Supporting Information

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SI Materials and Methods

Mice and Tumor Cell Lines. NOD scid gamma (NSG) mice were purchased from Jackson Laboratory and bred under pathogen-free conditions at Duke University. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee. The human glioma cell lines U87MG and D54, as well as their respective sublines, U87MG.ΔEGFR and D54-EGFRvIII, have been described previously (1, 2).

Expression and Purification of bscEGFRvIIIxCD3 in *Escherichia coli*. Synthesis of bscEGFRvIIIxCD3 was performed using standard recombinant technologies. The scFv directed against EGFRvIII was isolated from pMR1-1(scFv) (3), and the scFv specific for human CD3 was derived from the hybridoma cell line OKT3 as described previously (4). A hexahistidine tag was added to the carboxyl terminus to aid in the detection and purification of the final protein product. The resulting gene was cloned into the expression vector pRB199 and transformed into E. coli strain BL21 (λDE3). Bacterial cultures were inoculated into Terrific Broth containing 80 μg/mL chloramphenicol and grown at 37 °C to an A_{600} of 2.0. Expression of the recombinant bispecific antibody was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at 37 °C for 4 h. Cultures were centrifuged at 2000 × *g* for 15 min, and bacterial pellets were resuspended in TE50/20 buffer [50 mM Tris (pH 7.5) and 20 mM EDTA] for storage at −70 °C. Inclusion bodies were prepared by extensively washing bacterial material with TE50/20. Inclusion bodies were solubilized in 6 M guanidine-HCl, reduced with dithioerythritol, and then refolded by 100-fold dilution into renaturation buffer (0.1 M Tris, 0.5 M arginine-HCl, 0.9 mM oxidized glutathione, and 2 mM EDTA; pH 10.3) at 4 °C with rapid mixing, followed by a 72-h incubation at 4 °C. After renaturation, the solution was dialyzed 1:10 against 0.1 M Tris and 0.5 M NaCl three times and filtered (0.2 μm) to prepare for metal ion-affinity chromatography.

Refolded bscEGFRvIIIxCD3 was purified by fast protein liquid chromatography (BioLogic DuoFlow 10 System; Bio-Rad) using TALON metal-affinity resin (Clontech). In brief, a sample was loaded by constant controlled flow at 2 mL/min, and then the column was washed with 0.1 M Tris and 0.5 M NaCl until the 280-nm absorbance of the eluate returned to 0. Protein was eluted by stepwise imidazole gradient at a flow rate of 1 mL/min. The product was concentrated with 10,000 molecular weight cutoff columns (Sartorius Stedim Biotech), dialyzed against PBS, and sterile-filtered. Concentrations were determined by Bradford assay. The product was subjected to SDS/PAGE analysis (2 μg of total protein per lane) under reducing conditions for purity by silver stain.

Flow Cytometric Analysis. Lymphocytes were incubated with combinations of titrated antibodies to CD4 (RPA-T4), CD8 (RPA-T8), CD25 (M-A251), and CD69 (L78) and analyzed on a BD Biosciences FACSCalibur flow cytometer. In some experiments, bscEGFRvIIIxCD3 (10 μg/mL) was used in conjunction with Penta-His Alexa Fluor 488 conjugate (Qiagen) and analyzed for binding as above.

Surface Plasmon Resonance. Binding to antigen was measured by surface plasmon resonance using the GE Healthcare BIAcore 3000. Antigen was coupled to the surface of carboxylated dextran-coated CM5 research grade chips at pH 4.0 using the amide coupling reagents provided with the BIAcore system. Solutions of bscEGFRvIIIxCD3 (100 nM, 200 nM, and 300 nM) were then passed over the chip to measure binding. Regeneration was performed with 10 mM Glycine pH 2.0. Nonspecific binding was determined using a flow cell on a chip without antigen. Binding kinetics were analyzed by using BIAcore 3000 software.

Proliferation Assay. In experiments assessing proliferation, 1 μCi ^{3}H-thymidine was added to each well of a 96-well plate after 72 h incubation at 37 °C. Where indicated, recombinant EGFRvIII extracellular domain (ECD) was used as a source of cognate antigen, to eliminate the variability in ^{3}H-thymidine incorporation that otherwise would occur owing to proliferation of target glioma cells. Cells were cultured for an additional 24 h and collected by a cell harvester. Counts were performed using a Wallac 1450 Microbeta Trilux Liquid Scintillation/Luminescence Counter (Perkin-Elmer) and data were taken as means of triplicate wells.

T-cell Activation and Cytometric Bead Array. Freshly thawed PBMCs were placed in 96-well plates with bscEGFRvIIIxCD3 (10 μg/mL) and target cells at an E:T ratio of 20:1 in a total volume of 200 μL. Supernatant was removed after incubation for 48 h at 37 °C and analyzed by manufacturer's instructions. Cells were also harvested and analyzed for activation markers as above.

In Vitro Cytotoxicity. The ability for bscEGFRvIIIxCD3 to redirect cytotoxicity from previously unstimulated human peripheral blood mononuclear cells (PBMCs) was analyzed by a standard chromium release assay in 96-well round-bottom plates. In brief, target cells were labeled with ^{51}Cr and incubated with bscEGFRvIIIxCD3 at varying concentrations. PBMCs were then added to each well at various effector-to-target ratios (ranging from 1:1 to 20:1), after which the plates were centrifuged at 300 × *g* for 1 min and incubated at 37 °C for 18 h. The supernatant was then removed and measured by a gamma counter. Where noted, peptide blockade was performed by adding PEPvIII to wells at a 1:1 ratio by mass with bscEGFRvIIIxCD3. In certain experiments, target cells were labeled using a PKH26 red fluorescent cell linker kit (Sigma-Aldrich), and effector cells were labeled using intracellular carboxyfluorescein diacetate succinimidyl ester according to the manufacturer's instructions. Cells were then visualized across multiple time points using a Nikon Eclipse TE2000-E fluorescent microscope.

Tumor Implantation. U87MG and U87MG.ΔEGFR were grown in improved MEM zinc option media with 10% FBS (vol/vol). Tumor cells were collected in logarithmic growth phase, washed twice with PBS, and mixed with an equal volume of 10% methyl cellulose (vol/vol) before being loaded into a 250- μL syringe with an attached 25-gauge needle. The needle was positioned using a stereotactic frame at 2 mm to the right of the bregma and 4 mm below the surface of the skull at the coronal suture. The tumorigenic dose was 1×10^5 cells in a total volume of 5 μL. For some experiments, freshly thawed human PBMCs were resuspended with tumor cells before inoculation at a ratio of 1:1. Treatment with bscEGFRvIIIxCD3 was infused in a total volume of 250 μL by tail vein injection on the indicated days after tumor implantation. Where noted, peptide blockade was performed with the coinfusion of soluble PEPvIII via the tail vein at a 1:1 ratio with bscEGFRvIIIxCD3.

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