Supplementary Materials and Methods

Development and Optimization of a humanized M27 antibody

The cDNA sequences encoding the heavy and light chain variable regions of clone 7B3 that specifically bound to EGFRvIII and the EGFR epitope (aa287-aa302) were cloned by using SMARTer® RACE 5'/3' Kit (Clontech, USA) according to the manufacturer's instructions. The gene sequences of Fv fragments were cloned into the pGEM-T vector (Promega, Madison, WI) for sequencing. The antibody VL and VH domains of the 7B3 clone was humanized by CDR grafting. Two distinct principles were used to determine the appropriate human framework, including amino acid sequence homology of variable domains and canonical structure homology of CDR loops. The VH and VL sequences were searched against human germline sequence databases to identify the most similar human germline Fv sequence and J regions by using IMGT/V-QUEST program (heep://www.imgt.org/IMGT_Vquest/share/texts/). The residues within the KABAT CDR regions were grafted into the human framework template as well. The humanized antibody 7B3 was named hu7B3.

Affinity maturation of hu7B3 was performed twice. CDR3 that contributed the most towards binding affinity was chosen for affinity maturation at the starting point. Each residue in CDRL3 or CDRH3 was randomized by using NNK codons (all amino acids allowed). Two phage libraries of 1.9×10^9 and 6×10^9 variants were generated, and then four-round selection was performed according to the published protocols (Philippa M. O'brien and Robert Aitken). The best clone from the randomization of CDRL3 and CDRH3 was combined as the template for the second round of affinity

maturation. A soft randomization of CDRL1 and CDRL2 or CDRH1 and CDRH2 was performed for the second round of affinity maturation (i.e., the doped oligonucleotides were used such that each wild-type residue was retained 50% of the time and the other 19 amino acids were introduced the remaining 50% of the time). The nucleotide sequence was replaced with the following sequences for doping:

A replaced with N1 (a mix of 70% A, 10% C, 10% G and 10% T)

C replaced with N2 (a mix of 10% A, 70% C, 10% G and 10% T)

G replaced with N3 (a mix of 10% A, 10% C, 70% G and 10% T)

T replaced with N4 (a mix of 10% A, 10% C, 10% G and 70% T)

A library of 3.5×10^9 pooled diverse clones was generated, and two-round selection was performed. A clone M27 was selected with the best characteristics.

Real-time cytotoxicity assay (xCELLigence)

A 50μl target cell culture medium was added into xCELLigence E-plates (Acea, San Diego, CA) as a blank reference. Target cancer cells in 50μl medium were then seeded at a density of 1×10^4 cells per well. After 30-min recovery, the cell culture was analyzed by using the xCELLigence RTCA DP device. After 18–24 hours, M27-28BBZ CAR-T cells or Mock T-cells were added into the well containing target cells at 1:1 effector:target (E:T) ratio. Impedance measurement was performed every 30 min for up to 72 h. All experiments were performed in triplicates. Data were analyzed by following the manufacturer's manual.

Expression and purification of M27, 806 and C225 scFv-Fc fusion proteins

To express M27, 806 and C225 scFv-Fc fusion protein in 293F cells, three

expression vectors were constructed respectively. Specific primers with encoded restriction enzyme sites were used in the PCR amplification of the scFv fragment. After NheI and BamHI digestion, the gene of each scFv was cloned into the expression vector pCMV-V5-Fc that has a Fc (hinge-CH2-CH3) region of IgG1 (purchased from Shanghai Raygene Biotechnology, Shanghai, China). Proteins were produced transiently in FreeStyle™ 293F cells (Thermo Fisher Scientific, Waltham, MA). 293F cells were transfected with a 1:2 ratio of plasmid/ 293fectin^{TM} at a density at 1×10^6 cells/ml. The cells were placed into an incubator at 37°C with 8% CO_2 for 6-7 days. Proteins in culture supernatant were harvested by centrifugation, and then purified by using protein A affinity chromatography.

Western blot analysis

5×10^6 cells were lysed in 200 μl lysis buffer for 60 min on ice. Cell lysate was then removed by centrifugation at 12,000 × g for 10 min. Protein was quantified by using the BCA Kit (Pierce, Rockford, IL). Each sample was subjected to electrophoresis by using 10% denatured SDS-PAGE. After transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), the sample was immunoblotted with the rabbit anti-human EGFR antibody (sc-03, Santa Cruz, CA). β-actin was used as a loading control. The blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kangchen Biotech, Shanghai, China) and then detected by using the ECL western blot analysis system (Pierce, Thermo Scientific, Rockford, IL) in accordance with the manufacturer’s instructions.