# Supplementary Information

**Preclinical Assessment of AMG 596, a BiTE® (Bispecific T-Cell Engager) Immune Therapy Targeting the Tumor-Specific Antigen EGFRvIII**

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**Supplementary Table S1. Key resources**

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| **Resource** | **Source** | **Identifier** |
| **Cell lines**  All cell lines were cultured for a period of 2 to 3 months before being replaced by newly thawed cells of the same origin. All used cell lines were tested Mycoplasma negative. Testing was performed with Lonza’s MycoAlertTM Mycoplasma Detection Kit; Cat.: LT07-318. | | |
| Chinese hamster ovary cells | ATCC | Cat# CRL-9096; RRID:CVCL\_1977 |
| U-251 MG, U-251 MG/EGFRvIII | obtained from Dr. Darell Bigner (Duke University)\* | |
| U-87 MG | ATCC | Cat# HTB-14, RRID:CVCL\_0022 |
| DK-MG | DSMZ | Cat# ACC-277, RRID:CVCL\_1173 |
| GOS-3 | DSMZ | Cat# ACC 408, RRID:CVCL\_2050 |
| HPB-ALL | DSMZ | Cat# ACC 483, RRID:CVCL\_1820 |
| LnPx4119 | obtained from Dr. Helmut Fickenscher (*Hygiene Institute, Virology, Erlangen-Nuernberg*, Germany)\*\* | |
| **Effector cells** | | |
| Human blood | human buffy coats purchased from the “*Institut für Klinische Transfusionsmedizin und Immungenetik Ulm*” (Ulm, Germany) | |
| Cynomolgus monkey blood | purchased from Charles River Laboratories (former WIL Research, Lyon, France) | |
| Human Pan T cells | AllCells, Alameda, CA, USA | Cat# S-ISO004-11327 |
| **Antibodies and Fluorescent Conjugates** | | |
| **On cell binding of AMG 596** | | |
| Mouse-anti-His-Biotin | Dianova | Cat# DIA‐900‐BIOT |
| APC Streptavidin | BD | Cat# 554067, RRID:AB\_10050396 |
| **T cell–dependent cellular cytotoxicity assays** | | |
| Mouse-anti-human CD4-APC-Cy7 | BD | Cat# 341115, RRID:AB\_2868770 |
| Mouse-anti-human CD8-APC-Cy7 | BD | Cat# 557834, RRID:AB\_396892 |
| Mouse-anti-human-CD25-APC | Invitrogen | Cat# MHCD2505, RRID:AB\_10372046 |
| CBA Human Th1/Th2 Cytokine Kit II | BD | Cat# 551809, RRID:AB\_2868940 |
| CBA Non-Human Primate  Th1/Th2 Cytokine Kit | BD | Cat# 557800, RRID:AB\_2869098 |
| **Microvesicle assays** | | |
| Anti-EGFRvIII mAb | Internally generated by Amgen | |
| Anti-Actin Mouse mAb | Cell Signaling Technology | Cat# 3700, RRID:AB\_2242334 |
| IRDye® 800CW Goat anti-Human IgG Secondary Antibody | LI-CORE Bioscience | Cat# 926-32232, RRID:AB\_10806644 |
| Mouse-anti-His-PE antibody | MiltenyiBiotec | Cat# 130-092-691, RRID:AB\_1103227 |
| Mouse-anti-human-CD3-Alexa Fluor 488 | BD | Cat# 557705, RRID:AB\_396814 |
| Mouse-anti-human-CD4-BUV395 | BD | Cat# 564107, RRID:AB\_2738596 |
| Mouse-anti-human CD8a-Brilliant Violet 785 | BioLegend | Cat# 301046, RRID:AB\_2563264 |
| Mouse-anti-human-CD25 PE | BD | Cat# 557138, RRID:AB\_396584 |
| Fixable Viability Dye eFluor 780 | eBioscience | Cat# 65-0865 |
| **In vivo and ex vivo studies** | | |
| Anti-Mouse Asialo GM1, Ig Fraction (Polyclonal)(rabbit Ig) | BIOZOL | Cat# CL8955, RRID:AB\_10090118 |
| Anti-EGFR (vIII) Mouse Monoclonal Antibody | NewEast Bioscience | Cat# 26176, RRID:AB\_2629303 |
| Mouse-anti-human CD3 | Sigma Aldrich | Cat# C7048, RRID:AB\_476863 |
| MOUSE IgG1 NEGATIVE CONTROL | Bio-Rad | Cat# MCA928, RRID:AB\_322259 |
| EnVision+ System- HRP Labelled Polymer | Dako | Cat# K4001, RRID:AB\_2827819 |
| **Software** | | |
| FACSDiva | BD | RRID:SCR\_001456 |
| GraphPad Prism | BD | RRID:SCR\_002798 |
| FlowJo | BD | RRID:SCR\_008520 |

\*Hamblett KJ, Kozlosky CJ, Siu S, et al. AMG 595, an anti-EGFRvIII antibody-drug conjugate, induces potent antitumor activity against EGFRvIII-expressing glioblastoma. Mol Cancer Ther 2015;14:1614-24.

\*\*Lutterbuese R, Raum T, Kischel R, et al. T cell-engaging BiTE antibodies specific for EGFR potently eliminate KRAS- and BRAF-mutated colorectal cancer cells. Proc Natl Acad Sci U S A 2010;107:12605-10.

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| **Cell Line** | **PBMC Donor**  (Number, n) | **Mean EC50 ± SEM(pM)** |
| U-251 MG/EGFRvIII | Human  (n = 4) | 2.5 ± 0.3 |
| Cynomolgus Monkey  (n = 5) | 6.5 ± 1.7 |
| U-87 MG/EGFRvIII | Human  (n = 4) | 2.8 ± 0.2 |
| Cynomolgus Monkey  (n = 4) | 16 ± 4 |
| DK-MG | Human  (n = 4) | 14.4 ± 1.6 |
| Cynomolgus Monkey  (n = 5) | 38.8 ± 9.7 |
| GOS-3/EGFRvIII | Human  (n = 4) | 4.6 ± 0.4 |
| Cynomolgus Monkey  (n = 5) | 38.5 ± 12.4 |

**Supplementary Table S2. Mean AMG 596 EC50 concentrations required for lysis of EGFRvIII-expressing GBM cells by human and cynomolgus monkey effector cells**

EC50 values of sigmoidal dose response curves shown in **Figure 3** were calculated and averaged. PBMC, peripheral blood mononuclear cells. EGFRvIII, epidermal growth factor receptor variant III.



**Supplementary Fig. S1**. BiTE® molecule structure and mechanism of action to target and eliminate TAA-positive tumor cells. BiTE®, bispecific T-cell engager; mAb, monoclonal antibody; MOA, mechanism of action; TAA, tumor-associated antigen.



**Supplementary Fig. S2. Cynomolgus monkey EGFRvIII specific domain truncation within cynomolgus monkey EGFR**

The extracellular domain of cynomolgus monkey EGFRvIII was generated by deletion of 267 amino acids (aa; indicated by strike-through text) and insertion of glycine at the fusion site within the cynomolgus monkey EGFR aa sequence 1 to 665 (<https://www.ncbi.nlm.nih.gov/protein>, accession number XP\_005549616.1). For generation of an expression construct, EGFRvIII-modified cynomolgus monkey EGFR was fused to the human EGFR (<https://www.ncbi.nlm.nih.gov/protein>, accession number NP\_005219.2) coding sequence lacking aa 1–665.



**Supplementary Fig. S3. AMG 596 in vitro activity data in cytotoxicity assays with U-87 MG/EGFRvIII tumor cells**

U-87 MG/EGFRvIII tumor cells were co-cultured with human PBMC at effector to target cell ratios of 10:1 and increasing AMG 596 concentrations for 48 hours. (**A**) Redirected lysis was monitored via flow cytometric determination of PI uptake by target cells. (**B**) T‑cell activation was analyzed by flow cytometry after 48 hours. (**C**) AMG 596-induced cytokine secretion is exemplarily shown by the flow cytometric analysis of INF- in supernatants after 48 hours. EGFRvIII, epidermal growth factor receptor variant III; IFN, interferon; PBMC, peripheral blood mononuclear cells. Data points represent the mean of duplicate measurements. Error bars indicate the standard error of the mean (SEM).



**Supplementary Fig. S4. Detection of human T cells in brain sections of mice from the orthotopic tumor study**

Immunohistochemistry analysis of brain sections of mice from the orthotopic tumor study that were treated with (A) vehicle or (B) AMG 596 (0.5 mg/kg) were analyzed for the presence of human CD3 T cells at study days 44 and 19. Arrows indicate T cells. Images were recorded with a Leica LM2500 microscope (100 x magnification).



**Supplementary Figure S5. AMG 596-mediated lysis by CD3+, CD4+ and CD8+ T cells**

U-87 MG/EGFRvIII tumor cells were co-cultured with human (**A**) CD3+, (**B**) CD4+ and (**C**) CD8+ T cells at effector to target cell ratios of 10:1 and increasing AMG 596 concentrations for 48 hours. Redirected lysis was monitored via flow cytometric determination of PI uptake by target cells. T cells were untouched isolated (T cell isolation kits from Miltenyi) from indicated donor PBMC. EGFRvIII, epidermal growth factor receptor variant III; Data points represent the mean of duplicate measurements. Error bars indicate SEM.



**Supplementary Fig S6. AMG 596 TDCC assays with U-251 MG cells previously co-cultured in transwells with U-251 MG/EGFRvIII cells**

In transwell experiments, U-251 MG (bottom) and U-251 MG/EGFRvIII cells (cell impermeable membrane, BD Bioscience) were co-cultured for 72 hours. Thereafter, U-251 MG cells harvested from the bottom wells, U-251 MG cells (negative control) and U-251 MG/EGFRvIII cells (positive control) were co-cultured with PBMC at effector to target ratios of 10:1 with 1 µg/mL AMG 596 for 72 hours. (**A**) AMG 596‑mediated cytotoxicity was analyzed by flow cytometry as loss of target cell membrane integrity, reflected by the nuclear uptake of propidium iodide. (**B**, **C**) Upregulation of T-cell activation markers CD69 and CD25 on T cells was analyzed by flow cytometry using fluorescent antibodies against CD4, CD8 (identification of T cells) and CD25, CD69 (all antibodies from BD Bioscience). Cytotoxicity and T-cell activation were analyzed on a FACSCanto II flow cytometer (BD Bioscience) and data were evaluated using FACS Diva software (BD) and GraphPad Prism (GraphPad). EGFRvIII, epidermal growth factor receptor variant III.