Novel DNA-based T-Cell Activator Promotes Rapid T-Cell Activation and Expansion

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**Summary:** Autologous chimeric antigen receptor engineered T-cell therapies are beginning to dramatically change the outlook for patients with several hematological malignancies. Yet methods to activate and expand these cells are limited, often pose challenges to automation, and have biological limitations impacting the output of the injectable dose. This study describes the development of a novel, highly flexible, soluble DNA-based T-cell activation and expansion platform which alleviates the limitations of current technologies and provides rapid T-cell activation and expansion.

**Key Words:** T-cell activation, CD3/CD28 activation, soluble activator, DNA hybridization, rolling circle amplification, ssDNA

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Chimeric antigen receptor T (CAR-T) cell therapy is revolutionizing the treatment of hematological malignancies with unprecedented response rates reaching 80%–90% observed in acute lymphocytic leukemia and diffuse large B-cell lymphoma. With recent Food and Drug Administration (FDA) approvals of Kymriah and Yescarta and hundreds of clinical trials underway for various indications, including treatment of solid tumors, demand for these therapies is expected to rise significantly. The production of CAR-T therapies requires the transduction of a chimeric antigen receptor for tumor recognition followed by large scale expansion of autologous T cells to meet the dose requirements for therapeutic response. The transduction of viral vectors is more efficient when the T cells are activated using a combination of activating and costimulating antibodies in solution. The most commonly used platform for immobilized antibodies is bead-based due to the enhanced available surface area. While bead-based approaches require a debeading step postexpansion leading to significant cell loss, the expansion rates with soluble antibodies are much lower requiring longer expansion times. This reduction in expansion rate is related to a lack of significant receptor clustering with untethered antibodies. Soluble anti-CD3 has also been used in conjunction with antigen-presenting cells (APCs) or artificial APCs with impressive results. However, time and cost involved with the inclusion of another engineered cell line in the expansion process and variability in results associated with artificial APCs is not ideal. A more recent approach using colloidal nanoparticles that does not require a separate debeading step provides similar levels of expansion as Dynabeads has also emerged, though a trend towards higher programmed cell death protein-1 receptor expression in cells activated with the nanomatrix has been reported. Thus, an alternate approach that maintains robust expansion while eliminating the need for bead removal or utilizing additional cell lines is highly desirable. This report describes a novel T-cell activation platform [DNA-based T-cell activator (DBTA)] comprised of a linear single-strand DNA (ssDNA) scaffold prepared by rolling circle amplification (RCA) and scaffold-complementary oligo conjugated anti-CD3 and anti-CD28 antibodies.

**MATERIALS AND METHODS**

**DNA Scaffold Preparation**

All deoxyoligonucleotides [oligo(s)] were synthesized by Integrated DNA Technologies (Coralville, IA) and Table 1. Antibodies Used and Their Sources

| Antibody                  | Supplier   | Cat Code  |
|---------------------------|------------|-----------|
| Mouse anti-human CD3-PerCPCy5.5 clone UCHT1 | BD Biosciences | 560835    |
| Mouse anti-human CD4-V500 | BD Biosciences | 560768    |
| Mouse anti-human CD4-PE   | BD Biosciences | 555347    |
| Mouse anti-human CD8-AF488 clone RPA-T8   | BD Biosciences | 557696    |
| Mouse anti-human CD25-PE clone MA251      | BD Biosciences | 555432    |
| Mouse anti-human CD57-APC clone NK-1       | BD Biosciences | 560845    |
| Mouse anti-human CD45RO-PECy7              | BD Biosciences | 560608    |
| Mouse anti-human CD62L-V450 clone DREG-56  | BD Biosciences | 560440    |
| Mouse anti-human CCR7 clone 150503         | R&D Systems | FAB 197P-100 |
| Mouse isotype control IgG2a-PE             | BD Biosciences | 55574    |
FIGURE 1. A schematic of T-cell activation using the DBTA platform. The specific version used in the current study included: a DNA polymer produced via rolling circle amplification (RCA(\text{act})) consisting of repeating 43-base nucleotide segments which feature a 20-base sequence with full complementarity to the antibody (Ab)-oligo conjugates (A), anti-human CD3 monoclonal Abs conjugated to a 20-base DNA oligonucleotide derived from human beta-actin [o20b(+)act] and anti-human CD28 monoclonal Abs also conjugated with o20b(+act) (B), receptor clustering and T-cell activation (C).

FIGURE 2. T-cell activation and expansion with an early version of the DBTA reagent utilizing the anti-CD3 antibody-oligo conjugate with the ssDNA product and unmodified anti-CD28 antibody. A, Expression of activation marker, CD25, 24 hours postactivation (day 1), and day 4. In comparison to the DBTA (version 1) group, control samples with unmodified antibodies, ssRCA alone, unmodified antibodies with unconjugated oligo (free oligo), or ssRCA product and cells only show a very low level of activation. Data represents averages taken from 5 separate experiments and is shown as mean ± SD, 1-way ANOVA. The activation is also significantly lower than the benchmark Dynabeads at both the timepoints tested, t test. B, Fold expansion over 7 days of culture. T-cell expansion with the DBTA reagents was significantly higher than the component control groups but similar to the Dynabeads group, 1-way ANOVA. Data were shown as mean ± SD, 1-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001). Ab indicates antibody; ANOVA, analysis of variance; DBTA, DNA-based T-cell activator; ssRCA, single-strand rolling circle amplification.

FIGURE 3. DBTA reagent with immobilized activator and costimulator. A, Rapid activation as indicated by higher CD25 expression, is observed with the immobilized anti-CD3 and anti-CD28 antibodies in comparison to the immobilized anti-CD3 and unmodified anti-CD28, 24 hours postactivation. B, Fold expansion over 7-day culture. At day 7, fold expansion with the DBTA utilizing the modified anti-CD3 and anti-CD28 antibodies was higher in comparison to the unmodified anti-CD28 antibody but comparable to that with the Dynabeads. Data represent averages from 7 separate experiments and is shown as mean ± SD, t test (**P < 0.01, ***P < 0.001). DBTA indicates DNA-based T-cell activator.
purified by desalting. The ssDNA scaffold was prepared by a modification of the standard double-stranded RCA reaction conditions [supplementary information (SI)].

**Preparation of Oligo-modified Antibody Conjugates**

Antibodies were conjugated to scaffold-complementary oligonucleotides in a 2-step maleimide-thiol coupling strategy (SI). Oligonucleotide loading on each antibody was calculated by the method of Zhou et al<sup>6</sup> using A260 and A280 absorbance measurements.

**T-Cell Activation**

Activation studies were conducted in 6-well tissue culture plastic plates using commercially available Pan T cells (AllCells, Alameda, CA). Cultures were diluted as needed (1:3 or 1:4) to enable a 7-day expansion protocol (SI).

**Viral Transduction**

Clinical CAR-T vector transduction was carried out at day 2 at a multiplicity of infection of 40 using GMP-grade lentiviral particles harboring a chimeric antigen receptor construct specific for CD19 and CD227 (SI).

**Flow Cytometry Studies**

Antibodies used and their sources are listed in Supplementary Materials (Supplemental Digital Content 1, http://links.lww.com/JIT/A559, Table 1). Samples were analyzed using the CytoFLEX flow cytometer (Beckman Coulter, Brea, CA) for T-cell subsets, T-cell differentiation states, and homing and senescence markers.

**Data Analysis**

All data were expressed as the mean ± SD. Statistical comparisons were performed using Student *t* test or 1-way analysis of variance (*P* < 0.05 were considered to be statistically significant).

**RESULTS**

**Characterization of the Platform Components**

DBTA is a DNA hybridization-based platform (Fig. 1) used to cluster antibodies and their target receptors on T cells to initiate cell activation and subsequent clonal expansion. It consists of the following components: (1) a concatenated ssDNA polymer prepared by ligating a linear oligo into a circle (Supplementary Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A559) and using this circle as a template for RCA, and (2) activating and costimulating antibodies conjugated to an oligonucleotide with partial or complete complementarity to the ssDNA. Melting temperature analysis of the ssDNA demonstrated no hyperchromatic shift seen with double-stranded DNA (Supplementary Fig. 2, Supplemental Digital Content 1, http://links.lww.com/JIT/A559) as the hydrogen bonds between the bases are broken. The length of the RCA product was determined to be around 40,000 bases and a gel mobility shift assay was also accomplished that demonstrated hybridization of the oligo conjugated antibody to the ssDNA (data not shown).

**DBTA is a Flexible and Tunable Activation Reagent Platform**

We compared the T-cell activation and expansion potential of the novel DBTA reagent against Dynabeads CD3/CD28 CTS (Invitrogen, Carlsbad, CA) utilizing antibodies to the CD3 subunit of the T-cell receptor complex and the CD28 costimulatory molecule. Initial studies were carried out with an early version of the DBTA reagent which employed the anti-CD3 antibody-oligo conjugate immobilized on the ssDNA and an unmodified anti-CD28 antibody. Early 24 hours activation

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**FIGURE 4.** Degree of oligo modification and its effects on T-cell activation. A, Percentage of CD25<sup>+</sup> cells in response to changes in oligo loading on the anti-CD28 costimulator at constant oligo loading of anti-CD3 activator. The expression appears to maximize at an oligo/Ab ratio of 3 and remain unaffected at least to 4.5. B, Percentage of CD25<sup>+</sup> cells in response to changes in oligo loading on the anti-CD3 activator while maintaining constant loading of the costimulator. Expression continues to increase till an oligo/Ab ratio of ~3 (2.8). High loading, > 4, significantly affects antibody binding (from > 95% to ~60% CD25<sup>+</sup> cells) and hence was not tested for activation. Intermediate loadings were not tested. Data points in (A) and (B) represent averages of duplicate wells in a single experiment. C, CD25 expression, 24 hours postactivation. Using antibody conjugates having O/P ratios of 2.8 or higher, the initial 24-hour expression of CD25 postactivation was found to be ≥ 90%. The data represents averages taken from 8 separate experiments and is shown as mean ± SD, *t* test, **P* < 0.01. Ab indicates antibody; DBTA, DNA-based T-cell activator; O/P, oligo:antibody (protein).
kinetics (measured by CD25 expression) were significantly slower with the unmodified anti-CD28 in comparison to Dynabeads (Fig. 2A), with similar fold expansion by day 7 (Fig. 2B). When both the antibodies were modified with oligo and immobilized on the linear ssDNA, activation (Fig. 3A) and expansion (Fig. 3B) was higher than the unmodified anti-CD28 antibody version and comparable to Dynabeads. To further characterize the ability of the DBTA reagent to tune activation kinetics, we evaluated the effect of varying levels of conjugate antibody—ssDNA hybridization on activation kinetics by varying the oligo:antibody (protein) (O/P) ratios of the conjugates. Figure 4 shows the performance of DBTA where both anti-CD3 and anti-CD28 antibodies are conjugated to the Mal-o20b(+)act oligo (a 20-base oligo with maleimide functionality for conjugation and a modified β-actin sequence with full complementarity to ssDNA scaffold) but with varying O/P ratios. Using antibody conjugates having O/P ratios of 2.8 or higher, the initial 24-hour expression of CD25 postactivation was found to be ≥ 90% (Fig. 4C). This improved version of the DBTA reagent was utilized for T-cell viral transduction studies. Preliminary studies with a commercially available GFP lentiviral vector (LentiBrite GFP Particles, MilliporeSigma), showed high (> 90%) transduction with both DBTA and Dynabeads at day 5 (Supplementary Fig. 3, Supplemental Digital Content 1, http://links.lww.com/JIT/A559) for gating strategy. Data represent the average of duplicate wells in a single experiment. DBTA indicates DNA-based T-cell activator; MOI, multiplicity of infection.

**FIGURE 5.** Comparison of DBTA versus Dynabeads for chimeric antigen receptor T-cell transduction and expansion. A, Expansion of peripheral blood mononuclear cells in DBTA and Dynabeads: the number of T cells in culture are measured from days 2–8. B, Expression of activation marker, CD25, on day 8. C, Similar CD4:CD8 ratio in CD3+ T cells was observed in the DBTA and Dynabeads groups on day 8. D, The transduction efficiency of T cells in DBTA and Dynabeads. Chimeric antigen receptor T-cell vector transduction was carried out at day 2 at an MOI of 40 using a GMP-grade CD19/CD22 bispecific chimeric antigen receptor construct. In a single donor study, transduction efficiency (D) with DBTA was higher (21.6%) compared with Dynabeads (11.5%). E, CD8 expression in CD3+ T cells on day 8 of culture. F, Percentages of T-cell subsets on day 8. Despite having a higher fold expansion at day 8 in comparison to the Dynabeads, the expanded cell population in the DBTA group has a much higher percentage of central memory T cells (Tcm) for both, the CD4+ (11.5% vs. 3.43%, respectively) and CD8+ subpopulation of cells (12.2% vs. 2.89%, respectively). T-cell subsets were determined by the following fluorescent activated cell sorting isolations: CCR7+ CD45RA+ for naive T cells (Tn); CCR7- CD45RA+ for effector T cells (Teff); CCR7+ CD45RA+ for central memory T cells (Tcm) and CCR7- CD45RA+ for effector memory T cells (Tem). Refer to Supplementary Data (Supplemental Digital Content 1, http://links.lww.com/JIT/A559) for gating strategy. Data represent the average of duplicate wells in a single experiment. DBTA indicates DNA-based T-cell activator; MOI, multiplicity of infection.
24 hours activation with the DBTA reagent. The ratio of CD4/CD8 subsets within the final cell population was similar (Fig. 5C). In a single donor study, transduction efficiency (Fig. 5D) with DBTA was higher compared with Dynabeads. In our earlier studies with a commercial GFP vector (Lentibrite GFP) used at 20 multiplicity of infection, also in a single donor, >95% transduction efficiency was observed (data not shown) with both reagents. Despite having a higher fold expansion at day 8 in comparison to the Dynabeads, no major differences in the exhaustion (programmed cell death protein-1) marker expression (Fig. 5E) was apparent between the 2 groups. The expanded cell population in the DBTA group had a much higher percentage of central memory T cells (Tcm) in comparison to Dynabeads for both, the CD4+ and CD8+ subpopulation of cells (Fig. 5F, Supplementary Fig. 4, Supplemental Digital Content 1, http://links.lww.com/JIT/A559). Ex vivo T-cell expansion protocols generating a higher percentage of Tcm cells are critical to in vivo efficacy and persistence of the CAR-T product.8

**DISCUSSION**

In vitro expansion of cytotoxic T cells is an essential step in generating a CAR-T dose. Several recent studies have highlighted the effect of the choice of cytokines and costimulatory domains on the quality and quantity of the expanded CAR-T cells.9–12 Similarly, shortening the duration of T-cell activation by providing transient stimulation has been shown to enhance CD8+ T-cell expansion.13 We have developed a novel soluble, tunable, T-cell activator that is based on in situ DNA hybridization to cluster the T-cell surface receptors required for T-cell activation and provide costimulatory signals to increase proliferation. The noncovalent and reversible immobilization of anti-CD3 and anti-CD28 on the ssDNA-based scaffold provides higher activation than soluble antibodies and allows for unprecedented fine-tuning of T-cell response. Our results indicate that the activation and proliferation kinetics with the DBTA is robustly comparable to the CD3/CD28 Dynabeads and potentially scalable (Supplementary Fig. 5, Supplemental Digital Content 1, http://links.lww.com/JIT/A559). Reducing the in vitro expansion time for CAR-T cells is also being considered as a strategy to limit differentiation to effector T cells.14 Such protocols rely on the ability of the activation reagent to induce rapid activation and T-cell proliferation.

The in situ DNA hybridization strategy is particularly attractive offering many advantages. The scaffold used in this strategy provides a high degree of flexibility in controlling the strength of immobilization, amount of clustering, and multiple and flexible receptor/coreceptor targeting by changing length and composition of the complementary sequences and the types and loading of antibody conjugates. Finally, the ssDNA scaffold can be readily released or degraded, if required, by using DNases (Supplementary Fig. 6, Supplemental Digital Content 1, http://links.lww.com/JIT/A559) which are already FDA approved for use in clinical cord blood applications.15

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**Conflicts of Interest/Financial Disclosures**

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