Establishing reverse chimeric antigen receptor T cells for precise targeting of immune-mediated thrombotic thrombocytopenic purpura

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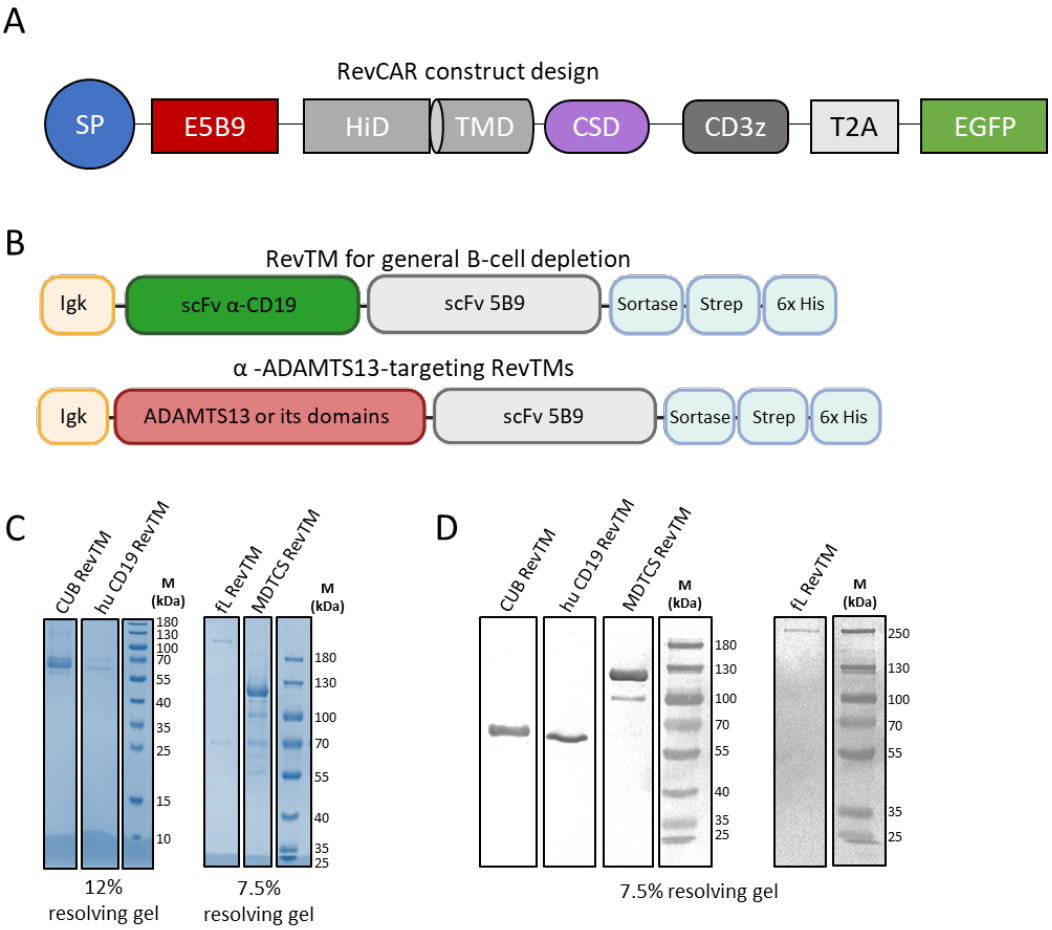
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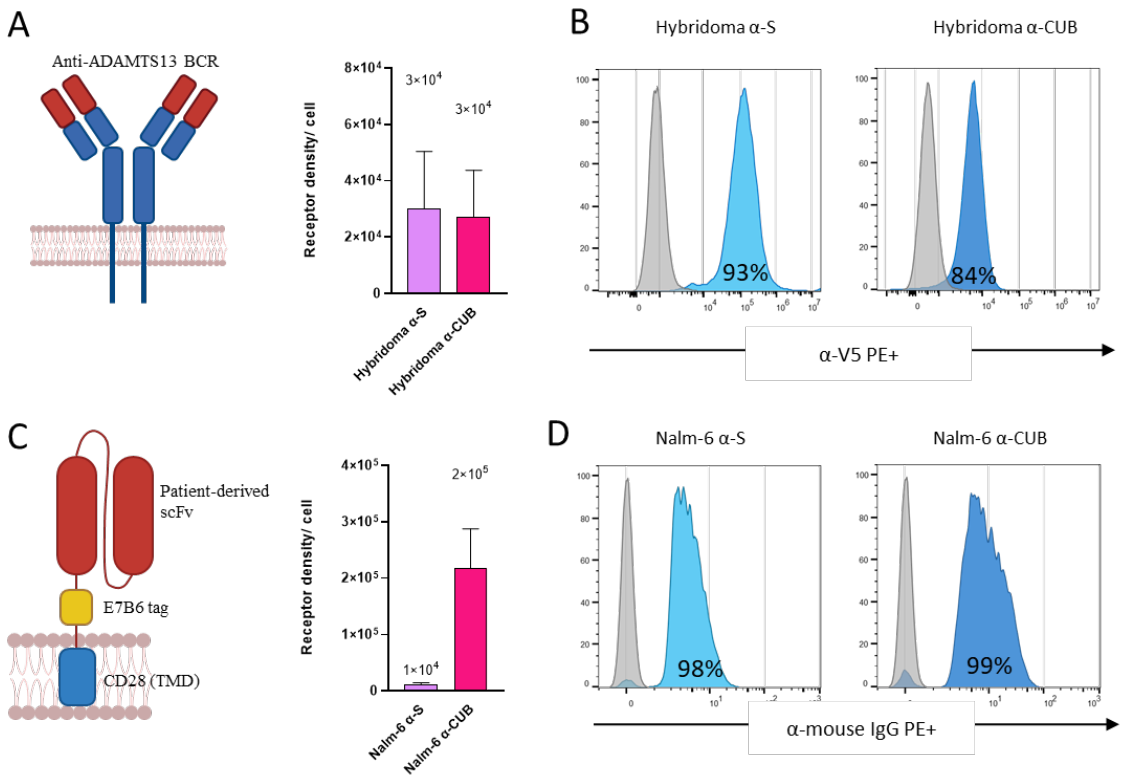
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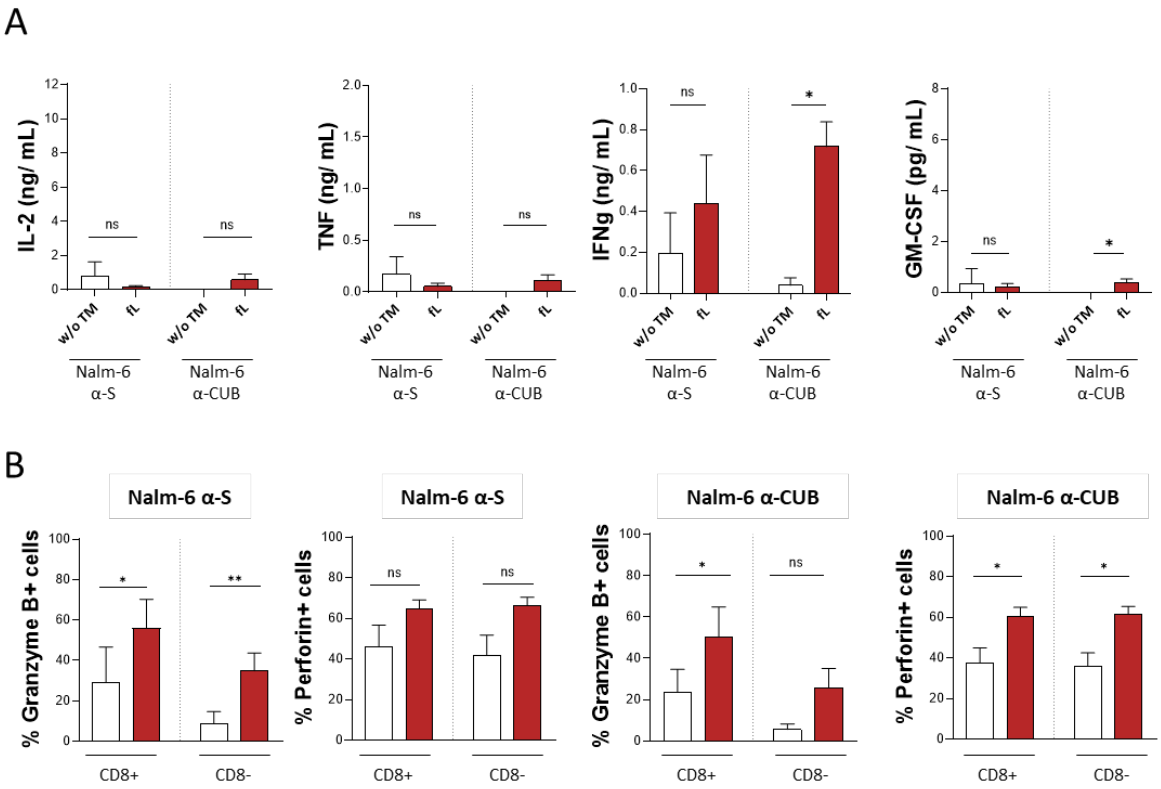
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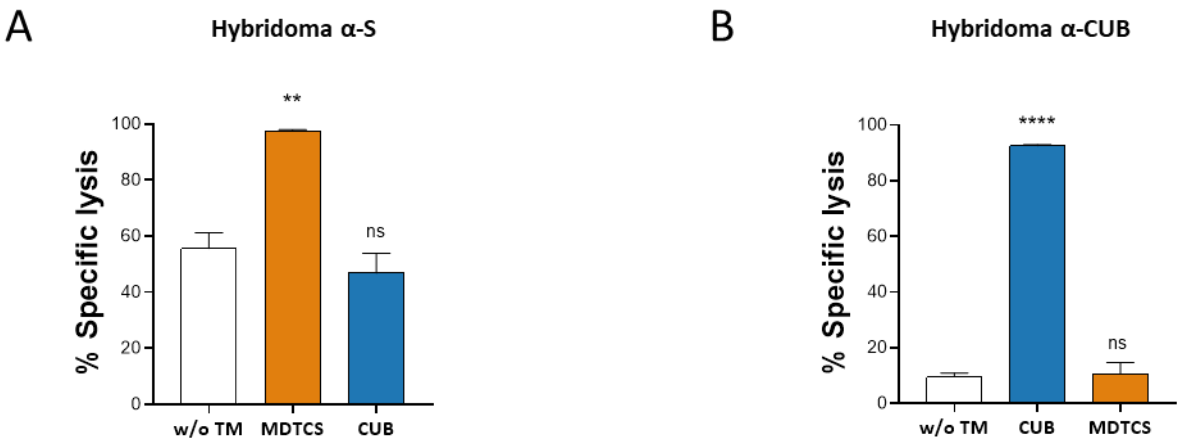
Supplementary Figure S1: (A) RevCAR construct design; SP: Signal peptide, HiD: Hinge domain, TMD: transmembrane domain, CSD: co-stimulatory domain, CD3: CD3 zeta, T2A: self-cleaving peptide sequence, EGFP: Enhanced green fluorescence protein. (B) Structure of RevTM targeting both α-ADAMTS13 autoreactive B cells and hu CD19. (B) Coomassie-stained SDS gel with purified CUB, MDTCS, ADAMTS13 and α-hu CD19 RevTMs. (C) Western blots with purified CUB, MDTCS, ADAMTS13 and α-hu CD19 RevTMs developed using α-His IgG + rabbit-α-mouse IgG Alkaline phosphatase. Each panel containing gel images was grouped from the same gel and paired with its corresponding marker. For panel D, non-linear adjustment using the gamma-contrast were made.



Supplementary Figure S2: (A) Left, pictorial representation of α-S/ α-CUB1 receptor on hybridomas; right, receptor density/ cell of each hybridoma used. (B) α-S (left) and α-CUB1 (right) BCR expression as confirmed by flow cytometry staining of α-S and α-CUB hybridomas respectively. (C) Left, pictorial representation of α-S/ α-CUB2 receptor on Nalm-6 cells; right. Patient-derived scFv (II-1: α-S, or z1-201: α-CUB) and La-derived E7B6 epitope on the extracellular part while CD28-derived transmembrane domain (TMD) for anchoring. Receptor density/ cell of each Nalm-6 cell line used. (D) α-S (left) and α-CUB (right) decoy receptor expression as confirmed by flowcytometry staining of Nalm-6 α-S and α-CUB respectively. Receptor density quantification done in three independent experiments. SEM represented.



Supplementary Figure S3: Cytokine secretion (A) and granzyme and perforin expression (B) profile of RevCAR T cells when co-cultured with fL RevTM and corresponding target Nalm-6 cells. Data generated using T cells isolated from 3 donors. Paired t test, one-tailed; *P < 0.05; **P< 0.001; ***P < 0.005, ****P < 0.0005 was performed by comparing all conditions with the respective condition without TM.



Supplementary Figure S4: RevTMs specifically redirect RevCAR T cells to kill target cells. In vitro co-culture assay between RevCAR T cells and α-S (A) or α-CUB (B) hybridomas. RevCAR T cells were co-cultured with hybridomas at 2.5:1 E:T ratio and 25 nM RevTM. Number of target cells were enumerated on flow cytometer 24 hours after co-culture. Data generated for T cells isolated from 3 healthy donors. Paired t test, two-tailed; *P < 0.05; **P< 0.001; ***P < 0.005, ****P < 0.0005 was performed by comparing all conditions with the respective condition without TM.

Supplementary Methods:

Cell culture

Hybridoma and Nalm-6 were the two target cell lines used for characterization and functional validation of the RevCAR system. In-house (KU Leuven) hybridoma 15D1 expressed the α-S BCR and 20D2 the α-CUB1 BCR[1,2]. These cells were cultured in DMEM medium supplemented with 10% bovine serum optimized for hybridomas (GE Healthcare), 1% MEM Non-Essential Amino Acids (NEA) solution (Invitrogen), 0.0004% β-Mercaptoethanol (Sigma Aldrich), 1% Penicillin-Streptomycin (Invitrogen), 2% HT-supplement (Invitrogen), 20 mM HEPES (Invitrogen). Nalm-6 cells were engineered to express a patient-derived α-S (II-1) and α-CUB2 (z1-201) single-chain fragment variable (scFv) on their surface via lentiviral transduction and were cultured in RPMI medium supplemented with 10% Fetal Calf Serum (FCS), 1% Penicillin-Streptomycin, 1% MEM NEA, 1% L-Alanine-L-Glutamine, 1% Sodium pyruvate (RPMI complete). Murine fibroblast 3T3 cells and HEK293T cells were cultured using DMEM medium supplemented with 10% FCS, 1% Pen-Strep, 1% MEM NEA (DMEM complete). All cells were maintained at 37 ºC and 5% CO_2.

Construction, expression and quantification of recombinant RevTMs

For construction of the pan B cell depleting α-CD19 RevTM, the α-human CD19 scFv (monoclonal antibody (mAb) clone HD-37) was fused with the α-La scFv (5B9) (Figure 1A). RevTMs targeting α-ADAMTS13 B cells were generated by linking full-length (fL) ADAMTS13, MDTCS or CUB1-2 domains to the α-La scFv (5B9) to form the fL, MDTCS and CUB RevTMs respectively. All RevTMs were equipped with C-terminal Strep and 6x His tags to aid protein purification (Figure 1A). Moreover, the murine Ig kappa leader was added as a signal peptide (SP) to the N-terminus of all RevTMs to enable their secretion in the cell culture supernatants (Supplementary Figure S1A).

For generation of persistent RevTM-producing 3T3s, 0.75 x 10^6 HEK293T cells were cultured in 90 mm cell culture dish (Greiner Bio-One) on day 0. On day 3, the cloned RevTM plasmids, along with packaging plasmids, were co-transfected in HEK293T cells as described [3]. The supernatant of the transfected HEK cells was then collected four times, every 12 hours, filtered and transferred onto 3T3 cells at each timepoint, and fresh DMEM complete medium was added onto HEK293T cells. After four transductions, the HEK293T cells were discarded, and the 3T3 cells were further treated with the selection antibiotic Zeocin at 0.3 mg/ mL concentration to enrich the transduced cells, until the enrichment was ~ 99%. The transduction efficiency was monitored via the reporter gene EGFP in the TM construct by flow cytometry.

The purified RevTMs were analyzed by SDS-PAGE followed by Quick Coomassie staining[4] or Western blotting. For immunodetection, proteins were transferred to nitrocellulose and probed using anti-His mAb (Qiagen, 1:2500) and rabbit-anti-mouse IgG alkaline phosphatase (Biozol, 1:1000).

Binding assessment using flow cytometry

Binding of the RevTMs to target cells (hybridoma and Nalm-6) and RevCAR T-cells was determined via flow cytometry. For this, 2 × 10^5 cells were incubated with the respective

RevTM (20 µg/ml) for 1 h at 4 ºC, washed with PBS/2% FCS, and then incubated again for 30 min at 4 ºC with α-His-PE (1:50) or α-His APC (1:100) mAb (Miltenyi Biotec). Shortly before analysis, cells were counterstained with propidium iodide to allow for live-dead-cell discrimination. The stained cells were measured with the MACSQuant10 Analyzer (Miltenyi Biotec), and the results were analyzed with MACSQuantify (Miltenyi Biotec) or FlowJo™ Software.

Surface expression of α-S BCR/scFv, α-CUB BCR/scFv and RevCARs on target or effector cells was determined in a similar way. For detection of α-S scFv and α-CUB scFv on Nalm-6, cells were stained with 20 µg/ml α-La 7B6 mAb (in-house) as primary antibody and goat α-mouse PE mAb (Invitrogen, 1:50) as the secondary antibody. RevCAR expression was detected by using first 20 µg/ ml α-La 5B9 mAb (Davids) and second goat α-mouse PE mAb (Invitrogen, 1:50). As negative control, cells were only stained with the secondary mAb in combination with the corresponding isotype control antibody (7B6: mouse IgG1, κ; 5B9: mouse IgG2b, κ (Biolegend). For detection of α-S or α-CUB BCRs on hybridomas, cells were incubated with MDTCS-V5 or T2-C2-V5 respectively and then stained with α-V5 PE mAb (eBioscience). To determine antigen density on the cell surface, the Quantitative Analysis Kit QIFIKIT® (Agilent) was used following the manufacturer's instructions. Data was plotted using GraphPad prism 10 software.

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