment anti-ELTD1 treated mice had an average tumor volume at 9 days post tumor detection of 70 mm<sup>3</sup> and an average survival of 18 days.

We determined if our anti-ELTD1 treatments had a direct effect on human tumor cells by staining brain tissue against the human mitochondrial antibody. **Figure 2A** is a representative untreated IHC slice showing that there is no mitochondrial antibody staining in the healthy contralateral tissue as shown by the black box. Additionally, the blue box and **Figure 2C** demonstrates the distinct tumor boundaries that can be seen as shown through the antibody positivity. Positivity quantification demonstrated that the untreated controls have high positivity staining against the human mitochondrial antibody within the tumor region (**Figure 2D, G**). **Figure 2E–G** demonstrates that the anti-ELTD1 treatments are effective in reducing the overall positivity staining for human mitochondria within the tumor region. The anti-ELTD1 treatments, therefore, directly target human tumor cells within mouse brain tissues. With molecular-targeted MRI we have previously demonstrated that both the mmAb and fragment directly bind onto and specifically target the tumor cells.<sup>14</sup> Additionally, we stained the tissue against ELTD1 for each group and quantified the IHC positivity within the samples. As seen in **Supplementary Figure 1**, both mmAb and fragment anti-ELTD1 treatments were successful in decreasing the overall positivity of ELTD1 within the tumor regions.

Once established that anti-ELTD1 treatments directly targeted human tumor cells, we then sought to assess cell proliferation within the tumor region. c-Met is a cell receptor for the Hepatocyte Growth Factor (HGF) and is crucial in regulating various cellular functions. c-Met has previously been shown to be associated with poorer overall survival because it promotes tumor cell growth, proliferation, and

angiogenesis.<sup>26</sup> Ki-67 is a nonhistone nuclear protein that is expressed during all active cell cycle phases.<sup>27</sup> Ki-67 is strongly associated with cellular proliferation and tumor growth and has been clinically correlated with metastasis.<sup>27</sup> Both of these biomarkers were used to investigate cellular proliferation. In normal, healthy tissue there is low expression of c-Met, however IHC analysis showed that c-Met expression (70% positivity) was elevated in tumor regions (**Figure 3D**). Both anti-ELTD1 treatments, mmAb and fragment, were successful in significantly decreasing c-Met expression within tumor regions ( $P = .0071$  and  $P = .0063$ , respectively) as shown through representative images in **Figure 3A–C**. There was no significant difference between the two treatments. When examining the Ki-67 expression we saw a similar trend. As shown in **Figure 3E**, there was 52% positivity Ki-67 expression in the tumor region of the untreated animals. The Ki-67 expression was significantly decreased in tumor regions of mmAb ( $P = .0002$ ) and fragment ( $P < .0001$ ) anti-ELTD1 treated animals (**Figure 3E**).

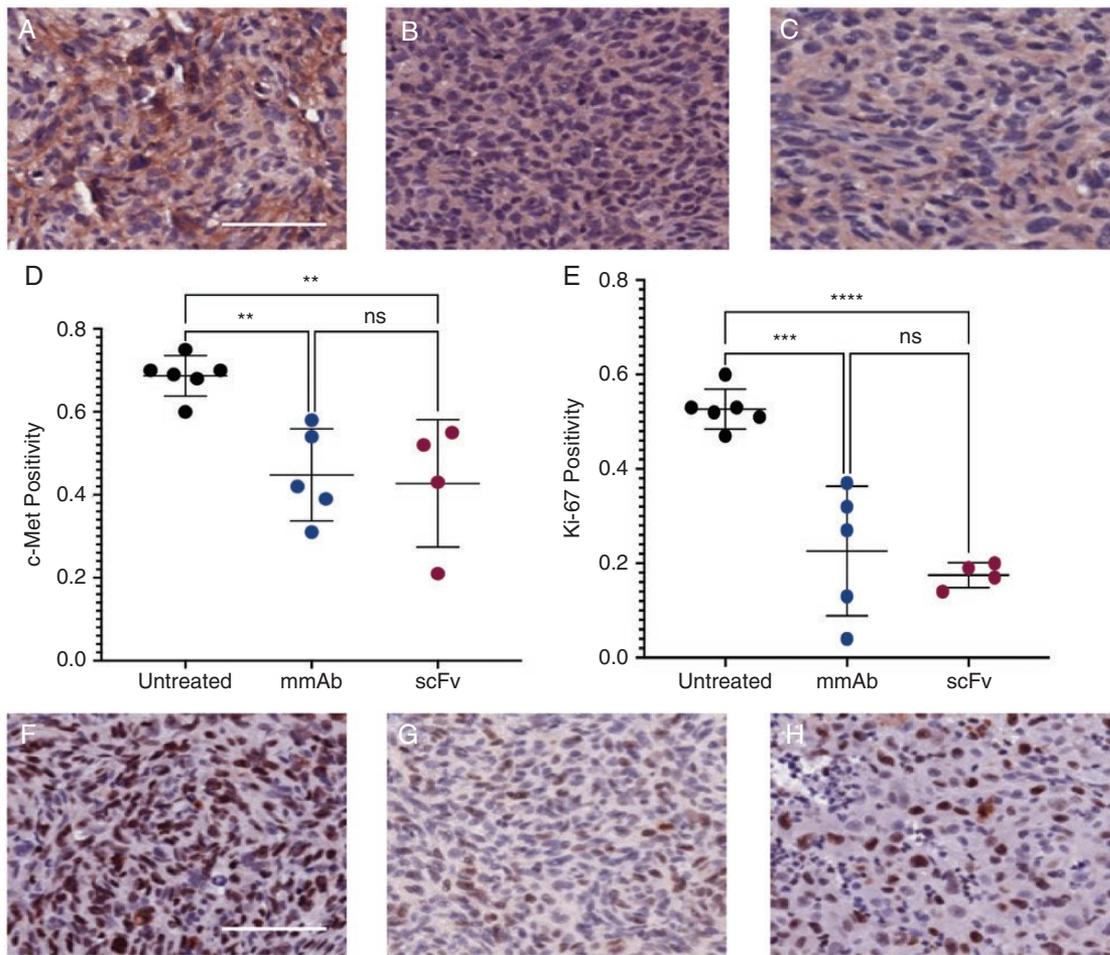
Previous RNA-sequencing analysis showed that multiple genes involved with migration were directly affected by our anti-ELTD1 treatment. Therefore, we assessed whether our anti-ELTD1 treatments had an effect on migration. Initially, we established if anti-ELTD1 treatment had an effect on tumor cell velocity, by using a cellular migration chamber assay, where G55 cells were seeded in the chamber on one end of each PDMS microchannel device, and were either left untreated, or were treated with either mmAb or fragment anti-ELTD1. Cells were then allowed to migrate through the microchannels, where the distance traveled by the G55 cells 16- and 44-h posttreatment were measured (**Figure 4A, B**). At 16 h posttreatment, both mmAb and scFv anti-ELTD1 therapies were successful in decreasing the velocities of the migrating cells, when compared to the untreated controls ( $P < .0001$  for both) (**Figure 4C**). There was no significant difference between the two



**Figure 2.** Anti-ELTD1 treatments, both mmAb and fragment are successful in decreasing human mitochondrial positivity. (A) Untreated tumor tissue showing human mitochondrial antibody staining. The black box corresponds to (B) and shows that there is no mitochondrial staining in the normal brain tissue (20×). (C) The corresponding 20× image from the red box showing the distinct tumor boundaries. Representative IHC images (20×) for human mitochondrial antibody from untreated ( $n = 6$ ) (D), mmAb ( $n = 5$ ) (E), and scFv ( $n = 4$ ) (F) anti-ELTD1 treated animals. (G) Quantification of human mitochondrial antibody positivity staining from the tumor regions of each corresponding group (UT vs mmAb \*\*\* $P = .0001$ ; UT vs scFv \*\*\* $P = .0004$ ; ANOVA  $F$  value = 23.86, significant) (bars = 100  $\mu$ m).

therapies. The anti-ELTD1 treatments were still effective in significantly decreasing the velocities of the migrating cells 48 h posttreatment ( $P < .0001$  for both) (Figure 4D). Additionally, at 48 h posttreatment we start to see that the scFv anti-ELTD1 treatment was more effective in decreasing the migrating cell velocity, compared to the

mmAb ( $P = 0.004$ ). Additionally, the *in-vitro* cell migration chamber assays were examined using both TMZ-sensitive and resistant GBM cell lines. As shown in Supplementary Figure 2, both anti-ELTD1 antibodies are effective against T98G (TMZ-resistant) and LN229 (TMZ-sensitive) at 16 h posttreatment administration. Both mmAb and fragment



**Figure 3.** Cellular proliferation is decreased with monovalent monoclonal and single chain variable fragment anti-ELTD1 therapies. Representative C-met IHC images (20×) of untreated (A), mmAb (B), and scFv (C) anti-ELTD1 treated animals (bars = 100 μm). Quantitative C-met (D) and Ki-67 (E) positivity staining in the tumor region of each group (C-met: UT vs mmAb  $**P = .0071$ ; UT vs scFv  $**P = .0063$ ) (Ki-67: UT vs mmAb  $***P = .0002$ ; UT vs scFv  $****P < .0001$ ; ANOVA  $F$  value = 10.1, significant) Representative Ki-67 IHC images (20×) of untreated (F), mmAb (G), and scFv (H) anti-ELTD1 treated animals (untreated ( $n = 6$ ), mmAb ( $n = 5$ ) (E), and scFv ( $n = 4$ )).

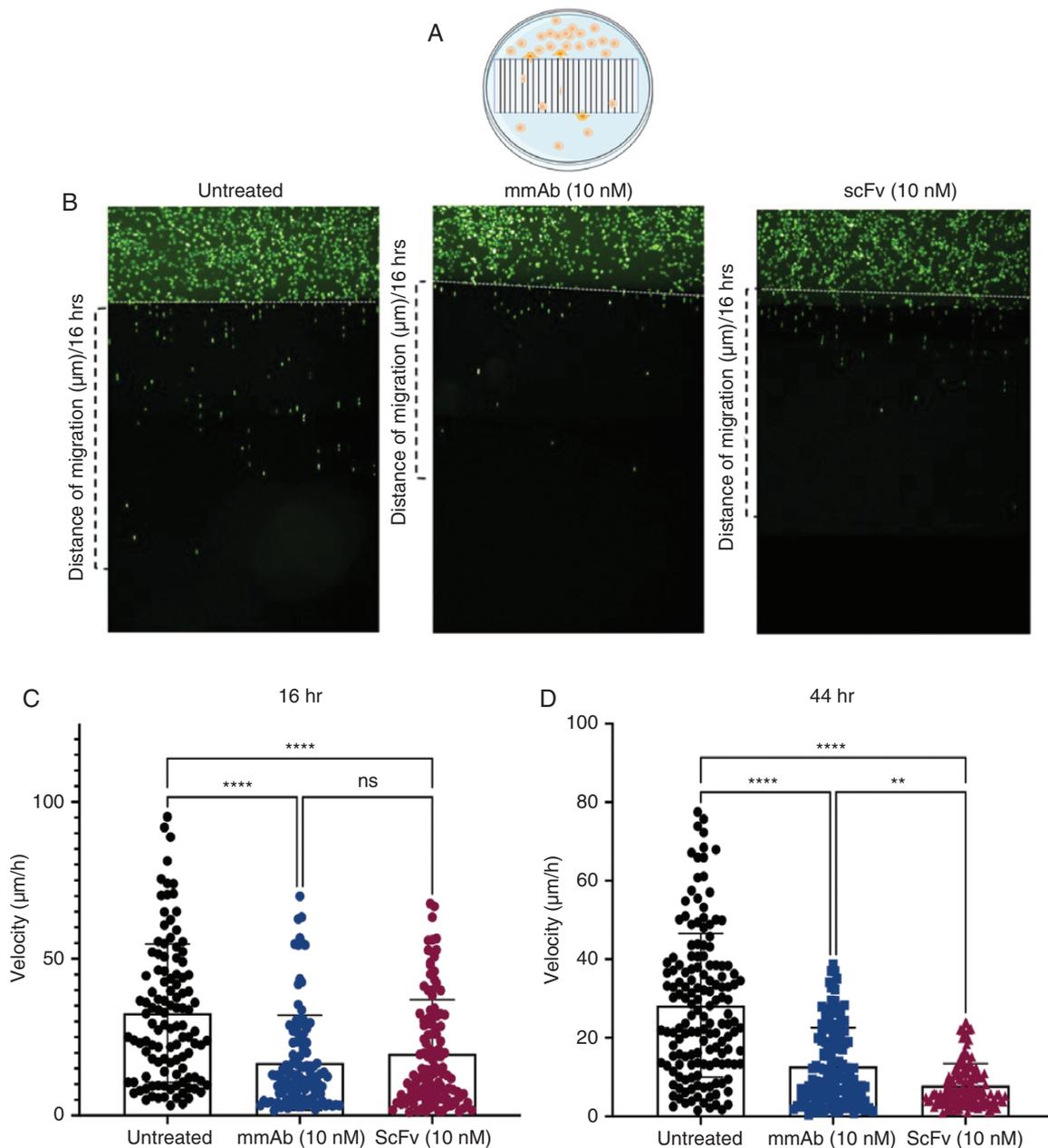
anti-ELTD1 treatments were effective against LN229 at 44 h, however the treatments did not seem to have an effect for the T98G cell line at 44 hours.

Our *in vitro* cell migration chamber assays demonstrated that our anti-ELTD1 therapies were successful in decreasing the velocities of the migrating cells. The next step was to examine how the anti-ELTD1 treatments affected tumor cells *in vivo*, by staining the tissues against CD44v6. CD44 has been a reliable marker for migration for various cancers, due to its key role in regulating metastasis, promoting migration, and enhancing invasion.<sup>28</sup> CD44v6 is also a marker of tumor metastasis. Overexpression of CD44v6 is associated with poorer prognosis in various cancers and is responsible for inducing cell adhesion, migration, and proliferation.<sup>29</sup>

Unfortunately, the IHC analysis demonstrated that there was no significant difference between any of our three groups; UT, mmAb, and fragment treated animals

when staining against CD44v6 (Figure 5A–D). Instead, we saw that the untreated tumor bearing animals had low CD44v6 positivity expression within the tumor regions. It is known that GBMs have increased CD44 expression.<sup>30,31</sup> This discrepancy may be because CD44 is differentially expressed across the GBM subtypes. For example, Pietras *et al.* identified higher CD44 expression in mesenchymal tumors compared to both classical and neuronal GBM subtype. Additionally, CD44 variant (v1–10) expression has also been suggested to differ based on GBM tumor type. For example, CD44v5 was seen to be more highly expressed in GBM.<sup>31</sup> Therefore, further examination of different CD44 variant expressions in human G55 cells is needed to better understand the anti-ELTD1 treatment effect on CD44.

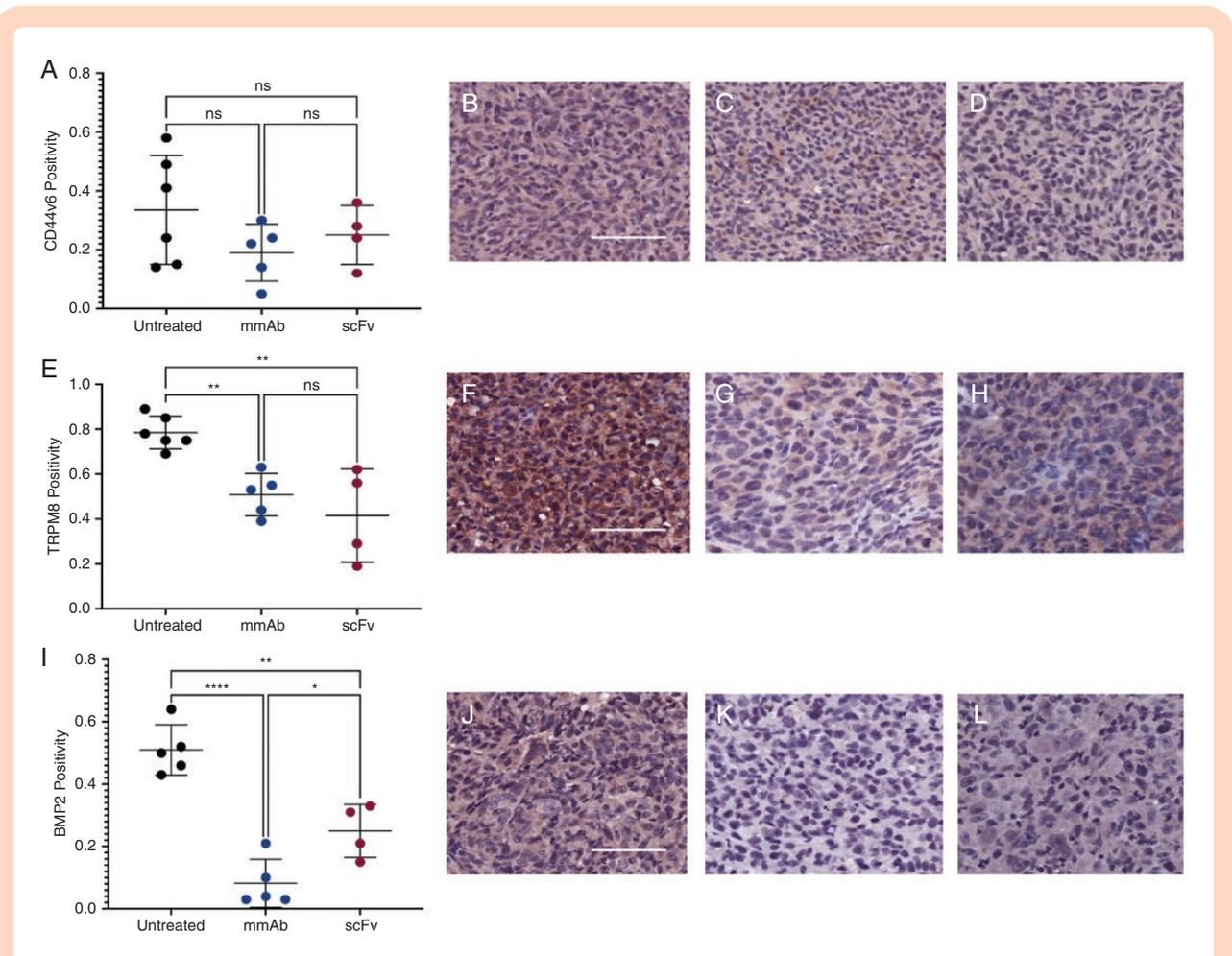
CD44v6 is not the only migration marker that is key in regulating cell migration and invasion. In a previous RNA sequencing study,<sup>13</sup> we found two downregulated genes,



**Figure 4.** Anti-ELTD1 treatments decreased *in vitro* cellular migration velocity. (A) Schematic of experimental design. The velocity of the migrating cells was calculated at 16- and 48-h posttreatment. (B) Representative images showing G55 cells (80–90) labelled with H2B-GFP are migrating in microchannels 16 h posttreatment of all groups. The dotted lines in the figure separate the cells in 2D with the cells migrating in 3D microchannels ( $5 \mu\text{m} \times 5 \mu\text{m}$ ). The images are obtained using Operetta high content imaging system (Perkin Elmer) at  $10\times$  and the images from multiple fields of the same migrating path were stitched together to show the entire distance of any cells that migrated at the time of measurement. (C, D) Plots showing migration velocity ( $\mu\text{m}/\text{h}$ ) of G55 cells quantified at 16- and 48-h posttreatment, respectively.

TRPM8 and BMP2, which are directly associated with glioma cell proliferation, migration, and invasion.<sup>32,33</sup> The transient receptor potential melastatin family member 8 (TRPM8) is upregulated in GBMs compared to normal brain tissue, and the elevated signaling is associated with

radio-resistance.<sup>32</sup> Additionally, TRPM8 promotes brain invasion and migration, and is required for survival of GBM cells.<sup>32,34</sup> Unlike CD44v6 staining, we saw high TRPM8 positivity signal within the tumor regions of untreated tumor bearing mice (Figure 5E). The positivity staining of TRPM8



**Figure 5.** Anti-ELTD1 treatments, both mmAb and scFv, decrease TRPM8 and BMP2 positivity staining, however they do not have an effect on CD44v6 (untreated ( $n = 6$ ), mmAb ( $n = 5$ ), and scFv ( $n = 4$ )). (A) Quantification of CD44v6 positivity staining within the tumor region. IHC representative images at 20 $\times$  of untreated (B), mmAb (C), and scFv(D) anti-ELTD1 treated animals. Quantification of TRPM8 (untreated ( $n = 6$ ), mmAb ( $n = 5$ ), and scFv ( $n = 4$ )) (E) and BMP2 (untreated ( $n = 5$ ), mmAb ( $n = 5$ ), and scFv ( $n = 4$ )) (I) positivity staining within the tumor region (TRPM8, UT vs mmAb  $**P = .0091$ , UT vs scFv  $**P = .0018$ ; ANOVA  $F$  value = 12.02, significant) (BMP2, UT vs mmAb  $****p < .0001$ , UT vs scFv  $**P = .0015$ , mmAb vs scFv  $*P = .0249$ ; ANOVA  $F$  value = 35.7, significant) TRPM8 IHC representative images at 20 $\times$  of untreated (F), mmAb (G), and scFv (H) anti-ELTD1 treated animals (bars = 100  $\mu$ m). BMP2 IHC representative images at 20 $\times$  of untreated (I), mmAb anti-ELTD1 (J), and scFv anti-ELTD1 (K) treated animals.

was significantly decreased with mmAb and fragment anti-ELTD1 treatments, as can be seen in representative images (Figure 5F–H).

The bone morphogenetic protein 2 (BMP2) belongs to the TGF- $\beta$  superfamily. BMP2 is commonly upregulated in high grade gliomas, and this high expression is correlated with decreased survival in patients.<sup>35</sup> Glioma initiating cells are sensitive to this superfamily, and in particular BMP2 is known to promote differentiation and growth in GBM cells.<sup>36,37</sup> Tissue samples of each treatment group were stained against BMP2 and Figure 5J–L shows representative images of each group. There was a significant decrease of BMP2 positivity staining within the tumor regions of both anti-ELTD1 treatments, compared to untreated controls (Figure 5I). This is also the only variable in which the mmAb anti-ELTD1 had a more profound effect than scFv anti-ELTD1 treatments. To further examine the tumor migratory phenotype, the invasion markers, both TRPM8 and

BMP2, were quantified beyond the xenograft tumor borders. IHC positivity quantification demonstrated that the pro-migratory phenotype was significantly decreased with both of the anti-ELTD1 treatments compared to untreated (Supplementary Figure 3).

Lastly, GBMs are widely known to evade apoptotic mechanisms. Caspase-3 is crucial in coordinating the destruction of cellular structures and degradation of cytoskeletal proteins. Decreased caspase-3 activation has also been shown to lead to increased cellular proliferation and decreased apoptosis which further promotes tumor growth.<sup>38</sup> In this study, we stained our tumor tissue against cleaved-caspase 3 which recognizes the activated form of caspase 3. The untreated animal samples had low levels of apoptosis, which is similar to previously published studies.<sup>39</sup> Both anti-ELTD1 treatments (mmAb and fragment) were successful in increasing the cleaved caspase 3 expression within tumor regions, compared to untreated controls

( $P = .0054$  and  $P = .035$ , respectively) (Figure 6D). This suggests, that there is increased apoptosis within tumor regions from the anti-ELTD1 treatments, which therefore stunts further tumor growth.

## Discussion

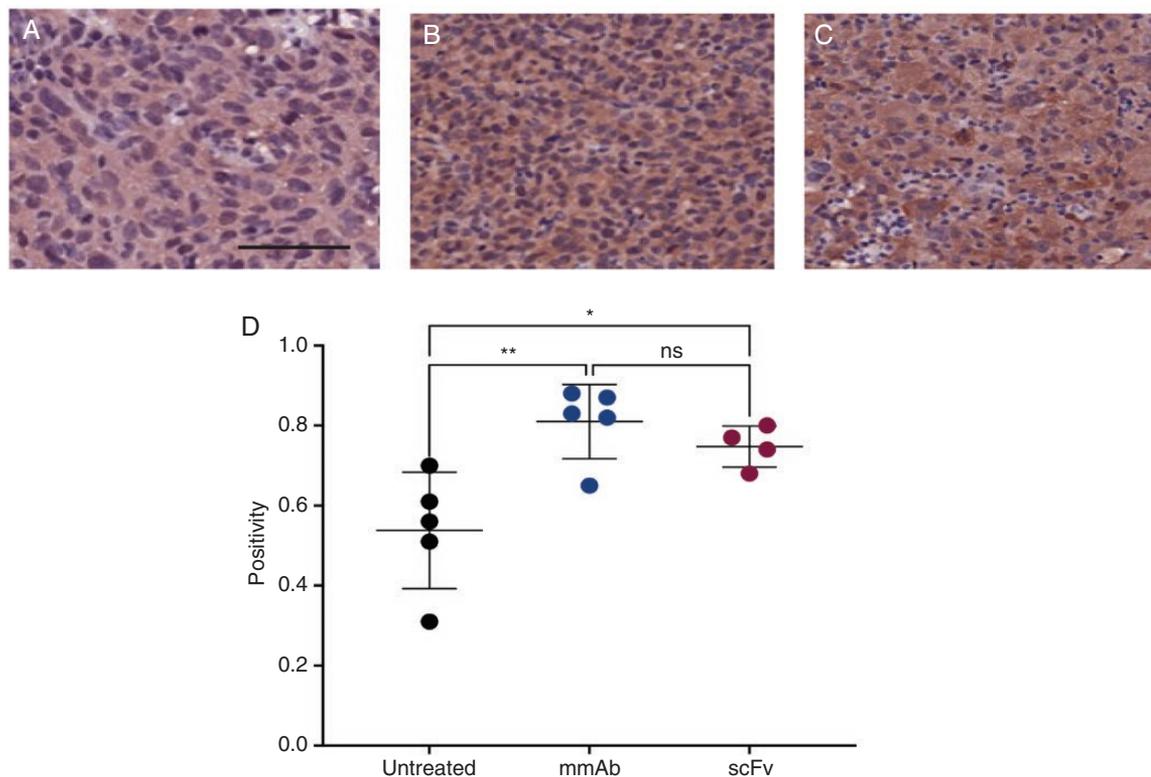
Glioblastoma is the most common malignant tumor of the central nervous system, characterized by a high recurrence rate, and despite current treatment strategies, there is a high mortality rate.<sup>40</sup> One of the main issues with treatments targeting GBMs is that they most commonly only target one pathway.

GBMs are extremely heterogeneous tumors that can adapt to new and hostile environments posing an important challenge against new and existing drug therapies.<sup>7</sup> Two commonly used chemotherapeutic agents in the standard treatment plan are TMZ and bevacizumab. Repeated use of TMZ has led to chemoresistance in 50% of the patients,<sup>41</sup> which may reflect innate resistance to TMZ in MGMT unmethylated tumors. Additionally, bevacizumab, an anti-angiogenic drug targeting VEGF-A, has not shown any promising results in the clinic,

resulting in a negative impact on the quality of life of patients, with tumors showing signs of resistance.

ELTD1 is known to be a biomarker of angiogenesis and in normal vasculature has been shown to be regulated by the two main angiogenic pathways, VEGF and Notch.<sup>12</sup> Additionally, in G55 tumors, anti-ELTD1 treatments were successful in decreasing both VEGFR2 and Notch1 levels within the tumor regions.<sup>13,14,25</sup> The decrease of pro-angiogenic factors, ELTD1 included, caused a complete normalization of the tumor associated vasculature within G55-tumor bearing animals treated with anti-ELTD1 therapies, both mmAb and single chain variable fragment.<sup>13,14,25</sup> RNA-sequencing results also indicated that anti-ELTD1 treatments affect genes directly associated with Notch signaling pathway.<sup>13</sup> This data demonstrated that ELTD1 has a much more complex relationship with the main angiogenic factors in the tumor environment than previously thought, and further validated anti-ELTD1 as an anti-angiogenic treatment.

In addition to being an anti-angiogenic treatment, the RNA sequencing data shed light on new areas that may be directly targeted with anti-ELTD1 therapy. The genes that were affected with our anti-ELTD1 treatment could be categorized into four main tumorigenic areas,



**Figure 6.** Anti-ELTD1 treatments increase apoptosis in tumor region. Representative IHC (20 $\times$ ) images of cleaved caspase 3 staining in each of the treatment groups, untreated (A), mmAb (B), and fragment (C) (bars = 100  $\mu$ m). (D) Quantitative cleaved caspase 3 positivity staining in each of the treatment groups (UT vs mmAb  $**P = .0054$ , UT vs scFv  $*P = .0354$ ; ANOVA  $F$  value = 8.668, significant) (untreated ( $n = 5$ ), mmAb ( $n = 5$ )) (E), and scFv ( $n = 4$ )).

1) angiogenesis, 2) cellular proliferation, 3) cellular migration/invasion, 4) apoptosis.<sup>13</sup> Based on the RNA sequencing genes, the anti-ELTD1 treatment worked to downregulate key glioma genes such as Sodium Voltage-Gated Channel Alpha Subunit 5 (SCN5A), TRPM8, and BMP2 that impact cell proliferation, migration, and invasion.<sup>34,42,43</sup> Anti-ELTD1 treatment also down regulated alkaline phosphatase (ALPL), a stem cell marker and L1CAM, a gene that regulates neural cell growth and migration.<sup>44,45</sup> Therefore, we further investigated the three remaining aspects of tumorigenesis and examined whether anti-ELTD1 treatments had an effect on them.

Through this study, we demonstrated that both mmAb and scFv anti-ELTD1 treatments directly decreased human tumor cells in the G55 human xenograft mouse model. It is important to note that although the G55 cell line was shown to display classic GBM behavior in respect to aggressive proliferation, angiogenesis, and migration, it was originally derived from an anaplastic astrocytoma. However, as indicated in the revised version of the 2000 WHO classification, microvascular proliferation (without necrosis) was determined to be sufficient for the diagnosis of glioblastoma, and the tumor was later re-classified as a glioblastoma.<sup>46</sup>

Anti-ELTD1 treatments also increased apoptotic activity within tumor regions. Together, this demonstrates that anti-ELTD1 treatments cause direct targeting and killing of GBM tumor cells. Additionally, our cell proliferation studies, supporting our previous RNA-sequencing results, demonstrate a significant decrease in cell proliferation positivity, as seen through two established migration markers. Preclinical and clinical bevacizumab data has shown that GBMs resist anti-angiogenic therapies by shifting into pro-migratory behavior. However, our *in vitro* and *in vivo* data demonstrate that this is not the case with our anti-ELTD1 treatments. Instead, we see a significant decrease in migratory behavior with our treatments when compared to the untreated controls.

### Limitations and Future Studies

It should be noted that from the T98G *in vitro* studies, that ELTD1 Ab treatment was effective at 16 h, but not at 44 h posttreatment, and that future studies should consider by-pass mechanisms. Previously we have demonstrated that there was no statistical significance between an isotype IgG-treated group and untreated controls,<sup>11</sup> however a more comprehensive study should include an IgG isotype control. Also of importance, cellularity between the treated tumors and untreated tumors appears to be similar, which may be due to the timing of when samples were processed for *ex vivo* studies, i.e. all obtained at maximum tumor volumes. A future study could involve obtaining tumor tissues at the same time as when the untreated mice are euthanized. Future *in vivo* studies could also be conducted on other established GBM cell lines such as U251.

### Conclusion

In preliminary assessments we saw that anti-ELTD1 treatments had possible effects on different

characteristics associated with tumorigenesis. In this paper, we validated those claims by demonstrating that anti-ELTD1 treatments decreased cellular migration, proliferation, as well as directly increased apoptotic signaling causing a decrease of human tumor cells. Overall, our results have expanded the scientific knowledge of ELTD1 by showing that it is not just an angiogenic biomarker, but that it also directly targets various aspects of tumorigenesis. This also increases the therapeutic potential of our anti-ELTD1 treatments over singular pathway drugs.

### Supplementary Material

Supplementary material is available at *Neuro-Oncology Advances* online.

### Keywords

apoptosis | glioblastoma (GBM) | ELTD1 | migration | proliferation

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