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Prosthetic Antigen Receptors

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Supporting Information

ABSTRACT: Chimeric antigen receptors (CARs) have shown great promise for the immunological treatment of cancer. Nevertheless, the need to genetically engineer a patient's T-cells has presented significant production and safety challenges. To address these issues, we have demonstrated that chemically self-assembled nanorings (CSANs) displaying single chain antibodies can bind to both the CD3 ε subunit of the T-cell-receptor/CD3 complex and the CD22 antigen on malignant B cells such as B-leukemias or lymphomas. We demonstrate that the multivalent and bispecific format allows the antiCD3/antiCD22 CSANs to stably bind to T-cell surfaces for greater than 4 days, while being easily disassembled on the cell membrane by treatment with the nontoxic FDA approved drug, trimethoprim. In the presence of CD22+ Raji cells, T-cells modified with antiCD3/antiCD22 CSANs were shown to selectively up-regulate the production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) and to initiate cytotoxicity. Taken together, our results demonstrate that antiCD3/antiCD22 bispecific CSANs offer a potential alternative to CARs, as prosthetic antigen receptors.

The potential to harness T cells for the elimination of tumor cells is one of the most compelling concepts in anticancer immunotherapy development. Nevertheless, tumors have developed a number of mechanisms to evade T-cell immunosurveillance.[1,2]

One approach to overcome the impotence of the immune system regarding cancer cells is to re-engineer T-cells with targeting molecules that are difficult for cancer cells to defend against.[3−6] For example, T-cells engineered to express an antiCD19 CARs have been shown to clinically eradicate B-cell leukemias.[7,8] Nevertheless, the preparation of the engineered cells is time-consuming, costly, and of variable efficiency, while the potential long-term risks of gene transfer methods on the expression of oncogenes and tumor-suppressor genes and of long-lived engineered T-cells to target normal tissues are major concerns.[9,10] Consequently, an attractive alternative to CARs would be the development of prosthetic antigen receptors (PARs) that could stably (days) and reversibly bind to the CD3 ε subunit of the T-cell-receptor/CD3 complex, while allowing targeting to a cancer cell surface receptor (Figure 1).

We have previously demonstrated that in the presence of the chemical dimerizer bisMTX, DHFR-DHFR (DHFR^2), and DHFR-DHFR-antiCD3 (DHFR^2-antiCD3) fusion proteins can spontaneously assemble into a range of chemically self-assembled nanorings (CSANs) whose size varies depending on the length and composition of the linker peptide between the DHFRs.[11−14] If the linker is a single glycine, we observed that rings containing 7−10 DHFR^2 fusion proteins with an average ring size composed of 8 monomers.[11,13] Therefore, the mixing of an equal proportion of two DHFR^2 linked by a single glycine and fused to two different antibodies should theoretically produce a stochastic mixture of CSANs that are approximately 99% bispecific (Figure 1). Consequently, we prepared fusion proteins of DHFR^2 linked by a single glycine either tethered to an antiCD3 scFv (DHFR^2-antiCD3, 1DDantiCD3) or an scFv targeting CD22 (DHFR^2-anti CD22, 1DDantiCD22), an antigen widely expressed on B-leukemias or lymphomas.

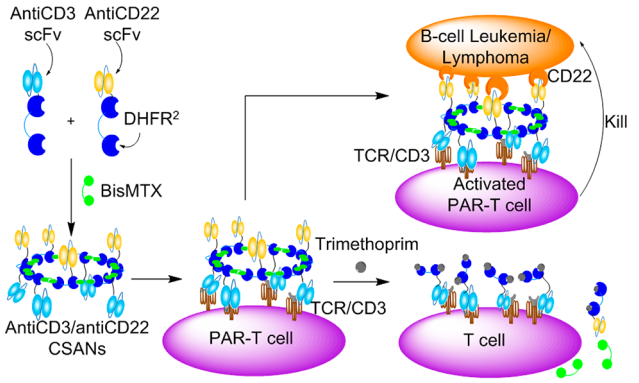

Figure 1. Prosthetic antigen receptor (PAR) T-cells.

The purified antibody fusion proteins, 1DDantiCD3 and 1DDantiCD22, were incubated for 1 h with bisMTX (1:1:2.2 equiv), and the assembled proteins were analyzed by size-exclusion chromatography (SEC; Figure S1A). Octavalent bispecific antiCD3/antiCD22 CSANs were eluted in a broad peak centered at 18.5 min with almost 100% oligomerization of 1DDantiCD3 and 1DDantiCD22 monomers eluted at 28.5 min. The bispecific CSANs have a similar retention time to the antiCD3 CSANs which were previously analyzed by SEC, indicating that they have similar hydrodynamic radius. Again, the major nanoring species formed were octamers.[13] The hydrodynamic diameter of purified bispecific antiCD3/antiCD22 CSANs was determined by dynamic light scattering which is 16.06 ± 0.01 nm (Figure S1B).

To determine quantitatively the binding affinity of the monovalent or octavalent DHFR^{2}antiCD22 to the CD22 positive B lymphoma Raji cells, the dissociation constant was

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evaluated by a flow cytometric competitive binding assay. Binding of nonlabeled antibodies to Raji cells was competed with a subsaturated amount of FITC-labeled mAb antiCD22 RFB-4, followed by quantitating the fluorescence intensities of cell bound FITC-labeled RFB-4 by flow cytometry (Figure S2). The lower binding affinity of the monomer 1DDantiCD22 (K_d = 104.81 ± 0.5 nM) compared with the parental monoclonal antibody RFB-4 (K_d = 1.05 ± 0.4 nM) is likely due to the transition from mAb to the single chain format in tandem with $DHFR^2$. Compared with the monomer 1DDantiCD22 (K_d = 104.81 ± 0.5 nM), the multivalent antiCD22 CSANs were found to have a lower K_d value of 68.77 ± 0.5 nM.

Since antiCD22 scFvs and immunotoxins undergo receptor-mediated endocytosis,[15] we tested the ability of antiCD22 CSANs prepared with fluorescein-labeled bisMTX (FITC-bisMTX)[14] to be internalized by CD22+ Daudi cells by confocal fluorescence microscopy. Consistent with receptor-dependent endocytosis, green puctates could easily be observed by Daudi cells incubated at 37 °C with the fluorescent-labeled antiCD22 CSANs, indicating internalization, while membrane bound green fluorescence was observed for incubations at 4 °C (Figure S3).

To confirm that the bispecific CSANs maintained the binding specificity and selectivity of the monospecific CSANs, FACS analysis was carried out with PBMCs (CD3+) and Raji cells (CD22+). Both antiCD3 and antiCD3/antiCD22 CSANs were found to bind to a similar extent to PBMCs, while no significant binding of antiCD22 CSANs was observed (Figure 2A).

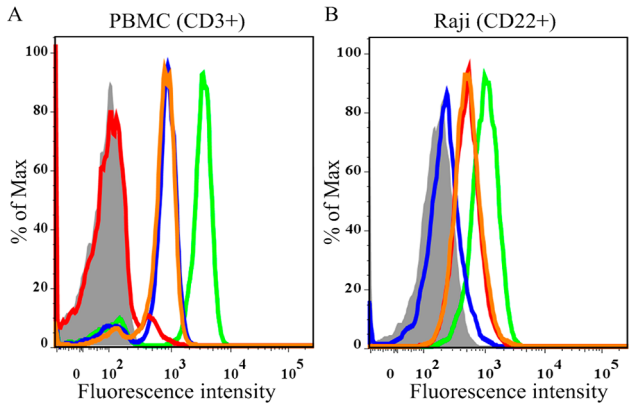

Figure 2. Flow cytometry-based binding assay of monospecific antiCD3 CSANs (blue), antiCD22 CSANs (red), and bispecific CSANs (orange) to PBMCs (A) and CD22 positive Raji cells (B). Positive controls: FITC-antiCD3 mAb UCHT-1 (green) (A) and FITC-antiCD22 mAb RFB4 (green) (B).

Similarly, both antiCD22 and antiCD3/antiCD22 CSANs were found to bind to a similar extent to Raji cells, while no significant binding of antiCD3 CSANs could be detected (Figure 2B).

Unlike monovalent bispecific antibodies, multivalent bispecific CSANs might be expected to have high avidity and thus exhibit stable binding to cells. Previously, we have demonstrated that antiCD3 CSANs bind to the surfaces of PBMCs and are not significantly internalized.[13] Having demonstrated that both monospecific and bispecific CSANs were stable for >72 h at 37 °C in PBS (data not shown), we investigated the stability of the bispecific CSANs on PBMCs in complete RPMI at 37 °C. To assess the binding of the CSANs to T-cells, with a flow cytometric competitive binding assay, we determined the ability of antiCD3/antiCD22 CSANs to compete with the FITC-labeled parental antibody UCHT-1 for binding to CD3 receptors on PBMCs. After incubating the PBMCs with antiCD3/antiCD22 CSANs and washing the cells, we found that >85% of the potential binding of UCHT-1 could be blocked over 3 days (Figure 3A). A small loss in the ability of the antiCD3/antiCD22

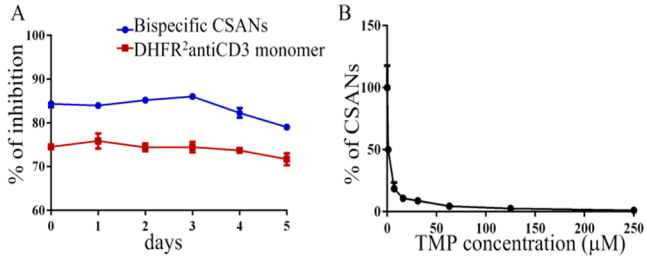

Figure 3. (A) Time course study of the stability of bispecific CSANs (blue) and 1DDantiCD3 monomer (red) on PBMC cells. (B) The disassembly of bispecific CSANs by trimethoprim.

CSANs to block UCHT-1 binding could be observed from day four to five, which may indicate the effects of cell division and thus dilution of the bispecific CSANs. In addition, the antiCD3/antiCD22 CSANs were found to have a significantly greater ability to block UCHT-1 binding than the $DHFR^2$antiCD3 monomer. Therefore, the bispecific CSANs bind cell surface CD3 and remain largely intact on the surface of T-cells.

One of the distinct advantages of our bispecific CSANs over other bispecific antibodies is the potential to initiate disassembly by the addition of the competitive inhibitor and FDA approved drug, trimethoprim.[12−14] Previously, we have demonstrated that the disassembly of antiCD3 CSANs prepared with FITC-bisMTX by incubation with trimethoprim by monitoring the loss of FITC fluorescence.[14] Consequently, we investigated the disassembly of antiCD3/antiCD22 CSANs prepared with FITC-bisMTX bound to PBMCs in the presence of variable concentrations of trimethoprim for 1 h at 37 °C (Figure. 3B). By monitoring the decrease of mean fluorescence intensity of FITC on PBMCs using flow cytometry, the IC_{50} of trimethoprim was determined to be 1.18 ± 1.1 μM, which is well below the plasma concentration of 5 μM typically found after a clinical oral dose of trimethoprim.[16]

To determine the ability of antiCD3/antiCD22 CSANs to activate T-cells, we investigated their ability to up-regulate the early activation marker, CD69, and the late activation marker, CD25 (IL-2R). As can be seen in Figure 4A, incubation of PBMCs with either media or $DHFR^2$ (1DD) octamer resulted in insignificant expression of CD69 by CD4+ or CD8+ cells, regardless of the presence of the target cells. In contrast, both antiCD3 CSANs and antiCD3/antiCD22 CSANs enhance CD69 expression to similar levels with or without incubation with Raji cells. Similar to the results observed for CD69, appreciable increases in the expression of CD25 (Figure 4B) were only observed in the presence of either antiCD3 CSANs or antiCD3/antiCD22 CSANs. However, a much greater increase (5- to 7-fold) in CD25 expression was observed in the presence of Raji cells when compared to incubations in their absence. When a set of similar experiments was carried out with UCHT-1 $F(ab')_2$, compared to the antiCD3 CSANs and antiCD3/antiCD22 CSANs, only a modest level of CD69 and CD25 expression with or without Raji cells was observed. Thus, the multivalent CSANs are able to facilitate the preactivation of CD4+ and CD8+ T cells to greater extent than bivalent antiCD3 UCHT-1 $F(ab')_2$.

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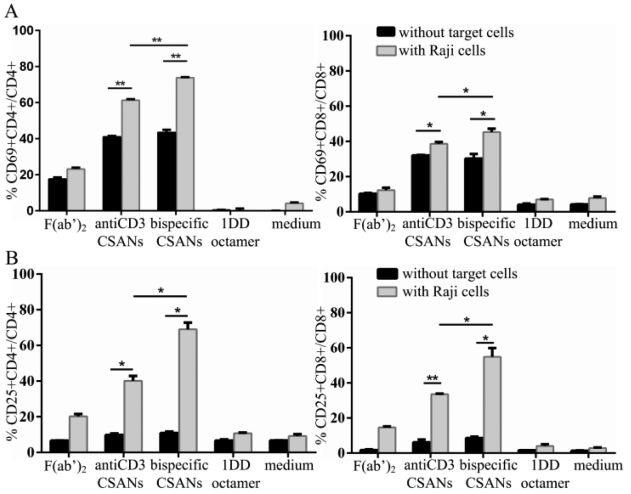

Figure 4. Expression of CD 25 and CD 69 on PBMCs. Unstimulated PBMCs were co-cultured with different treatments in the presence or absence of CD22+ Raji cells for 24 h. The expression of the activation markers CD69 (A) and CD25 (B) on CD8+ T cells or CD4+ T cells was analyzed by flow cytometry. Data were obtained from one donor and are representative of data from three donors. * $P < 0.05$ or ** $P < 0.005$ with respect to the absence of Raji cells or with antiCD3 CSANs treatment.

To further investigate the specificity of the activation of T-cells by antiCD3 CSANs and antiCD3/antiCD22 CSANs, we determined the ability of purified CD4+ and CD8+ T cells to undergo activation in the presence of the Raji (CD22+, MHCI+) and K562 (CD22−, MHCI−) cell lines (Figure 5). Consistent

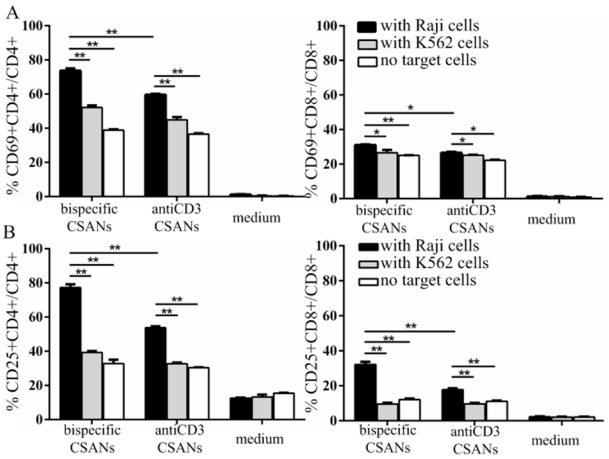

Figure 5. Expression of CD25 and CD69 on redirected CD4+ and CD8+ T cells. Unstimulated CD4+ or CD8+ T cells were co-cultured with different treatments in the presence or absence of CD22 positive Raji cells or CD22 negative K562 cells for 24 h. The expression of the activation markers CD69 and CD25 on CD8+ T cells (A) or CD4+ T cells (B) was analyzed by flow cytometry. * $P < 0.05$ or ** $P < 0.005$ with respect to the absence of Raji cells or with antiCD3 CSANs treatment. Student t test to compare mean ± SD of two groups was used.

with our results with PBMCs, activation of the CD4+ and CD8+ cells was observed to a similar extent after treatment with either antiCD3 CSANs or antiCD3/antiCD22 CSANs. Only a modest increase in the expression of CD69 by CD8+ T cells in the presence of Raji cells was observed. In contrast, no significant difference was detected in the presence of K562 cells relative to no cells. Again, similar to the results with PBMCs a significant increase was observed in CD25 expression for CD4+ and CD8+ T cells in the presence of Raji cells relative to K562 cells and no cells.

The presence of cytotoxic granules and evidence of degranulation are a hallmark of cytotoxic CD8+ T cells activation. The level of degranulation can be assessed by determining the amount of the degranulation marker CD107a on the surface of CD8+ cells. Similar to our findings for CD69 and CD25 (Figure S4), treatment of CD8+ cells with either antiCD3 CSANs or antiCD3/antiCD22 CSANs resulted in increased expression of CD107a, with a modest increase found for the bispecific over antiCD3 CSANs in the presence of Raji cells.

A hallmark of T-cells activation and their interaction with targeted cells is the production of the pro-inflammatory cytokines, IL-2 and IFN-γ. The cell media concentration of IL-2 or IFN- γ was determined by ELISA. As can be seen in Figure 6A, treatment of Raji cells with PBMCs bound to antiCD3

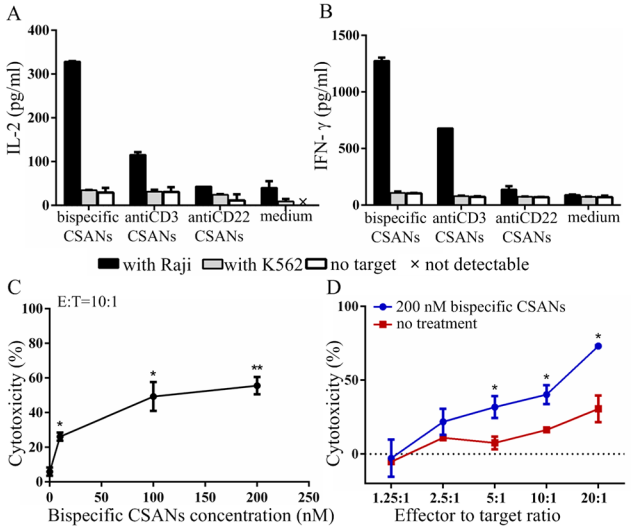

Figure 6. (A) Effects of CSANs on the cytokine production of IL-2 and (B) IFN-γ by PBMCs. Tumor cells Raji or K562 cells and PBMC cells were co-cultured in the presence of different CSANs treatments (E:T = 10:1) . (C) Killing capacity of bispecfic CSANs functionalized T cells. Pre-activated T cells were incubated with variable concentrations of bispecific CSANs at E:T ratio 10:1. (D) Killing capacity of T cells against Raji cells in the presence of 200 nM bispecific CSANs at different E:T ratios.*$P < 0.05$ or ** $P < 0.005$ with respect to the absence of treatment.

CSANs resulted in a 2-fold increase in the amount of secreted IL-2, when compared to treatment with either antiCD22 CSANs or media control, while in the presence of antiCD3/antiCD22 CSANs a >8-fold increase in IL-2 production was observed relative to the same controls. When compared to incubations with K562 (CD22−) cells or no target cells, a >13-fold increase in IL-2 production was observed. Interestingly, no difference in IL-2 production was observed for PBMCs treated with either antiCD22 CSANs or media, whether in the presence of Raji cells or not, indicating that the CSANs do not inherently lead to T-cell activation.

The increase in IL-2 was found to be consistent with an even greater effect found for both antiCD3/antiCD22 CSANs and antiCD3 CSANs on IFN-γ production (Figure 6B). IFN-γ production was only observed when the PBMCs were incubated with antiCD3/antiCD22 CSANs and antiCD3 CSANs in the presence of Raji cells, with a >2-fold increase observed for the

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bispecific over the antiCD3 CSANs. Taken together, these results demonstrate that the bispecific CSANs were able to selectively redirect T-cells to CD22+ Raji cells. The observed enhanced ability of T-cells functionalized with antiCD3 CSANs to release cytokine production in the presence of Raji cells is likely due to their known expression of MHCI. The inability of the antiCD3 CSANs to induce cytokine expression by PBMCs in the presence of K562 cells (CD22−, MHCI−) is consistent with this conclusion.

To further assess the role of MHCI expression by Raji cells on the induction of cytokine release by T cells treated with antiCD3/antiCD22 CSANs, purified CD8+ T cells were co-cultured with Raji cells that were pretreated with either the parental mAb antiCD22 or antiMHCI in the presence of antibody treatment. As can be seen in Figure S5, treatment with antiCD22 mAb reduced the level of IFN-γ production to the same level observed for treatment with antiCD3 CSANs. Treatment with antiMHCI further reduced the level of IFN-γ by CD8+ T cells incubated with antiCD3/antiCD22 CSANs by 50% and for CD8+ T cells treated with antiCD3 CSANs by 60%. Further studies demonstrated that the inclusion of excess amounts of the $DHFR^2$ octamer (Figure S6), which does not contain either of the antiCD22 or antiCD3 scFvs, did not affect the production of IFN-γ by PBMCs functionalized with antiCD3 CSANs in the presence of Raji cells. Thus, consistent with our prior results, PBMCs treated with antiCD3 CSANs appear able to induce IFN-γ production in the presence of Raji cells because of their ability to engage MHCI on Raji cells, thus augmenting the effect observed for the bispecific CSANs.

Given the effects of the antiCD3/antiCD22 CSANs on cytokine production, we determined the ability of activated PBMCs to target and carry out the lysis of Raji cells. We first determined the concentration dependence of the bispecific CSANs on cell lysis and found that maximal killing was observed at a concentration of antiCD3/antiCD22 CSANs of 100−200 nM (Figure 6C). Choosing a concentration of 200 nM (Figure 6D), we then varied the effector/tumor cell ratios from 1.25:1 to 20:1 and measured the degree of cell lysis after 24 h. In each case the presence of the bispecific CSANs enhanced cell killing at least 2-fold greater than activated PBMCs alone. These results are consistent with the enhanced level of cytokine production induced by antiCD3/antiCD22 CSANs, relative to antiCD3 CSANs.

In summary, we have demonstrated that antiCD3 bispecific CSANs can stably bind but not fully activate T-cells. Once engaged with their target, however, cytokine release is rapidly enhanced, and the modified T-cells have an enhanced ability to initiate targeted cytotoxicity. Thus, antiCD3 bispecific CSANs can be viewed as PARs whose presence on the cells can be easily removed by a nontoxic FDA approved drug, trimethoprim. The results of on going studies comparing the ability of PARs to serve as a potential, nongenetic compliment to CARs will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b06166.

Additional experimental methods and Figures S1−S6 (PDF)

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Notes

The authors declare the following competing financial interest(s): Carston Wagner is a founder of Tychon Bioscience, Inc., to whom this technology has been licensed. The other co-authors have no competing conflicts of interest.

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