SUPPLEMENTAL FIGURES AND LEGENDS:

Population	# of Events	% Parent	% Total
All Events	30,000		100%
Not debris	17,996	60%	60%
Single cells	13,016	72.3%	43.3%
Live cells	12,464	95.8%	41.5%
EGFRvIII+/CD133+	847	6.8%	2.8%
EGFRvIII+/CD133-	6533	52.4%	21.7%
EGFRvIII-/CD133+	28	0.2%	0.08%
EGFRvIII-/CD133-	5056	40.6%	16.8%

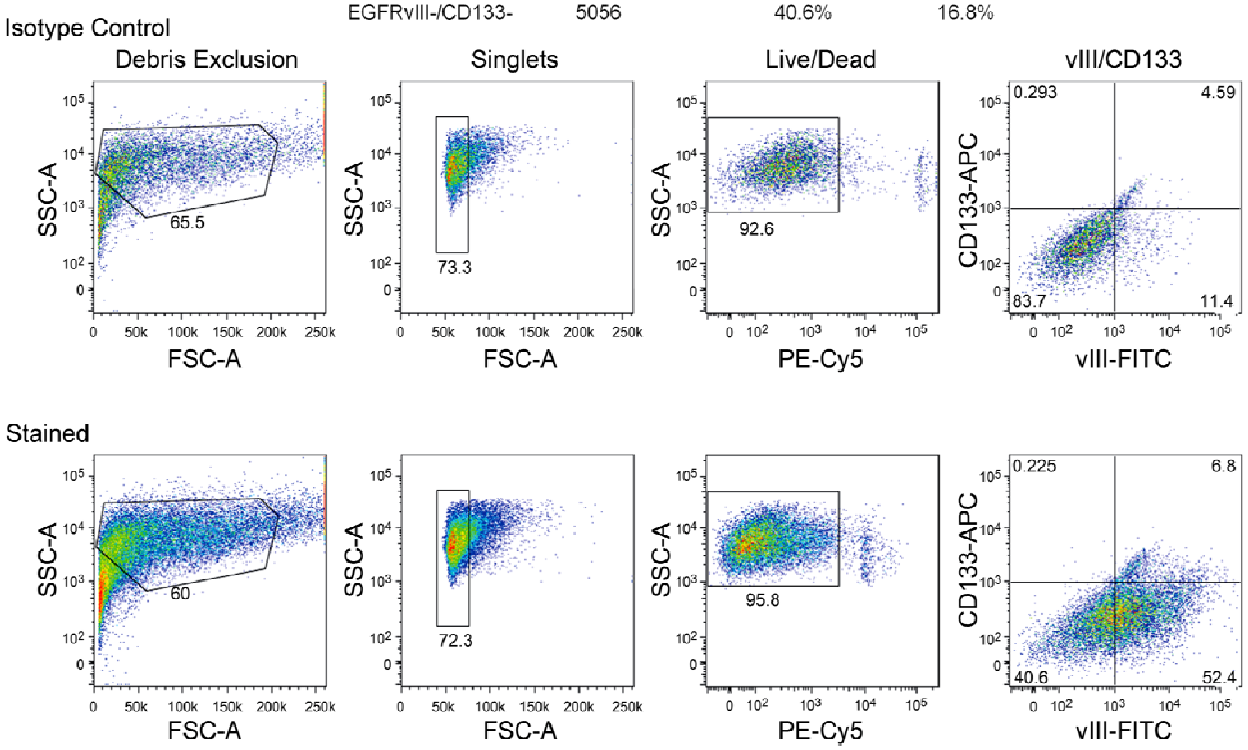

Figure S1, related to Figure 1. Gating Tree for Fluorescence Activated Cell Sorting (FACS) Analysis. Representative GBM sample gating tree to illustrate the process used to select cells for final analysis of EGFRvIII and CD133 expression. The gating tree for the corresponding isotype control is also shown as is a control for non-specific isotype background. Gating was done for debris exclusion (far left column), single cell isolation (left middle column), and removal of dead cells (right middle column) prior to sorting/analysis for EGFRvIII and CD133 expression (far right column). Each successive plot depicts only the cells carried forward from the previous step (% parent).

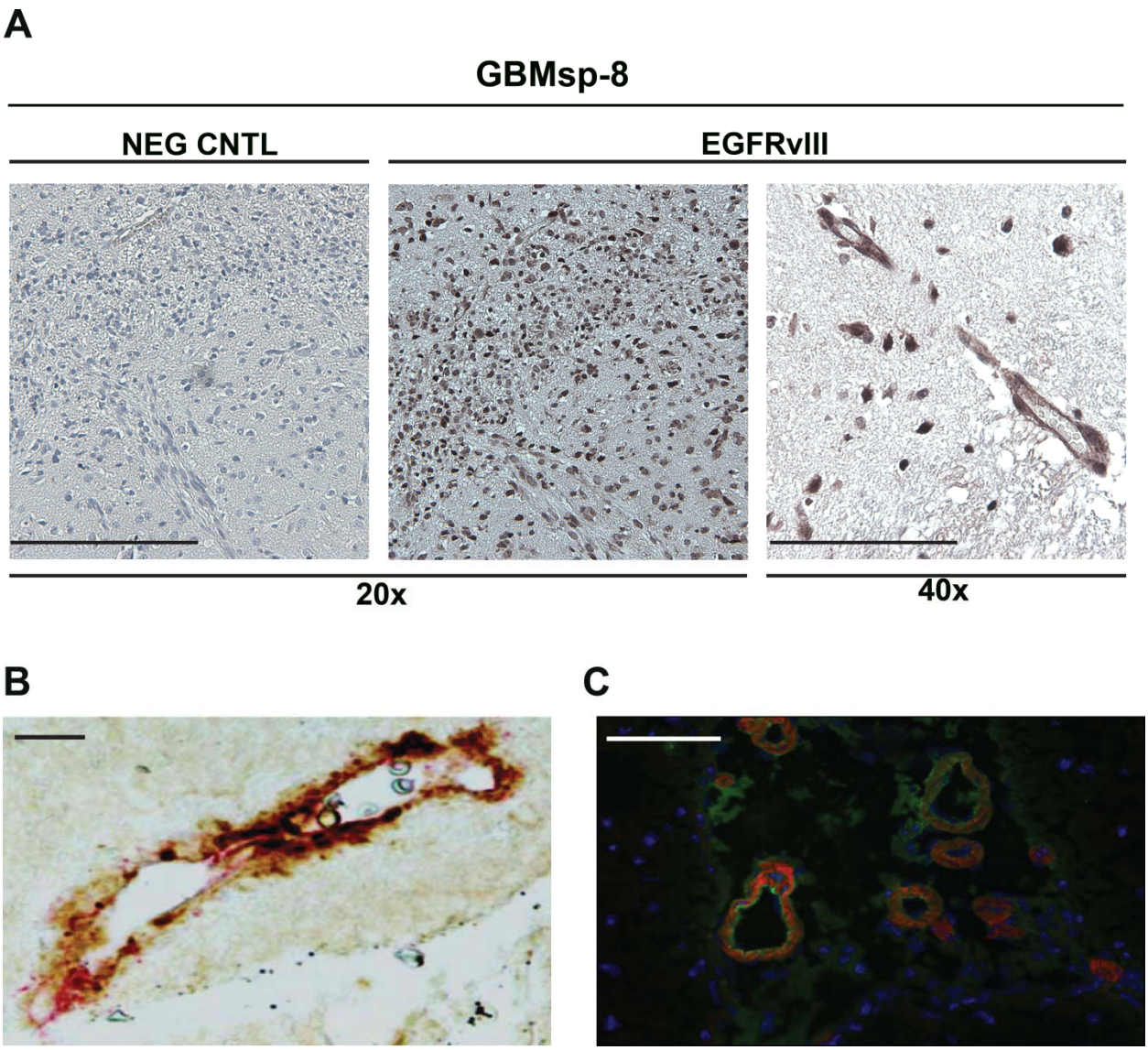

Figure S2, related to Figure 1. Vessel Staining of EGFRvIII in GBM Xenografts. EGFRvIII and CD31 expression were assayed in Primary human GBM samples and mouse xenografts. A) Cultured human GBMsp cells were intracranially injected into NOD-SCID mice and Xenografts were assayed for EGFRvIII expression by IHC. Shown is a representative experiment from GBMsp-8. Comparison of serial 20x images of secondary only (NEG CNTL, 20x) and staining with an anti-EGFRvIII polyclonal antibody (EGFRvIII, 20x) demonstrate EGFRvIII staining in a tumor with very high EGFRvIII expression. A 40x image (EGFRvIII, 40x) demonstrates the occasional vessel staining of the EGFRvIII antibody. (Scale bars = 250μM and 125μM). B) EGFRvIII (brown) and CD31 (pink) co-staining in primary human GBM samples verifies EGFRvIII staining around vessels. C) EGFRvIII (red) and CD31 (green) immunofluorescent co-staining in mouse xenografts.

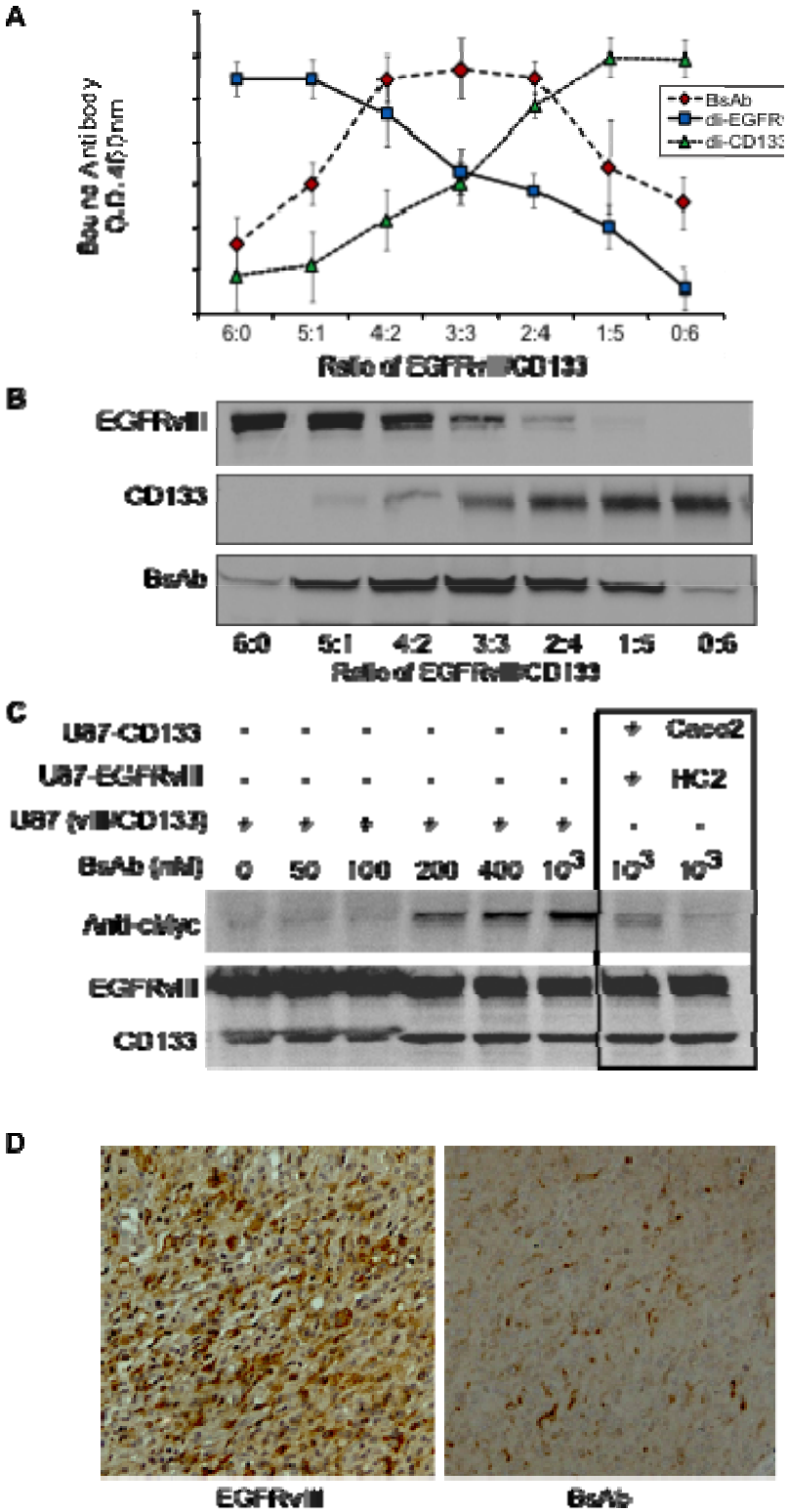

Figure S3, related to Figure 5. Characterization and Affinity Analysis of the Recombinant Antibodies. A) ELISA assay of recombinant antibody binding (◆) BsAb, (■) di-EGFRvIII, (▲) di-CD133 to membrane fractions of NIH3T3 cells co-transfected with varying concentrations of CD133 and EGFRvIII cDNA. Experiments were carried out in triplicate and error bars indicate ± SEM. B) Live cell pull down assay using BsAb to precipitate cells co-transfected with varying concentrations of CD133 and EGFRvIII cDNA. Whole cell lysate was analyzed for EGFRvIII , CD133, and BsAb using anti-cMyc (bottom panel). C) Equal numbers of live U87MG cells expressing either EGFRvIII or CD133 were mixed and incubated with the BsAb to test for crosslinking of cells. For controls, CD133 expressing CACO2 cells and EGFRvIII expressing HC2 cells were used. D), IHC analysis comparing ant-EGFRvIII staining to BsAb staining in serial sections of a primary human GBM sample.

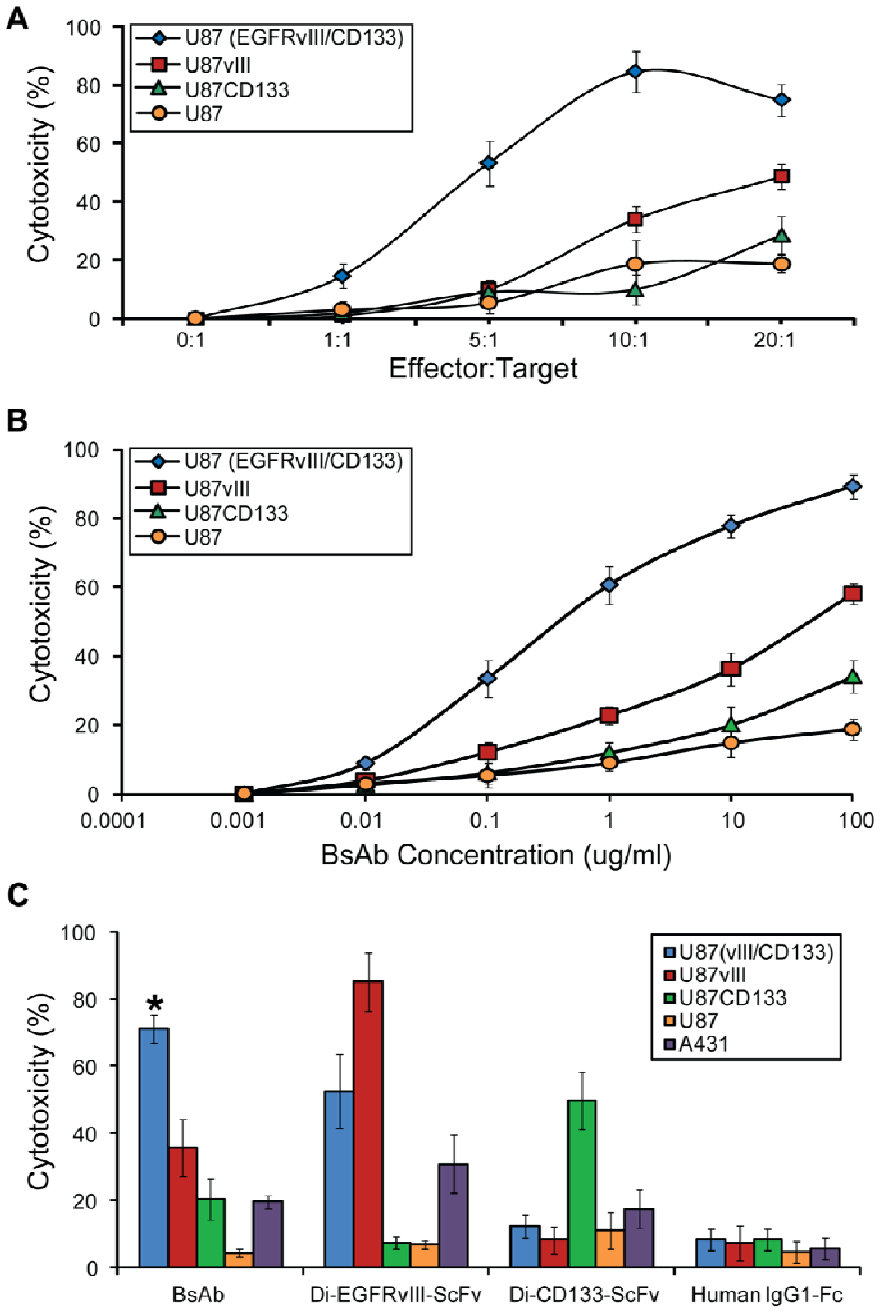

Figure S4, related to Figure 6. Antibody Dependent Cellular Cytotoxicity Induced by BsAb. A coupled luminescence ADCC assay using purified human NK cells as effectors was used to determine BsAb induced cytotoxicity. A) Effector: target ratios 0:1 to 20:1 were tested at a BsAb concentration of 1ug/ml on cells expressing neither receptor (●), either EGFRvIII (■), or CD133 (▲) or co-expressing both (◆). B) Using a constant E:T ratio of 10:1, BsAb was titrated from 0.001-100µg over cells expressing neither receptor (●), either EGFRvIII (■), or CD133 (▲) or co-expressing both (◆). C) Efficiency of the various recombinant antibodies to induce targeted cytotoxicity on cell lines expressing different surface epitopes. An E:T ratio of 10:1 and antibody concentration of 1ug/ml was used. *= statistical significance ($p<0.001$). All experiments were carried out in triplicate and error bars indicate ± SEM.

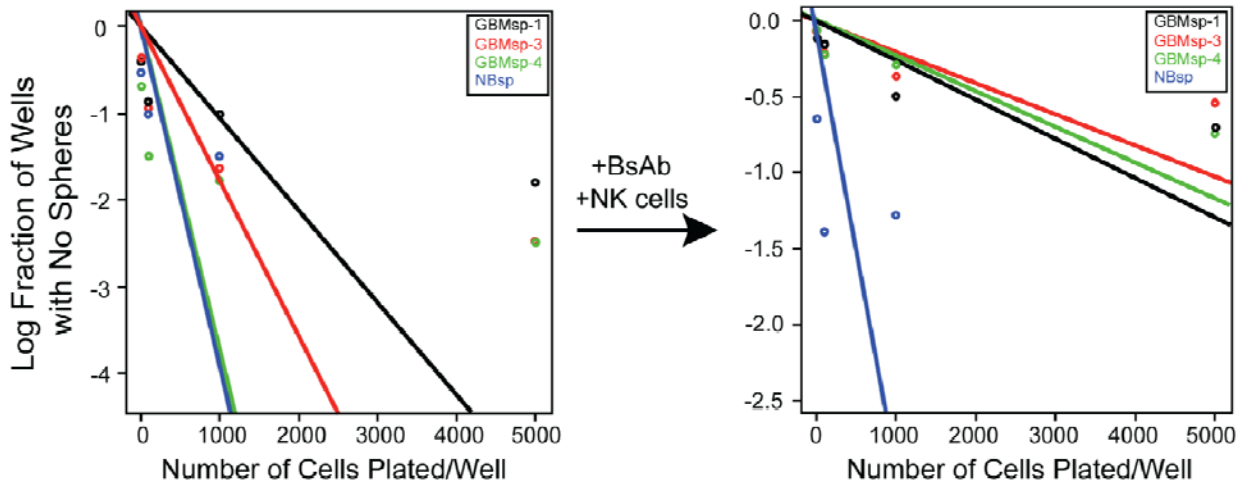

Figure S5, related to Figure 6. Limiting Dilution Analysis of Unsorted Tumor Spheres Before and After BsAb Induced ADCC. GBMsp-1 (black), GBMsp-3 (red), GBMsp-4 (green), and a normal epileptic sphere line NBsp (blue) were dissociated and either not treated (left graph) or treated with BsAb and human NK cells (right graph) for 4 hours. NK cells were depleted from the mix using lineage depletion antibody cocktail (Miltenyi) on a MACS column. Live cells left behind were subject to limiting dilution analysis to identify any change in frequency of sphere renewing cells.