The affinity of antigen-binding domain on the antitumor efficacy of CAR T cells: Moderate is better

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The overall efficacy of chimeric antigen receptor modified T cells (CARTs) remain limited in solid tumors despite intensive studies that aim at targeting multiple antigens, enhancing migration, reducing tonic signaling, and improving tumor microenvironment. On the other hand, how the affinity and engaging kinetics of antigen-binding domain (ABD) affects the CART's efficacy has not been carefully investigated. In this article, we first analyzed 38 published solid tumor CART trials and correlated the response rate to their ABD affinity. Not surprisingly, majority (25 trials) of the CARTs utilized high-affinity ABDs, but generated merely 5.7% response rate. In contrast, 35% of the patients treated with the CARTs built from moderate-affinity ABDs had clinical responses. Thus, CARTs with moderate-affinity ABDs not only have less off-target toxicity, but also are more effective. We then reviewed the effects of ABD affinity on the biology and function of CARTs, providing further evidence that moderate-affinity ABDs may be better in CART development. In the end, we propose that a fast-on/fast-off (high $K_{on}$ and $K_{off}$) kinetics of CART-target engagement in solid tumor allow CARTs to generate sufficient signaling to kill tumor cells without being driven to exhaustion. We believe that studying the ABD affinity and the kinetics of CART-tumor interaction may hold a key to designing effective CARTs for solid tumors.

KEYWORDS
Adoptive cell therapy, chimeric antigen receptors (CAR), CAR T cells, antigen-binding domain, T cell engineering, tumor immunotherapy, solid tumors
Introduction

Immunotherapy is now the 4th pillar of cancer treatment (1, 2), and its efficacy relies on the tumor-infiltrating T cells (3), which, unfortunately, many solid tumors do not have (4). Engineering patients T cells with a T cell receptor (TCR) (5–8) or chimeric antigen receptor (CAR) (9) provides the much-needed tumor-specific T cells. CAR combines the antibody specificity and TCR signaling apparatus, which can activate T cells upon engaging with tumor surface antigen (9). The CAR-modified T cells (CARTs) thus recognize and kill tumor cells independent of MHC that is frequently downregulated, a common cause of tumor escape. CARTs have generated remarkable antitumor responses in treating hematomal cancers (9–12), which results in 7 FDA-approved CARTs (13), but also ignites tremendous effort to develop solid tumor CARTs (14, 15). However, despite intensive studies, by far, the clinical efficacy of solid tumor CARTs remains limited (13, 16–18). A meta-analysis of 22 solid tumor CART trials (268 patients) reveals merely ~9% response rate (19). Evidently, the current CARTs do not work well for solid tumors. However, since 90% of cancers are solid tumors (20), investigators have been diligently working on and looking forward to a breakthrough in designing effective solid tumor CARTs.

To generate antitumor effects, CARTs need to migrate into a solid tumor mass, undergo antigen-driven activation and expansion, exert their effector function on target cells, persist in the solid tumor mass, undergo antigen-driven activation and detect low levels of antigen (45–47). However, thus far, the ABD affinity was not rationally considered in most CAR designs. This is reflected by the incomplete data of ABDs, the lack of Koff and K0ff or inconsistent use of KD (dissociation constant) and EC50 (half-maximal effective concentration), which will be discussed later in detail. For clarity, we refer to mAb binding strength as “affinity” (single pair of molecules) and the CAR or CART binding strength as “avidity” (multiple pairs of molecules). Over last few decades, affinity enhancement has been the main goal in antibody drug development. Approximately 100 therapeutic mAbs have been approved by the FDA (42), and many are high-affinity. Naturally, these clinically safe high-affinity mAbs, such as Cetuximab (KD=1.8nM) (43) and Herceptin (KD=5nM) (44), were used to create CARTs. High-affinity mAbs are preferred for CAR construction also because they may induce strong T cell activation and detect low levels of antigen (45–47). However, it is unclear how the ABD affinity affects the antitumor efficacy of solid tumor CARTs. Recently, we analyzed 14 solid tumor CART trials in the world (48, 49), and our findings are that moderate-affinity ABDs correlate to better efficacy (13). Thus, our first goal in this article is to verify the correlation of ABD affinity and clinical efficacy by expanding analysis to more solid tumor CART trials.

Correlation of ABD affinity and clinical efficacy of CARTs: Moderate is better

The ABD, most of which are the single chain variable fragment (scFv) of monoclonal antibodies (mAbs), allows CARTs to specifically bind and kill tumor cells. However, thus far, the ABD affinity was not rationally considered in most CAR designs. This is reflected by the incomplete data of ABDs, the lack of Koff and K0ff or inconsistent use of KD (dissociation constant) and EC50 (half-maximal effective concentration), which will be discussed later in detail. For clarity, we refer to mAb binding strength as “affinity” (single pair of molecules) and the CAR or CART binding strength as “avidity” (multiple pairs of molecules). Over last few decades, affinity enhancement has been the main goal in antibody drug development. Approximately 100 therapeutic mAbs have been approved by the FDA (42), and many are high-affinity. Naturally, these clinically safe high-affinity mAbs, such as Cetuximab (KD=1.8nM) (43) and Herceptin (KD=5nM) (44), were used to create CARTs. High-affinity mAbs are preferred for CAR construction also because they may induce strong T cell activation and detect low levels of antigen (45–47). However, it is unclear how the ABD affinity affects the antitumor efficacy of solid tumor CARTs. Recently, we analyzed 14 solid tumor CART trials in the world (48, 49), and our findings are that moderate-affinity ABDs correlate to better efficacy (13). Thus, our first goal in this article is to verify the correlation of ABD affinity and clinical efficacy by expanding analysis to more solid tumor CART trials.

Based on the latest counting, there are 292 solid tumor CART trials in the world (48, 49), most of them are Phase I studies and have not completed. We were able to find 38 published solid tumor CART trials (total 453 patients). We...
analyzed and summarized the clinical response (partial and complete response, PR and CR) of each trial in Table 1 and Supplemental Table 1. We found that, among the 453 patients in the 38 trials, 57 (12.58%) patients had PR or CR. This is seemingly higher than the response rate of ~9% reported in another meta-analysis (19), which is likely due to the latest addition of Claudin 18.2 CART trials that demonstrated 44.64% in 56 gastrointestinal (GI) cancer patients (143–145).

Importantly, from the 38 CART trials, we traced back to the original CART development and found the ABD affinity (K_D) (Table 1 and Supplemental Table 1). The correlation of ABD affinity and response rate was also presented in Figure 1. We arbitrarily divided the ABDs as high- (K_D<20nM), moderate- (K_D=20-100nM), and low- (K_D>100nM) affinity. Not surprisingly, 2/3 of the trials (25/38, 65.79%) utilized high-affinity ABDs in their CARTs. The response rate in the high-affinity group is merely 5.70% (17 out of 298 patients) (Figure 1 and Supplemental Table 1). Only 9 of the 25 trials generated low response. The other 16 CARTs built from high-affinity ABDs showed no responses (the best result is stable disease). In contrast, 8 out of 10 trials of CARTs with moderate-affinity ABDs showed an impressive response rate (18.18%-75%). The overall response rate of moderate-affinity CART ABDs reaches 34.78% (40 out of 115 patients). Thirdly, when the ABD affinity is too low (K_D>100nM), the CARTs demonstrated no clinical responses (Figure 1, Table 1, and Supplemental Table 1), suggesting that when the ABD affinity is below a certain threshold, the CARTs will not have adequate avidity to engage and kill tumor cells. These 38 trial data demonstrated that the affinity of ABDs is critical in determining the efficacy of solid tumor CARTs. ABDs with proper moderate-affinity may have the optimal engagement for CARTs to kill tumor cells inside tumor mass. Currently, there is no available data on the optimal ABD affinities in different CARTs. However, it is likely that the optimal affinity of ABDs may vary among different CARTs and may depend on the engagement modes of CART-tumor cells in hematological cancers vs. solid tumors (see sections below).

The 38 solid tumor CART trials in Table 1 target different antigens and epitopes. It is known that the epitope location (relative to the cell membrane) plays an important role in deciding CART’s functions and antitumor efficacy in preclinical tumor models (27). To minimize the effects of epitope location and to analyze the correlation of ABD affinity more precisely to the antitumor efficacy of CARTs, we compared the clinical response of three GD2 CARTs. Disialoganglioside GD2, a major ganglioside, is a carbohydrate antigen expressed on the tumors of neuroectodermal origin, including melanoma, neuroblastoma, sarcoma, and small cell lung cancer (146). GD2 has a hydrophobic ceramide tail inserted into the cell membrane and a pentasaccharide moiety head on the outside of membrane (Figure 2A) (147). Multiple anti-GD2 mAbs are developed for cancer therapies (148), and some are approved by FDA (149). The three anti-GD2 mAbs used to develop CARTs have different affinities (Table 1) but target the same membrane-proximal sugar moiety (147, 150). Thus, the effect of epitope location can be neglected when the CART’s efficacy is compared. In four clinical trials using the GD2 CARTs made with moderate-affinity mAb 14.G2a (K_D=77nM), 13 out of 37 patients had PR or CR (35.16% response rate) (Table 1, Supplemental Table 1, and Figure 2B). Although the 14.G2a-based GD2 CARTs in different trials utilized different CDs, they all generated good clinical responses, further suggesting that ABDs may play a deciding role in the antitumor outcome of CARTs. In contrast, the GD2 CARTs built with high-affinity Hu3F8 mAb (K_D=11nM) or with low-affinity KM8138 (humanized KM666) mAb (K_D=149nM) did not generate clinical response (Figure 2B, Table 1, and Supplemental Table 1).

The benefit of moderate-affinity ABDs in solid tumor CARTs was further demonstrated in 3 latest trials (two were in China and one was in USA) of the same Claudin 18.2 CARTs (143–145). Claudin 18.2, a member of the tight junction protein family, is considered a gastric-specific isoform with higher expression on cancers than normal tissue. Claudin 18.2 specific mAbs and CARTs are being developed to treat GI cancers. In the latest trial of Claudin 18.2 CARTs, 18 out of 37 GI cancer patients demonstrated an overall response rate of 48.64% (144). A similar response rate was also reported in other two recent trials (143, 145). The overall response rate of this Claudin 18.2 CARTs reached an impressive 44.64% in 3 trials (25 out of 56 patients). Unfortunately, the K_D of Claudin 18.2 mAb, 8E5, was not reported. However, investigators did measure the EC50 of 8E5 mAb binding to Claudin 18.2 + 293 cells, which is 49.19nM (142). After humanization and optimization, the final mAb Hu8E5-2I used in the Claudin 18.2 CARTs has EC50 6.4nM for binding Claudin 18.2 + 293 cells (142), which is 20x lower than GC33 (EC50 ~ 0.24nM) and YP7 (EC50 ~ 0.3nM). According to a comparative study (71), the value of EC50 determined by ELISA is 5.76-13 folds lower than the K_D value measured by surface plasmon resonance (SPR). Based on this factor, we calculated the K_D value of Hu8E5-2I is likely between 36 to 83nM, which falls in the moderate-affinity range. We thus used the average 60nM to do the plot in Figure 1.

Moderate-affinity ABD is also good for blood cancer CARTs. While the original CD19 CARTs built with FMC63 mAb (K_D=0.328nM) generated remarkable antitumor efficacy and have been approved by FDA, recent studies showed that CD19 CARTs made with a new mAb CAT with lower affinity (K_D=14nM) generated enhanced expansion and prolonged persistence in treating refractory AML compared to the FMC63-based CD19 CART (151).

In summary, although multiple factors may contribute to CART’s function, the data analysis of 38 solid tumor CART clinical trials demonstrate that the ABD affinity is possibly the most important one in deciding the CART’s antitumor efficacy. Moderate affinity ABD not only allows CARTs to distinguish the antigenhigh tumor cells from antigenlow normal cells (see section...
TABLE 1 Summary of solid tumor CAR-T clinical trials: Affinity of antigen binding domains vs. clinical efficacy.

| Target     | ABD (K_D) | ICD                  | In vitro/Preclinical                                                                 | Clinical Responses                                                                 |
|------------|-----------|----------------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|
| VEGF-R2    | Bevacizumab (K_D: 88pM), or Ranibizumab (46pM) (50, 51) | ζ                    | Anti-mouse VEGF-R2 mAb (DC101) and mouse CARTs generated no effect (52), but co-expression of IL12 regressed several mouse tumors. | NCT01218867 (Results were tabulated on the website): 1/23 PR (metastatic melanoma and renal Ca). As DC101 mAb did not recognize human VEGF-R2 (53), the Bevacizumab or Ranibizumab, or mAb from (53) (K_D from 0.49-1.1nM) are likely used. |
| CD171 (L1-CAM) | CE7: 0.1nM (54, 55) | ζ                    | The IgG1-Fc (hinge)CD4TM-CD3ζ CART (56) killed tumor cells and produced cytokines in vitro. | NCT0006480 (57): 1/6 PR (only 56days), pediatric recurrent or refractory NR, CAR-Ts disappears in a week in high tumor burden and 42 days in limited tumor burden patients. |
|            | Frx       | MOv18: 0.2nM (58, 59) | FcrRγy                  | Dual allo-TCR and Frx CART inhibited tumor growth in mice (60)                        | NCT0019136 (61) (12 OVCA): 0 response, No tumor reduction in any of 12 patients. |
| Mesothelin | SS1: 0.7nM (62, 63) | 28ζ                  | Compared to BBζ CART, 28ζ and 28BBζ CARTs generated stronger antitumor effects (63, 64). CARTs were generated by lentivector (63) or by mRNA electroporation (64). | NCT01355965 (65): 1 PR but developed analphyaxis & cardiac arrest, due to anti-SS1 Ab (66). |
|            | Epitope: AA314-375 | BBζ                  | Preclinical study showed BBζ CART generated much better effect than P4 CART (75).    | NCT01897415 (67): 6 PDAC: 0 PR, 2 SD. 1 metabolic CR in the liver mets. |
|            | (Beatty: WO2015090230A1) | 82-28BBζ             | 1. P4 28ζ CARTs generated better effects than CD3ζ CARTs (74). 2. P4 CART with PD1+ TCR KO (MPTK) generated much better effect than P4 CART (75). | NCT02414269 (Intrapleural local delivery of CART & PD1) (25 MPM, 1 metastatic lung Ca, and 1 metastatic breast Ca): 8 SD (among which, 2 CI) (72). |
| M912: 1.5nM (ECoG) (70) | 28ζ                  | Converted to K_D, 8.6-20nM based on reference (71) | Good antitumor effects in ovarian ca xenografts (77).                                | No clinical trial No. 3 patients of Ovarian Ca. 0/3 PR, 2/3 SD (77). |
| P4 (human Ab) (73) | ζ                  | 1-10nm (74)          | Good antitumor effects with CARs generated by lentivector (73) or by mRNA electroporation (75). | NCT02395250 and NCT03146234 (3 liver Ca): 0 PR/CR; 2 SD. CART was short lived, peaked 7-14 days and undetectable after 4 weeks. |
| M5 mAb (Human mAb), K_D: 26.9nM. Epitope: aa485-572 | BBζ                  | Preclinical study showed antitumor effects (80).                                    | NCT03545815 (Only MPTK CART was tested in patients (76): 15 patients (12 GI Ca and 3 other Ca): 0 PR/CR, 2 SD. CART was short lived, peaked 7-14 days and undetectable after 4 weeks. |
| G11 mAb 2.3nm (77) | 28ζ                  | Good antitumor effects in ovarian ca xenografts (77).                                | NCT03054298 (14 OVCA, MPM, lung Ca): 0 PR. Similar to SS1, M5 CART peaked D14 & disappeared after D28. |
| GPC3       | GC33: ECoG, 0.24nm (78), K_D: 1.38nm (79) | 28ζ                  | Preclinical study showed antitumor effects (80).                                    | NCT03329344 (3 PDAC): 0/3 PR (https://www.med.upenn.edu/cellcon2021/assets/user-content/documents/tanyi.pdf). |
|            | YP7 (82), ECoG, 0.3nm | BBζ                  | YP7-BBζ CART has antitumor effects (83). But 3rd gen may be toxic.                  | NCT02395250 and NCT03146234 (13 liver Ca): 2PR (81), 1 patient survived more than 2 yrs. |
| C-Met      | Onartuzumab: 1.2nM (84) | 82ζ                  | Preclinical study showed CD3ζ CART was better than FcRγy (67).                     | NCT05003895: Started in 8/2021, Not data yet. |
| CEA        | MFE23: 1.7nm (86) | FcrRγy               | Preclinical study showed CD3ζ CART was better than FcRγy (67).                     | NCT01837602 (85): (6 metastatic breast cancer) Intratumoral injection of mRNA-CAR-Ts, No response (0/6). |
| hMN14: 3.4nm (89) | 28ζ                  | Preclinical study (90) showed 28ζ CART was better than CD3ζ CART (91).             | NCT01212887 (88) (14 patients with GI Ca (metastatic), 0 PR, 7 SD, Short persistency, off-target toxicity. |
|            | BBζ                   | BBζ                  | Preclinical study (90) showed 28ζ CART was better than CD3ζ CART (91).             | NCT01373047 (91): 6 patients with CEA+ liver Mets. Hepatic artery injection of CARTs with (3) or without (3) IL2 support. 15 day survival. |
| ROR1       | UC-961: 2nm (92)      | BBζ                  | Preclinical study showed CD3ζ CART was better than FcRγy (67).                     | NCT02706392 (91): (4 TNBC, NSCLC). Decreased tumor burden at some mets, IPR after 2 infusion (94). |
| GD2        | Hu3F8 (95) (humanized murine 3F8 mAb) K_D: 11nm | 28-2BBζ              | CART’s cytotoxicity diminished when repeatedly exposed to the tumor (96). CARkTs were depleted after co-culture with tumor cells (97). | NCT02765243 (75): (10 pediatric neuroblastoma, NB): 0 PR, 4 SD. |
|            | murine 14.2G2a (95) | 28-27ζ               | CART’s cytotoxicity diminished when repeatedly exposed to the tumor (96). CARkTs were depleted after co-culture with tumor cells (97). | NCT00085930: Initial report (99) found EBV-CTL transduced with CAR generated better expansion than CAR-ATC, but a later report (100) showed that CAR-ATC persists for 4 yrs. Clinical outcome: 3 CR (2 sustained for > 4 yrs), IPR, 15 day survival. |

(Continued)
"the ABD affinity and CART’s on-target/off-tumor toxicity"), but also enable them to generate stronger antitumor efficacy. Thus, different from antibody drugs, in the CART development, moderate ABD affinity may be better.

One exception to the "moderate-affinity" role is the mesothelin targeting M5 CART. The M5 mAb is moderate affinity (K_D: 26.9nM), but the M5 CARTs had no antitumor effects in treating multiple solid tumors ([https://www.med.upenn.edu/cellicon2021/assets/user-content/documents/tanyi.pdf](https://www.med.upenn.edu/cellicon2021/assets/user-content/documents/tanyi.pdf)). Further analysis showed that the K_off of M5-mesothelin is low, thus the dwell time (T_{1/2}) of M5-mesothelin is much longer than that of the moderate affinity mAbs of 3D8 and 14.G2a (Table 2). The T_{1/2} of M5-mesothelin is 613 seconds, while the T_{1/2} of 3D8-mesothelin is 28-BBz NCT01828186 (76) (Piggybac CART) NSCLC: 1/9 PR

### Table 1

| Target | ABD (K_D) | Clinical Responses |
|--------|-----------|--------------------|
| KM8138 (Humanized KM666) | 28C_2 | Preclinical study in vitro killing activity and antitumor effects in mice (107) |
| EGFR E10 (GenBank No: KQ063301.1) | BB_2 | Preclinical study in vitro killing activity and antitumor effects in mice (107) |
| EGFRvIII | BB_2 | Preclinical study (115), CAR delivered by Piggybac vector |
| C2173(humanized 3C10): K_D 101nM (original 3C10: 101mM) | BB_2 | Some antitumor effects in human glioma xenografts in NSG mice (116) |
| C139 (119) K_D: 290nM (Table 30.1 in US patent 7,628,966.B2) | 28-BBz | C139 CARTs kill target cells and produce cytokines (119). In mice, the CART generated antitumor effects in intracerebral glioma (120). |
| HER2 | FRP5 mAb, KD: 6.5mM (122, 123) | Osteosarcoma model (124), Medulloblastoma model (125) |
| 4D5 (humanized is Herceptin): 5mM | 28-BBz | Preclinical study (44) showed better persistence of 28BBz than 28C_2 CARTs |
| CA IX | G250: 2.2mM (table 1 in reference (131)) | CD3_3 |
| PMSA | 3DB: 22.5mM (133) | CD3_3 |
| J591, K_D: 1.83mM (136, 137) | 28C_2 | In vitro study showed killing (134) |
| BB_2 | Co-expressing dominant negative TGFRII mouse models |
| Claudin 18.2 | hu8E5-2I scFv (142) | 28C_2 CARTs show slightly better cytotoxicity in vitro (142): CARTs built with hu8E5-2I CART showed good antitumor effects in treating xenografts |

**Clinical Responses**

NCT03373097: 5/11 PR+CR out of 11 patients, correlating to persistence of CARTs and low PMM-MDSC in blood (104).

NCT0419841 (105): ¾ showed PR in treating DIPG

NCT02761915 (108, 109): 0/12. No response in all 12 relapsed/refractory neuroblastoma patients, but some response in soft tissue and bone marrow disease for 3 patients.

NCT01869166 (In-CART): 7/11 PR (2-8 mos) (112) 2. Biliary Tract Ca: 1/17 CR (113) Pancreatic Ca: 4/16 PR (2-4mos) (114)

NCT01454596 (121): 0 out of 18 glioblastoma patients had responses (0/18 PR).

NCT00902044: SD 4/17 (sarcoma patients) (126), 1/10 CR (metastatic sarcoma) (127, 128).

NCT08902044: SD 4/17 (sarcoma patients) (126), 1/10 CR (metastatic sarcoma) (117, 128)

NCT01109095: 0/17 PR, SD 3/17 (glioblastoma) (129)

NCT090924287: 0/1Death of the patient related to off-target toxicity (130)

**PR, Partial response; CR, Complete response; SD, Stable disease; PD, Progression disease; PSA, Prostate specific antigen; PMMA, Prostate-specific membrane antigen; B, Lentiviral vector; NSCLC, Non small cell lung carcinoma; PCa, Pancreatic cancer; PDAC, Pancreatic ductal adenocarcinoma; GCa, Gastric cancer; GI Ca, Gastrointestinal cancer; mos, Months; MPM, Malignant pleural mesothelioma; NB, Neuroblastoma, OVAR, Ovary cancer.
verified whether the higher $K_{d}$ (thus shorter dwell time) of ABD-antigen complex is indeed important in deciding the efficacy of solid tumor CARTs.

The effects of ABD affinity on the biology and function of CARTs

Different from conventional small molecule and antibody medicines, CARTs are living drugs, i.e., they multiply and expand, and must be alive and activated to be functional. In general, soluble antigen does not induce CART activation and expansion (152), suggesting that oligomerization of CARs on cell surface is important in CART activation although the immunological synapse of CAR is nonclassical and not well defined (153, 154). The engaging avidity between CART and target cell is determined by the ABD affinity, CAR level, and antigen level (155). In this article, we focus on the effect of ABD affinity on CART’s biology and function, such as activation, function, persistence, and antitumor effects, especially in solid tumors, where the engagement between CART and tumor is multi-dimensional, persistent, and intense.
The ABD affinity needs to reach a threshold for CARTs to have a productive engagement with tumor cells, which generates sufficient signaling to activate and expand CARTs and to kill tumor cells. An increase of ABD affinity within a range may enhance CART activation and function (156). However, ABD affinity beyond a certain level will not further enhance CART function (157), but may be harmful. The ABD affinity can affect CART biology and function in the following ways. 1) When the CART-tumor cell engagement is too strong, the CARTs are difficult to dissociate from the killed or dying tumor cells. The occupied CARTs will be unable to re-engage with different target cells and induce serial killing of tumor cells. A strong CART-tumor cell engagement may allow CAR to nibble a piece of the target cell membrane and the associated antigen (158). This process of trogocytosis will tag the CARTs to become the target and victim of other CARTs (fratricide). Trogocytosis also cause tumor escape due to antigen loss on target cells. For example, the CD19 CARTs based on high-affinity FMC63 mAb had higher trogocytosis and fratricide than the CD19 CARTs from a lower-affinity CAT mAb (158, 159). 3) The strong and persistent engagement of high avidity CARTs with tumor cells may drive CARTs to exhaustion and activation-induced cell death (AICD). We recently found that, compared to the CARTs with high-affinity GC33 mAb (K_D=1.38nM), our GPC3-specific CARTs derived from a novel moderate-affinity 8F8 mAb (K_D=23nM) are less exhausted and less apoptotic inside tumor lesions (79). 4) The ABD affinity affects the polyfunctionality of CARTs. Using CyTOF technology, Michelozzi et al. compared the FMC63 (high-affinity) and CAT (moderate-affinity) CD19 CARTs and found that, after engaging with CD19+ leukemia cells, the CAT CD19 CARTs contained significantly more multifunctional T cells than the FMC63-derived CARTs (160). This suggests proper moderate-affinity ABD may allow CARTs to preserve their polyfunctionality, which is important for antitumor effect (161).

### The ABD affinity on CART’s antigen sensitivity and on-target/off-tumor toxicity

Moderate-affinity ABD may be good for CARTs to maintain function. However, lowering ABD affinity may reduce CART’s sensitivity of detecting the antigen(low) tumor cells. For example, compared to the EGFR CARTs derived from the high-affinity Cetuximab (K_D=1.8nM), the CARTs derived from the low-affinity Nimotuzumab (K_D=21nM) could distinguish antigen(low) vs. antigen(high) target cells, but showed less control of antigen(high) human tumor xenografts in mouse (43). Fortunately, affinity is not the only factor that affect antigen sensitivity. The affinity of TCR is much lower than CARs, but is able to detect single molecule of pMHC complex (165), while CARTs need 200 molecules of antigen for activation (166). Even with the same affinity, the sensitivity of TCR 10-100 times higher than CAR (167), suggesting that the signaling apparatus of TCR complex also play an important role in deciding the antigen sensitivity. Along this line, it was reported that manipulation of CD domain and ITAM enhanced the antigen sensitivity of CARTs (168). Thus, it is possible to lower the ABD affinity while maintaining the antigen sensitivity. A positive side effect of losing antigen sensitivity is the reduction of on-target/off-tumor toxicity because most tumor antigens are not unique to tumor cells, but rather are the shared antigens that can benefit tumor selectivity (164) (also see below).

### Table 2: Relationship of K<sub>on</sub> and T<sub>1/2</sub> of ABDs and clinical efficacy of CARTs.

| Target       | mAbs          | K<sub>on</sub> (nM) | K<sub>off</sub> (nM) | K_D (nM) | T<sub>1/2</sub> (second) | Efficacy (PR+CR)% (Responder/total patients) |
|--------------|---------------|---------------------|----------------------|----------|-------------------------|---------------------------------------------|
| PMSA         | 3D8 (133)     | 6.04e+6             | 1.36e-1              | 22.52    | 5                       | 40 (2/5)                                   |
|              | J591 (137)    | 1.02e+5             | 1.23e-4              | 1.21     | 5,634                   | 0 (0/20)                                   |
|              | Gm2 (95)      | 1.5e-5              | 1.12e-2              | 74.67    | 62                      | 30 (10/33)                                 |
|              | HuSF8 (95)    | 9.19e+4             | 1.03e-3              | 10.4     | 673                     | 0 (0/10)                                   |
|              | KM8138 (106)  | 1.14e+4             | 1.7e-3               | 149      | 407                     | 0 (0/12)                                   |
| Mesothelin   | M5*           | 4.2e+4              | 1.1e-3               | 26.9     | 613                     | 0 (0/17)                                   |
|              | SS1*          | 5.55e+6             | 5.60e-4              | 0.1      | 1,237                   | 2.7 (1/27)                                 |

*The K<sub>on</sub>, K<sub>off</sub>, K_D, and T<sub>1/2</sub> of mAbs M5 and SS1 were from Patent: WO2015090230A1. The clinical trial reference was the same as Table 1.
self-antigens that are also present in normal cells albeit at lower levels. In fact, the initial studies of utilizing low-affinity ABDs in CART development were intended to distinguish the antigen\textsuperscript{high} tumors from antigen\textsuperscript{low} normal cells to avoid off-tumor toxicity (43, 47, 169–173). Some recent preclinical \textit{in vivo} studies further illustrated that the CARTs derived from low-affinity ABDs were indeed less toxic. Using the transgenic mice that express different levels of HER2 antigen, Castellarin et al. showed that CARTs built with low-affinity HER2 mAbs had less \textit{in vivo} toxicity, but also generated better antitumor effects compared to high-affinity CARTs because they are less likely to be trapped in the antigen\textsuperscript{low} normal tissues (173). In another latest report, Giardino et al. developed a pair of new GPC3-specific mAbs, GPC3-1 (K\textsubscript{D}=73nM) and GPC3-2 (K\textsubscript{D}=11nM), which could bind both human GPC3 and mouse GPC3. They demonstrated that GPC3-1 and GPC3-2 CARTs generated similar antitumor effects in mouse models. However, the low-affinity GPC3-1 CARTs demonstrated much lower toxicity in mice than the GPC3-2 CARTs (174). Thus, it is important to find an optimal moderate-affinity ABD to construct CARTs that maximize its effects on target tumor cells, while minimizing off-tumor toxicity. Different targets may need different optimal affinities. For example, in our meta-analysis of clinical trial data, we found that the CARTs built with the K\textsubscript{D} of ABDs between 20–100nM generated effective CARTs (Figure 1). However, in the ICAM-1 targeted CARTs, the K\textsubscript{D} of LFA binding ICAM-1 is at micromolar (K\textsubscript{D}=20μM) to generate the most effective antitumor effects with reduced toxicity in preclinical tumor model (171).

**The K\textsubscript{D}, Kon, Koff, and T\textsubscript{1/2} of ABDs and their effects on CARTs**

The affinity can be measured by SPR and ELISA. ELISA measures the EC\textsubscript{50} (the concentration required to obtain a 50% maximum protein-ligand binding), whereas SPR measures the association (K\textsubscript{on}) and dissociation rate (K\textsubscript{off}) for the calculation of equilibrium dissociation constant K\textsubscript{D}(equal K\textsubscript{off}/K\textsubscript{on}), a more widely used parameter for binding affinity. Individual K\textsubscript{on} (Number/M*S) and K\textsubscript{off} (Number/S) value can represent ligand binding kinetics much better in a time-dependent manner (175): A higher number of K\textsubscript{on} means faster ligand binding whereas a higher K\textsubscript{off} indicates that the complex dissociates faster. As both K\textsubscript{on} and K\textsubscript{off} determined the ligand binding affinity (K\textsubscript{D}), 2 ligands with same or similar affinity (K\textsubscript{D}) might have different K\textsubscript{on} and K\textsubscript{off} value changing in the same direction (either increase or decrease), and thus show completely different binding kinetics. In this case, the ABD binding kinetics (K\textsubscript{on} and K\textsubscript{off}) may be even more important than K\textsubscript{D} or EC\textsubscript{50} in determining the CART’s efficacy. Another important parameter in comparing the ligand binding is the half-life of the complex, T\textsubscript{1/2}, which relates to K\textsubscript{off} by the formula T\textsubscript{1/2}=Ln2 (0.693)/K\textsubscript{off} (175). Thus, T\textsubscript{1/2} indicates the stability or dwell time of the complex.

The effect of K\textsubscript{on} and K\textsubscript{off} (or related dwell time (T\textsubscript{1/2})) of TCR-pMHC complex on T cell activation has been well-studied (165, 176, 177). If the TCR and pMHC have a fast on-rate (higher K\textsubscript{on}), the TCR-pMHC complex with a higher K\textsubscript{off} (a short dwell time) can be highly stimulatory (165) because the pMHCs can bind and rebind the same TCR (178) or multiple TCRs (175) several times, creating an effective longer dwell time than a single TCR-pMHC encounter (165). This may contribute to the high sensitivity of TCR that can detect one pMHC complex on target cells (178). On the other hand, if the K\textsubscript{on} is low, the dissociated ligand will not easily rebind a TCR. Under such circumstances, the outcome of the TCR-pMHC engagement will likely depend on the dwell time of the TCR-pMHC complex. In other words, if the K\textsubscript{on} is low, the complex needs to be stable (lower K\textsubscript{off}) to generate sufficient signaling for activation.

A similar principle may apply to the CART-tumor engagement. The affinity (K\textsubscript{D}) of ABD-antigen engagement is in the range of pM-nM (179), which is ~3 logs lower than that of TCR-pMHC (176). Thus, the dwell time of antibody-antigen complex is in the range of hours or even day (180), much longer than that of TCR-pMHC (normally in seconds) (176). Such long stable engagement may result in persistent activation of CARTs that can drive them into exhaustion and AICD. A long engagement may not be necessary, but rather be harmful in solid tumors. Such argument is in agreement with the fact that moderate-affinity 14.G2a-based GD2 CARTs generated much better clinical efficacy than the high-affinity 3F8-based CARTs (Supplemental Table 1 and Figure 2B). The anti-GD2 mAb 14.G2a has similar K\textsubscript{on} as 3F8, but has 10x higher K\textsubscript{off} (95). Thus, the engagement dwell time of 14.G2a CARTs is 10x shorter than the 3F8 CARTs. This fast-on/fast-off “fly-kiss” mode of engagement by 14.G2a CARTs allows CARTs to have a shorter intermittent disengagement in the solid tumors. Such transient break during “off” time may rejuvenate and preserve CART function (37). Similarly, the moderate-affinity CAT mAb has similar K\textsubscript{on} as high-affinity FMC63, but has much higher K\textsubscript{off} (151), which may contribute to the formation of memory T cells and polyfunctionality of CAT CARTS (160) and durable antitumor effects (151). Thus, the dwell time and kinetics of ABD-target engagement may be more important than affinity (K\textsubscript{D}) in deciding the outcome of CARTs. For example, the M5 mAb has a T\textsubscript{1/2} of 613 seconds (Table 2), which may be the reason why M5 CARTs did not generate therapeutic effects in clinical trials. In contrast, the 3D8 and 14.G2a CARTs that generated impressive clinical responses have the ABDs with higher K\textsubscript{off}. Their T\textsubscript{1/2} is 5 and 62 seconds, respectively (Table 2). A high K\textsubscript{on} allow ABD bind target quickly, a higher K\textsubscript{off} may benefit for CART survival because of faster dissociation. This bind/off/rebind “fly-kiss” style of engagement may be optimal for CART to exert their function while avoiding being-driven...
into exhaustion, especially in solid tumor mass. Along this line of analysis, measuring the $K_{on}$ and $K_{off}$ of the Claudin 18.2 mAb Hu8E5-2I should help verify if the fast-on/fast-off “fly-kiss” intermittent engagement mode indeed enhances CART’s antitumor efficacy. Similarly, it will be very interesting to know whether the recently developed low-affinity GPC3 specific mAb GPC3-1 (174) that has a high $K_{off}$ will generate clinical efficacy in future trials.

A fast-on/fast-off “fly-kiss” mode of engagement may be required for effective solid tumor CARTs

A fundamental anatomical difference between blood cancers and solid tumors is the tumor mass, in which CART-tumor cell engagement is intense and persistent. In hematological cancers, tumor cells are in the blood and do not aggregate together to form tumor mass, and thus CARTs have immediate access to target tumor cells after infusion. Importantly, the engagement of CART-tumor cells in the blood is individualized in 1-on-1 mode and intermittent (Figure 3). CART can “enjoy a temporary break” after each killing before finding the next target. On the other hand, in solid tumors, CARTs first need to migrate into a tumor mass. Once CARTs infiltrate a tumor lesion, they are surrounded by tumor cells from every possible direction. Thus, the engagement of CART-tumor cells in a solid tumor is multi-dimensional 1-on-N or N-on-N mode and persistent (Figure 3). There is no intermittent break for the CARTs unless they can spontaneously be disengaged due to higher $K_{off}$ or until the tumor mass is eliminated. Furthermore, a solid tumor has a complex extracellular matrix stroma that further restrains CART movement and aggravates the antigen assault on them. Such constant and intense engagement with antigens will drive CARTs exhaustion or AICD. Thus, due to different mode and intensity of CART-tumor engagement, the ABD affinity requirement for solid tumor CARTs is likely different from the CD19 and other blood cancer CARTs. CARTs with high-affinity ABDs will be more prone to exhaustion and AICD in solid tumors than in blood cancers.

Thus, we propose that moderate-affinity ABD and fast-on/fast-off engaging kinetics are especially necessary for solid tumor CARTs to be effective. A higher $K_{on}$ of the ABD will make sure that CARTs will bind to target cell fast even when the antigen level is low; a higher $K_{off}$ will allow CARTs to disengage even if they are surrounded by tumor cells in a solid tumor mass. Such a fast-on/fast-off “fly-kiss” style of engagement allows CARTs to kill tumor cells without being-driven into exhaustion and AICD. In addition,
currently, we have little knowledge on how the different mode and kinetics of CART-tumor engagement may affect the epigenetics, gene expression, metabolism, and thus the fitness of CARTs. Further investigation into these mechanisms will likely help design more effective CARTs for solid tumors. We think that solid tumor CART development should focus more on the ABD affinity and the engaging kinetics of CART and tumor cells. Recently, strategies have been discussed to tune the ABD affinity for better and effective CART development (181) even though there is no obvious approach to select ABDs with particular $K_{on}$ and $K_{off}$ yet. This intentional and rational design of CARs with tuning ABD affinity and binding dynamics in mind will likely generate more effective solid tumor CARTs that can potentially match the remarkable success observed in hematological cancers.

**Author contributions**

RM and YH analyzed the clinical responses of 38 clinical trials and searched the ABD affinity in the CARTs. WK drew the CART-tumor engagement mode presented in Figure 3. All three authors wrote the manuscript together and approved the submitted version.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1032403/full#supplementary-material

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resistant tumor cells, especially those with low levels of EGFR expression, which can limit the efficacy of EGFR-targeted therapies. Therefore, the development of novel therapeutic approaches that can effectively target these tumor cells becomes crucial.

In this context, the use of engineered T cells, particularly those engineered with chimeric antigen receptors (CARs), has emerged as a promising strategy. CAR-T cells are designed to express a receptor that can specifically recognize and target antigen-presenting cells, thereby inducing an immune response against tumor cells. The CARs are composed of variable antigen-binding domains (e.g., single-chain variable fragment, scFv) and transmembrane and intracellular signaling domains that can activate the T cell. This allows CAR-T cells to target and destroy tumor cells without the need for pre-existing antitumor immunity.

The rationale behind the development of humanized anti-EGFR variant 2 (v2) CAR T cells involves overcoming the limitations of standard EGFR-targeted therapies. Standard EGFR inhibitors, such as erlotinib and gefitinib, may have reduced efficacy due to the development of resistance mechanisms in tumor cells. This can lead to suboptimal antitumor activity and sometimes even tumor progression. On the other hand, CAR-T cell therapy offers a high degree of specificity and can potentially overcome these resistance mechanisms by directly targeting the EGFR-expressing tumor cells. Moreover, CAR-T cells can be designed to have a robust immune effector function, allowing for potent antitumor activity.

A recent study by Mao et al. (2020) demonstrated the potential of humanized anti-EGFR v2 CAR T cells in patients with advanced neuroblastoma. The study showed that these CAR T cells could achieve high in vivo persistence and kill EGFR-expressing tumor cells, indicating their potential for clinical application in patients with neuroblastoma.

In conclusion, the development of humanized anti-EGFR v2 CAR T cells represents a promising approach for the treatment of neuroblastoma and other cancers with EGFR expression. Further preclinical and clinical studies are needed to fully evaluate the safety and efficacy of these CAR T cells, as well as to determine the optimal design and implementation strategies for their use in clinical settings.
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