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LETTER TO THE EDITOR

Bifunctional αHER2/CD3 RNA-engineered CART-like human T cells specifically eliminate HER2$^+$ gastric cancer

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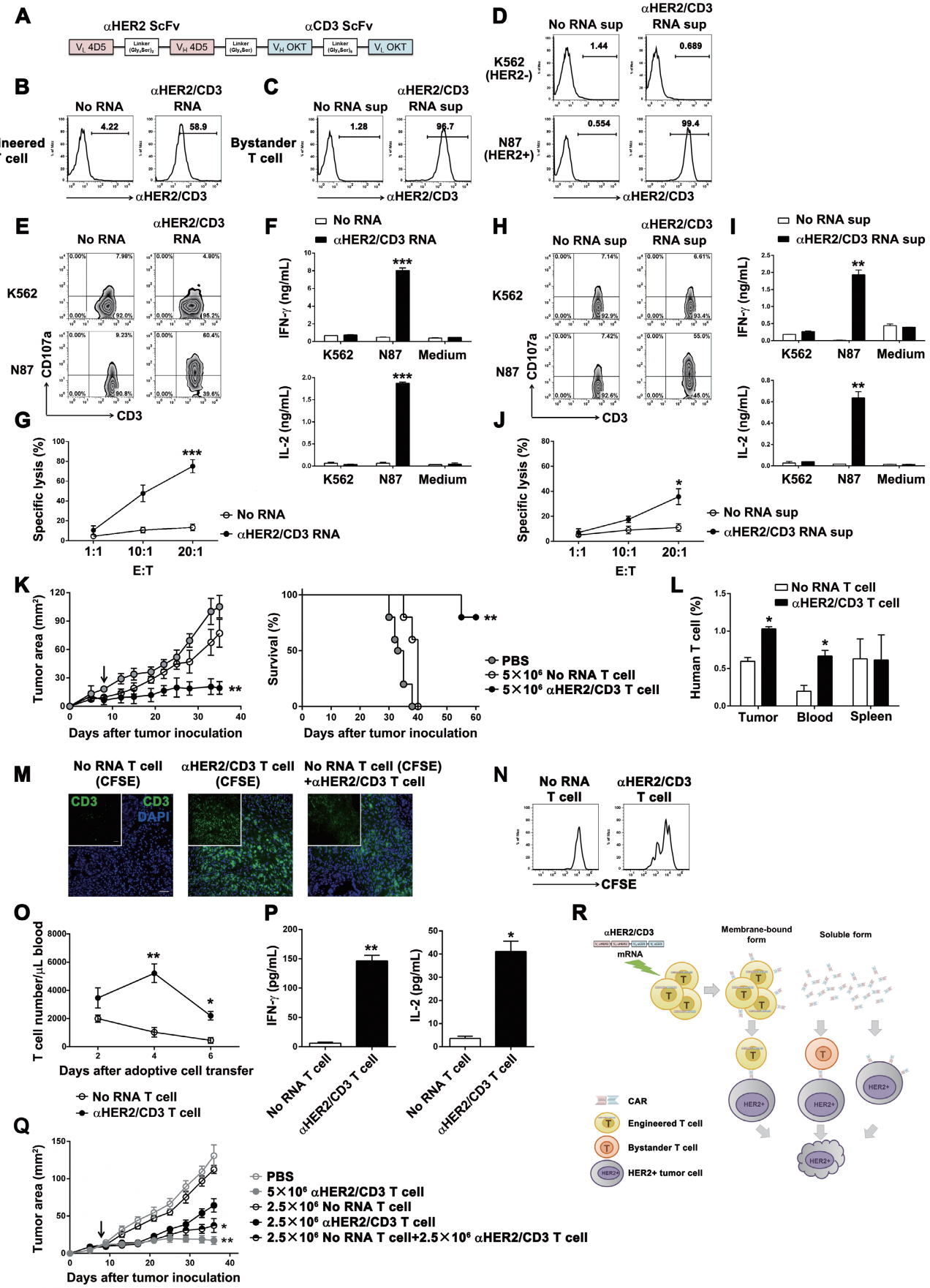
Dear Editor,

Genetically engineered T cell therapy is a promising new strategy to combat cancer. The chimeric antigen receptor-expressing T cell (CART) approach has achieved success in clinical trials of patients with non-solid tumors [1, 2]. Some limitations nevertheless have emerged. Among them are restriction of the CARs to the cell surface and the risks associated with retroviral integration in the genome [3, 4]. A recent development is the design of bispecific T-cell engagers (BiTEs), a class of artificial bispecific antibodies that are composed of two single-chain variable fragments (scFv), one specific for a T-cell-specific molecule, usually CD3, and the other specific for a tumor-associated antigen [5-8]. BiTE therapy is nevertheless limited by the short half-life of antibodies, the lack of endogenous effector T cells in patients with advanced cancer, and severe adverse effects such as cytokine release syndrome caused by T cell activation [9-11]. In the current study, we present a novel secretable BiTE, αHER2/CD3, which consists of HER2-specific scFv 4D5, CD3-specific scFv OKT3, and flexible linkers (Figure 1A). We show that αHER2/CD3 specifically targets HER2$^+$ tumor cells, such as those found in gastric cancer and breast cancer [12], and CD3$^+$ human T cells. αHER2/CD3 can be introduced into CD3$^+$ human T cells via electroporation of the *in vitro* transcribed capped αHER2/CD3 RNAs instead of lentiviral or retroviral transduction, circumventing the liabilities resulted from viral integration in the host genome.

αHER2/CD3 RNA-engineered CART-like T cells released αHER2/CD3 fusion protein to the culture medium and the secreted αHER2/CD3 can bind to T cell surface via CD3 recognition. 58.9% of the engineered T cells bore αHER2/CD3 on their surface 2 days after the electroporation (Figure 1B). Interestingly, secreted αHER2/CD3 BiTEs could affect bystander T cells (i.e., T cells that were not electroporated with αHER2/CD3 RNA). After incubation with αHER2/CD3-containing supernatant for 30 minutes at 4°C, 96.7% of bystander T cells acquired αHER2/CD3 on their surface (Figure 1C). These results suggest that secreted αHER2/CD3 can lodge onto bystander T cells. When human tumor cells, N87 (gastric cancer cell line; HER2$^+$) or K562 (chronic myeloid leukemia cell line; HER2$^-$) cells (Supplementary information, Figure S1A), were cultured with αHER2/CD3-containing supernatant, the binding of αHER2/CD3 was detected only on N87 cells, demonstrating the specificity of αHER2/CD3 (Figure 1D).

To determine the optimal dose of αHER2/CD3 RNA in order to maximize its efficiency and minimize its toxicity, we electroporated 0, 2.5, 5, 10, and 20 μg of αHER2/CD3 RNA into T cells. We found 10 μg of αHER2/CD3 RNA had little impact on the viability of electroporated cells and led to high levels of IFN-γ and IL-2 production when the cells were co-cultured with N87 cells (Supplementary information, Figure S1C and S1D). We next determined whether αHER2/CD3 RNA-engineered CART-like T cells have the potential to kill HER2$^+$ tumor cells by analyzing the mobilization of CD107a to plasma membrane of the T cells (indicative of cytotoxic phenotype) using flow cytometry. CART-like T cells were co-cultured with tumor cells at a 1:1 ratio. Four hours later, αHER2/CD3 RNA-engineered T cells, but not the non-engineered T cells, upregulated cell surface expression of CD107a only when co-cultured with the HER2$^+$ N87 tumor cells (Figure 1E). After an additional 12 h, markedly elevated levels of IFN-γ and IL-2 were detected in the supernatant of αHER2/CD3 RNA-engineered T cells co-cultured with N87 cells (Figure 1F). Furthermore, the engineered T cells lysed N87 cells much more efficiently than non-engineered T cells (Figure 1G), and the cytotoxicity increased in a ratio-dependent manner (effector vs target). These data suggest that αHER2/CD3 RNA-engineered T cells exhibit greater HER2-specific cytotoxicity than non-engineered T cells. More importantly, we found that bystander T cells that had been cultured with αHER2/CD3-containing supernatant exhibited similar effects against HER2$^+$ tumor cells (Figure 1H-1J). This observation indicates the ability of secreted αHER2/CD3 BiTE to direct bystander T cells against HER2$^+$ tumor cells. In addition, αHER2/CD3-en-


A
αHER2 ScFv
αCD3 ScFv
V_L 4D5
Linker (Gly4Ser)3
V_H 4D5
Linker (Gly4Ser)
V_H OKT
Linker (Gly4Ser)3
V_L OKT
B
No RNA
αHER2/CD3 RNA
Engineered T cell
4.22
58.9
αHER2/CD3
C
No RNA sup
αHER2/CD3 RNA sup
Bystander T cell
1.28
96.7
αHER2/CD3
D
No RNA sup
αHER2/CD3 RNA sup
K562 (HER2-)
1.44
0.689
N87 (HER2+)
0.554
99.4
αHER2/CD3
E
No RNA
αHER2/CD3 RNA
K562
7.98%
92.0%
4.80%
95.2%
N87
CD107a
8.23%
90.8%
60.4%
39.6%
CD3
F
No RNA
αHER2/CD3 RNA
IFN-γ (ng/mL)
***
K562
N87
Medium
IL-2 (ng/mL)
***
K562
N87
Medium
G
Specific lysis (%)
***
No RNA
αHER2/CD3 RNA
1:1
10:1
20:1
E:T
H
No RNA sup
αHER2/CD3 RNA sup
K562
7.14%
92.9%
6.61%
93.4%
N87
CD107a
7.42%
92.6%
55.0%
45.0%
CD3
I
No RNA sup
αHER2/CD3 RNA sup
IFN-γ (ng/mL)
**
K562
N87
Medium
IL-2 (ng/mL)
**
K562
N87
Medium
J
Specific lysis (%)
*
No RNA sup
αHER2/CD3 RNA sup
1:1
10:1
20:1
E:T
K
Tumor area (mm²)
**
Days after tumor inoculation
Survival (%)
**
PBS
5×10⁶ No RNA T cell
5×10⁶ αHER2/CD3 T cell
Days after tumor inoculation
L
No RNA T cell
αHER2/CD3 T cell
Human T cell (%)
*
*
Tumor
Blood
Spleen
M
No RNA T cell (CFSE)
αHER2/CD3 T cell (CFSE)
No RNA T cell (CFSE) +αHER2/CD3 T cell
CD3
CD3 DAPI
N
No RNA T cell
αHER2/CD3 T cell
CFSE
O
T cell number/μL blood
**
*
Days after adoptive cell transfer
No RNA T cell
αHER2/CD3 T cell
P
IFN-γ (pg/mL)
**
No RNA T cell
αHER2/CD3 T cell
IL-2 (pg/mL)
*
No RNA T cell
αHER2/CD3 T cell
Q
Tumor area (mm²)
*
**
Days after tumor inoculation
PBS
5×10⁶ αHER2/CD3 T cell
2.5×10⁶ No RNA T cell
2.5×10⁶ αHER2/CD3 T cell
2.5×10⁶ No RNA T cell+2.5×10⁶ αHER2/CD3 T cell
R
αHER2/CD3
mRNA
Membrane-bound form
Soluble form
CAR
Engineered T cell
Bystander T cell
HER2+ tumor cell
HER2+

gineered T cells could execute HER2-specific killing and produce IFN-γ and IL-2 for at least 6 days *in vitro*, indicating a continuous secretion of αHER2/CD3 from the engineered T cells (Supplementary information, Figure S1E-S1H). This observation suggests that in contrast to conventional BiTEs, such as blinatumomab (αCD19/CD3 BiTE), which has a half-life of only ~2 h [11], our new strategy may be long-lasting *in vivo*.

To explore the effect of the αHER2/CD3-containing supernatant on $HER2^+$ tumor cells, we first measured the proliferation of N87 cells cultured in αHER2/CD3-containing supernatant for 24, 48 and 72 h using the CCK8 (Cell Counting Kit-8) assay, and found it was inhibited in a time-dependent manner (Supplementary information, Figure S1I). Further cell cycle analysis revealed that N87 cells were arrested at the G1 phase after the supernatant treatment. Consistently, the supernatant treatment also reduced both the basal and EGF-induced expression levels of the cell cycle-associated proteins cyclin D1 and PCNA (Supplementary information, Figure S1J and S1K). Taken together, these data indicate that the αHER2/CD3-containing supernatant can inhibit the proliferation of N87 cells by downregulating the expression of cyclin D1 and PCNA to induce G1 arrest.

To determine whether αHER2/CD3 RNA-engineered T cells can eliminate $HER2^+$ tumors *in vivo*, we used a tumor model in nude mice subcutaneously (s.c.) implanted with 1×10^6 N87 tumor cells. On day 8, the tumor-bearing mice were intravenously (i.v.) transferred with 100 μl of PBS or 5×10^6 carboxyfluorescein succinimidyl ester (CFSE)-labeled human non-engineered or αHER2/CD3 RNA-engineered T cells every six days (three times in total). Compared with the PBS and control T cell treatments, αHER2/CD3 RNA-engineered T cells significantly inhibited tumor growth, and 80% of the mice (8/10) survived to 60 days post-implantation (Figure 1K). The distribution of adoptively transferred human T cells in the tumor and blood, as well as in the spleen, was analyzed one day after the 3rd injection. The percentage of human T cells in the tumor and blood from the αHER2/CD3 group markedly increased relative to that of the non-engineered T cell-treated group (Figure 1L). Fluorescence microscopy further confirmed the infiltration of CFSE-labeled αHER2/CD3-engineered T cells in the tumor tissues (Figure 1M). In addition, we found αHER2/CD3-engineered T cells, compared with non-engineered T cells, expanded significantly (Figure 1N) and expressed much lower levels of the inhibitory receptors PD1, LAG3 and TIM3, markers for T-cell exhaustion (Supplementary information, Figure S2A), at the tumor site, supporting the notion that infiltration of αHER2/CD3 T cells caused tumor reduction. The adoptively transferred T cells survived for at least 6 days *in vivo*, as demonstrated by the presence of transplanted T cells in the blood and the elevated serum levels of human IFN-γ and IL-2 in the αHER2/CD3 group (Figure 1O and 1P). To determine whether αHER2/CD3 RNA-engineered T cells could promote the anti-tumor ability of bystander T cells, we transplanted CFSE-labeled bystander T cells with αHER2/CD3 RNA-engineered T cells into N87 tu-

Figure 1 αHER2/CD3 RNA-engineered CART-like T cells specifically eliminate $HER2^+$ tumor cells. **(A)** A schematic of αHER2/CD3 construct that contains an αHER2 scFv, an αCD3 scFv, and linkers. **(B)** Activated human T cells were electroporated with or without 10 μg of αHER2/CD3 RNA. Two days later, the binding of the secreted αHER2/CD3 on these T cells was detectable by FACS. **(C**, **D)** The supernatant of these engineered T cells was harvested and used to culture bystander T cells and N87 or K562 tumor cells. The binding of αHER2/CD3 on these cells was analyzed by FACS. **(E-J)** αHER2/CD3 RNA-engineered T cells **(E-G)** or bystander T cells that had been incubated with supernatant harvested from non-engineered T cells (No RNA) or αHER2/CD3 RNA-engineered T cells **(H-J)** were co-cultured with K562 or N87 tumor cells at a 1:1 ratio. CD107a translocation to the plasma membrane in the T cells was analyzed by FACS 4 h after the co-culture **(E**, **H)**. The levels of IFN-γ and IL-2 in the culture media were measured by ELISA 16 h after the co-culture **(F**, **I)**. For the cytotoxicity assay, CFSE-labeled N87 cells and CMRA-labeled K562 cells were mixed at a 1:1 ratio. Effector T cells were co-cultured with the mixed tumor cells at the indicated ratio for 4 h and the specific lysis was determined by FACS **(G**, **J)**. **(K-P)** 1×10^6 N87 cells were transplanted into female nude s.c. Seven days post-inoculation, these mice were treated i.v. with PBS or CFSE-labeled αHER2/CD3 RNA (10 μg)-engineered $CD3^+$ human T cells every six days (three times in total). Tumor size was measured using digital calipers every 2-3 days, and the arrow indicates the starting time (day 8) of the injection (**K**, left). Mouse survival was monitored regularly and statistically analyzed using a log-rank test (n = 10; **K**, right). One day after the final treatment, tumor, blood and spleen samples were collected and analyzed for the distribution of adoptive T cells by FACS **(L)**. Tumors were also stained for DAPI and observed under a fluorescent microscope. Images in the white square show single CFSE staining (green) without DAPI counterstaining. Magnification 200×; scale bar, 50 μm **(M)**. Three days after the final treatment, the proliferation of implanted T cells infiltrated into the tumor site was analyzed by FACS. The cell proliferation is reflected by the CFSE dye dilution **(N)**. On the indicated days after the final treatment, blood samples were collected, and adoptively transferred T-cell numbers were counted by FACS **(O)**. Human IFN-γ and IL-2 levels in mice serum were measured by ELISA six days after final treatment **(P)**. **(Q)** Seven days post N87 cell inoculation, tumor-bearing mice were treated i.v. with PBS, No RNA T cells, αHER2/CD3 T cells or No RNA T cells plus αHER2/CD3 T cells every six days for a total of three times. The arrow indicates the starting time (day 8) of injection. Tumor size was measured using digital calipers every 3-4 days. Data are presented as mean ± SEM and represent results of one of three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001. **(R)** Model of αHER2/CD3 RNA-engineered T-cell function.

mor-bearing mice and found that bystander T cells infiltrated into the tumor site and exhibit anti-tumor activity (Figure 1M and 1Q). These data together indicate the effectiveness of αHER2/CD3 RNA-engineered T cells in eliminating $HER2^+$ tumors *in vivo*.

Finally, we investigated whether this new CART-like T-cell therapy would cause any side effects. We measured the serum levels of the inflammatory cytokines IL-6 and TNF-α after the final injection of αHER2/CD3 RNA-engineered T cells in N87 tumor-bearing mice. There was no difference in cytokine release between the experimental and control groups (Supplementary information, Figure S2B).

In summary, the αHER2/CD3 RNA-engineered T cells developed here may provide a new and attractive alternative to current CART therapy. The engineered T cells are bifunctional as they can not only kill $HER2^+$ tumors but also pass this ability to bystander T cells (Figure 1R). The αHER2/CD3 RNA-engineered CART-like T cells can persist and remain functional *in vivo* for at least 6 days with few cytokine release-related side effects. The data presented in this study should provide rational for further study of αHER2/CD3 RNA-engineered T cells as a potential treatment of $HER2^+$ malignancies.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)